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PDCD4 gene silencing in gliomas is associated with 5′CpG island methylation and unfavourable prognosis

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Abstract

Programmed cell death 4 (*PDCD4*) is a newly described tumour suppressor that inhibits oncogenesis by suppressing gene transcription and translation. Loss of *PDCD4* expression has been found in several types of human cancers including the most common cancer of the brain, the gliomas. However, the molecular mechanisms responsible for *PDCD4* gene silencing in tumour cells remain unclear. Here we report the identification of 5′CpG island methylation as the predominant cause of *PDCD4* mRNA silencing in gliomas. The methylation of the *PDCD4* 5′CpG island was found in 47% (14/30) of glioma tissues, which was significantly associated with the loss of *PDCD4* mRNA expression ($\gamma = -1.000$, $P < 0.0001$). Blocking methylation in glioma cells using a DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine, restored the *PDCD4* gene expression, inhibited their proliferation and reduced their colony formation capacity. Longitudinal studies of a cohort of 84 patients with gliomas revealed that poor prognosis of patients with high-grade tumours were significantly associated with loss of *PDCD4* expression. Thus, our current study suggests, for the first time, that *PDCD4* 5′CpG island methylation blocks *PDCD4* expression at mRNA levels in gliomas. These results also indicate that *PDCD4* reactivation might be an effective new strategy for the treatment of gliomas.

Keywords: *PDCD4* • tumour suppressor gene • glioma • methylation

Introduction

Human gliomas are the most frequent primary tumours of the central nervous system. Because nearly half of them are malignant, their treatment represents one of the most formidable challenges in clinical medicine. Over the past two decades, the overall survival rate of patients with gliomas has hardly improved, with only ~2% of patients aged 65 years or older survived for more than 2 years [1, 2]. Therefore, new diagnostic and therapeutic strategies are needed to control this devastating disease.

Recently, considerable progress has been made in understanding the genetic alterations that are associated with gliomas, which involve both oncogenes and tumour suppressor genes [3]. We previously reported that one of the newly described tumour suppressor genes, designated programmed cell death 4 (*PDCD4*), was silenced in the vast majority of human gliomas, 77% at protein level and 47% at mRNA level [4]. *PDCD4*, or MA3, was first cloned as a gene whose expression was elevated in mouse cell lines undergoing apoptosis [5]. Its homologues of human (*H731*; *TIS*), chicken and rat (*DUG*) were soon described [6–8]. Previous studies have shown that *PDCD4* directly interacts with the eukaryotic initiation factor (eIF) 4A complex to inhibit protein translation. It prevents tumour transformation in the mouse JB6 model system through inhibiting activating protein 1 (AP-1) function [9–11]. Consequently, *PDCD4*-deficient mice develop spontaneous tumours of the lymphoid origin [12], and *PDCD4* transgenic mice

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showed significant resistance to tumour induction [13]. Furthermore, *PDCD4* exerted its anti-tumour roles *via* regulating signal transduction pathways [14, 15].

Human *PDCD4* was first cloned from a human glioma library, which is highly homologous to the mouse *PDCD4* [16]. *PDCD4* is expressed ubiquitously in normal tissues, but its loss or reduction was found in several types of human cancer cell lines [17], and some primary tumours such as human gliomas [4], lung cancer [18], hepatocellular carcinomas [19] and pancreatic cancer [20]. Asangani *et al.* indicate that MicroRNA-21 (miR-21) may post-transcriptionally regulate *PDCD4* expression in colorectal cancer [21]. Dorrello *et al.* find that S6K1 and betaTRCP may control the levels of *PDCD4* protein through an ubiquitination-dependent mechanism [22]. However, the mechanisms of *PDCD4* silencing at the transcriptional level are unknown.

In recent years, epigenetic studies have revealed that DNA modifications, *e.g.* 5′CpG island hypermethylation, can cause tumour suppressor gene inactivation and therefore, the development of cancer [23–25]. We therefore reasoned that epigenetic changes of the *PDCD4* gene may be involved in the development of human primary gliomas. We report here that *PDCD4* gene silencing in gliomas was associated with 5′CpG island methylation and unfavourable prognosis of patients.

Materials and methods

Tumour specimens and cell lines

A total of 84 glioma specimens including 30 frozen [4] and 54 paraffin-embedded tissues were obtained from patients aged between 30 and 60 years (median = 40 years) who underwent operations at the Department of Neurosurgery in Qilu Hospital of Shandong University from October 2003 to March 2006. The pathological diagnoses were carried out according to the latest World Health Organization (WHO) criteria for gliomas. The patients' profiles are presented in Table 1. None of the patients studied had received adjuvant immunosuppressive treatments such as radiotherapy or chemotherapy prior to surgery in order to eliminate their effects on gene expression. Three non-tumour brain tissue samples were excised from tumour-adjacent sites. U251 and U87 cell lines derived from gliomas were purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). They were respectively cultured and maintained in Dulbecco's modified Eagle's medium and modified Eagle's medium (Gibco-BRL, Carlsbad, CA, USA) containing 10% foetal calf serum (Gibco-BRL). This study is in full compliance with the national legislation and the ethical standards of the Chinese Medical Association.

RNA isolation and RT-PCR

Total RNAs were extracted from 30 frozen glioma specimens, 3 frozen non-tumour brain tissue samples and 2 glioma cell lines (U251 and U87) using a modified TRIzol® one-step extraction method (Sangon Biotech Co., Ltd., Shanghai, China) [26, 27]. cDNA was synthesized using the Reverse-Transcribe Kit (Promega Co., Madison, WI, USA) according to manufacturer's instructions. PCR was performed with *PDCD4* specific primers (sense 5′-CCAAAGAAAGGTGGTGCA-3′, and anti-sense 5′-TGA GGTACTTCCAGTTCC-3′) for 35 cycles (95°C for 90 sec., 66°C for 90 sec. and 72°C for 90 sec.). Human β-actin was amplified as an internal control. The RT-PCR was performed at least twice for each sample.

SDS-PAGE and Western blot

The proteins were extracted from tissue samples and glioma cell lines using a modified TRIzol® one-step extraction method [26, 27]. The protein extract was dissolved in loading buffer (1 mM Tris Cl, 3% SDS, 60% glycerol, 75 mM DTT), and each sample was analysed by SDS-PAGE on a 12% gel. The nitrocellulose membrane was incubated with rabbit anti-human PDCD4 polyclonal (1:10,000) or rabbit anti-human β-actin polyclonal (1:1000) at 4°C overnight. The antibodies separately were from Abcam, Inc. (Cambridge, MA, USA) and Cell Signaling Technology, Inc. (Danvers, MA, USA). Immunoreactive bands were visualized using the enhanced chemiluminescence method according to the manufacturer's instructions (ECL, Amersham Biosciences, Buckinghamshire, UK). The concentration of protein was determined by Bradford analysis.

DNA bisulphite modification and bisulphite sequencing

To differentiate methylated CpGs from unmethylated CpGs, 2 μg of purified genomic DNA was treated with sodium bisulphite in a CpGenome™ Fast DNA Modification kit (Chemicon International, Temecula, CA, USA) at 50°C for 16–20 hrs according to manufacturer's instructions. The position of CpG island was predicted and primers for bisulphite genomic sequencing PCR (BSP) were designed by the CpG software provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/emboss/cpgplot/index.html) and by MethPrimer software [28]. PCR was done using the following primers: 5′-TTTTTATTTTTTAGTATTGTTTTTTTT-3′ (sense) and 5′-CTATT-TATTTTTATTTTCTTCTACCCAATA-3′ (anti-sense). PCR mixture contained 12.5 μl of 2× Master Mix (0.1 U Taq DNA polymerase, 500 μM dNTP, 3 mM $MgCl_2$, 100 mM KCl, 20 mM Tris-HCl), 0.5 μl of forward primer (5 μM), 0.5 μl of reverse primer (5 μM), 1 μl of the bisulphite-modified DNA sample and 10.5 μl of H_2O. For bisulphite sequencing, PCR products were gel-extracted and cloned into the PMD18-T Vector (Takara Biotechnology, Dalian, China), and 10 clones for each sample were sequenced using M13F or M13R primer by the Boya Biotechnology Sequencing Co. Ltd. (Shanghai, China). Cytosines in CpG dinucleotides that remained unconverted after bisulphite treatment were considered to be methylated.

Methylation-specific PCR (MSP)

The bisulphite modificated genomic DNAs were used as templates for MSP. PCR amplification was done using HotStar DNA polymerase (Takara Biotechnology) under the following conditions: 95°C for 5 min. (hot start), 40 cycles of 30 sec. at 95°C, 1 min. at 58°C and 1 min. at 72°C, and 5 min. of final extension at 72°C. DNA from adjacent normal glial tissues was used as negative controls. Five groups of primers for methylation and unmethylation were designed using MethPrimer software. One group of *PDCD4* primers which worked best was 5′-TTTAGTTTCGGTTTCGTCGTTAC-3′ (sense) and 5′-GAAAAATCTCTAACCCTTCTCGC-3′ (anti-sense) for the methylated sequence, and 5′-TTTAGTTTTGGTTTTGTTGTTATGA-3′ (sense)

Table 1 *PDCD4* expression in human glioma tissues

Parameters		*PDCD4*$^-$	*PDCD4*$^+$	*P*-value
Total number of patients		68	16	
Gender	Male	39	14	0.0245*
	Female	29	2	
Age (years)	>40	38	10	0.6303
	≤40	30	6	
Histological types	Astrocytoma	39	8	0.8557
	Glioblastoma	19	5	
	Anaplastic astrocytoma	10	3	
Grade	Low grade (I, II)	28	5	0.4645
	High grade (III, IV)	40	11	

*$P < 0.05$.
PDCD4 expression in human glioma tissues. The clinical parameters include gender, age, histological types and grade.
PDCD4 expression: loss, PDCD4−; expression, PDCD4$^-$.

and 5′-CAAAAAATCTCTAACCCTTCTCACT-3′ (anti-sense) for the unmethylated sequence. The DNA methylation status of the *PDCD4* CpG island was determined in glioma samples and cell lines by bisulphite conversion of unmethylated, but not methylated, cytosine to uracil as previously described [29].

5-aza-2′-deoxycytidine treatment

The glioma cell lines, U251 and U87, were seeded at a density of 1×10^5 cells/well in culture medium and allowed to attach over a 24-hr period. 5-aza-2′-deoxycytidine (Sigma, St. Louis, MO, USA) was then added to final concentrations of 0, 2, 5, 10 μM and the cells were allowed to grow for 48 hrs. At the end of the treatment, the medium was removed; and the RNA, DNA and protein were extracted for RT-PCR, methylation analysis, and protein analysis.

Immunohistochemistry

Paraffin sections (5 μm) were stained with anti-*PDCD4* antibody (Cat. 3975, ProSci Incorporated, Poway, CA, USA) by incubating overnight at 4°C. Secondary staining with biotin-conjugated anti-rabbit IgG and tertiary staining with HRP-conjugated streptoavidin, were performed with an ABC kit and DAB Peroxidase Substrate kit (Maixin Co., Fuzhou, China). The nuclei were counterstained with the haematoxylin. Negative controls for the specificity of immunohistochemical reactions were performed by replacing the primary antibody with phosphate-buffered saline. All slides were independently analysed by two pathologists. Cells were cultured in the Lab-Tek chamber glass slide (Nalgen Nunc International, Rochester, NY, USA), fixed by incubating with 4% paraformaldehyde in 100 mM phosphate buffer for 10 min. and then followed by treatment for 1 min. with cold acetone. The fixed cells were stained in the same way as the paraffin sections described above.

Apoptosis and cell cycle analysis

U251 and U87 cell lines were transfected with pDsRed2-N1 and pDsRed2-N1-PDCD4 for 24–72 hrs. Cells were stained with Annexin V-FITC and/or propidium iodide (Jingmei Biotech Co. Ltd., Shenzhen, China) according to the manufacturer's protocols. Apoptosis and cell cycle progression were analysed by flow cytometry using the Beckman Coulter Cytomics™ FC 500 (Beckman Coulter, Fullerton, CA, USA).

Colony formation assay

U251 (1.25×10^5 cells) cells and U87 (1.1×10^5 cells) were plated in 24-well plates for 24 hrs and transfected with pDsRed2-N1 and pDsRed2-N1-*PDCD4* vector using Lipofectin (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's protocol. After 24 hrs, cells were reseeded in six-well plates with a density of 5000 cells per plate in the presence of G418 (400 μg/ml) for 2–3 weeks. The colonies were fixed with 20% methanol and stained with 1% crystal violet.

Statistical analysis

Pearson's coefficient test was used to analyse the correlation between the hypermethylation of *PDCD4* 5′CpG island and the loss of *PDCD4* expression. The χ^2 test was used to compare the expression of *PDCD4* with clinicopathological parameters. Cumulative survival time was calculated by the Kaplan–Meier method and analysed only for 84 gliomas by the log-rank test. Multivariate survival analysis was performed with the Cox regression model for all 84 gliomas. $P < 0.05$ was considered statistically significant. All calculations were performed with the SPSS statistical software.

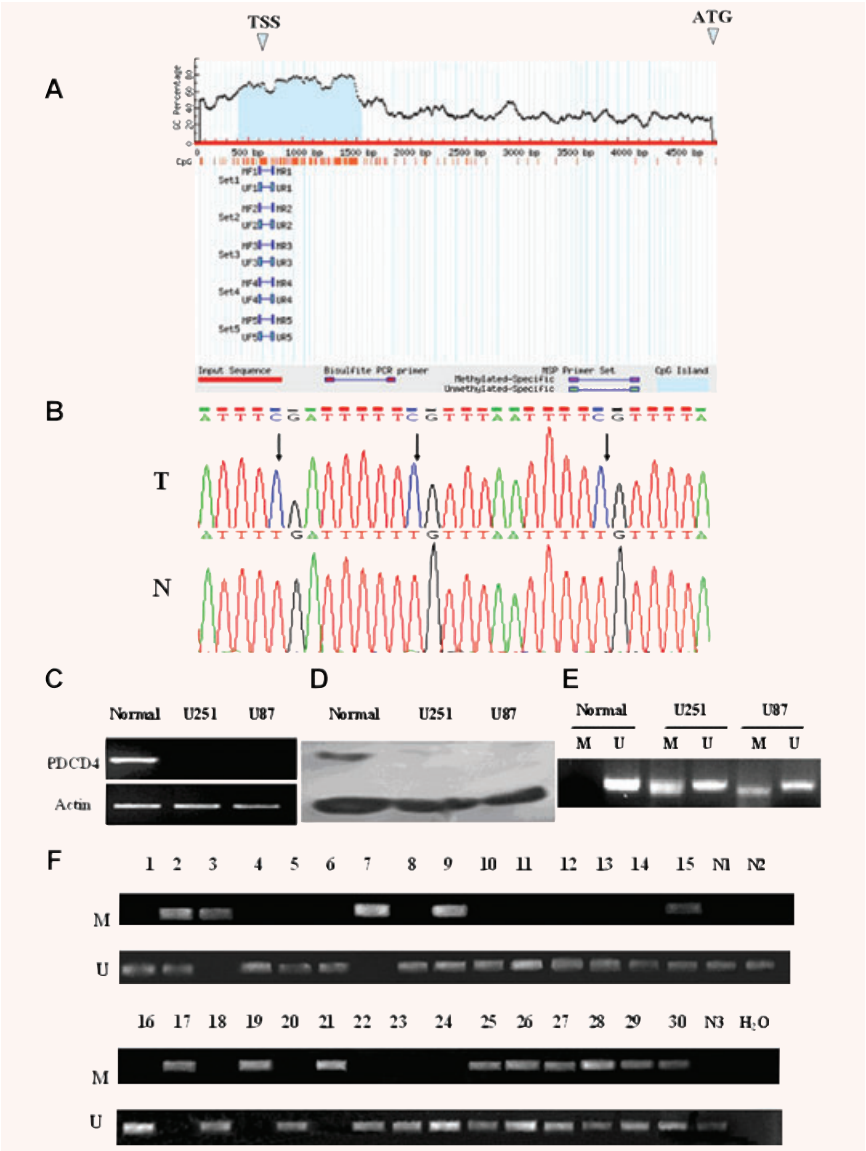

Fig. 1 Methylation of *PDCD4* 5′CpG island in glioma cell lines, non-tumour brain and glioma tissues. (**A**) Schematic representation of the CpG island of human *PDCD4* gene. (**B**) Representative figure of methylation. T: tumour tissue; N: non-tumour brain tissue. Arrows, the methylated CpGs. (**C**)–(**F**). *PDCD4* mRNA (**C**) and protein (**D**) expression and *PDCD4* gene methylation (**E**) as determined by RT-PCR, Western blot and methylation-specific PCR, respectively. U251 and U87, two glioma cell lines; normal, non-tumour brain tissue; M, methylated; U, unmethylated. (**F**) Methylation status of the *PDCD4* cytosine island in non-tumour brain and primary glioma tissues. 1–30, primary glioma tissues from 30 patients; N, non-tumour brain tissues; H_2O, untransfected control

Results

Methylation of the *PDCD4* 5′CpG island is common in glioma cell lines and primary gliomas

Of the 30 frozen glioma samples examined previously, 47% (14/30) of them lacked the *PDCD4* mRNA expression [4]. However, the mechanisms of the *PDCD4* silencing at mRNA levels are unclear. 5′CpG island methylation is a common epigenetic mechanism implicated in the silencing of tumour suppressor genes in human cancers [24]. To determine whether 5′CpG island methylation is responsible for *PDCD4* silencing in gliomas, we first searched for 5′CpG islands in the *PDCD4* gene as described in the section 'Materials and methods'. A typical CpG island (−251 to +918 bp) was found around the transcription start site of the *PDCD4* gene including the first exon and part of the upstream transcription start site sequence (Fig. 1A). This suggests that *PDCD4* may be vulnerable to methylation-mediated silencing. To test this theory, genomic DNAs from glioma cell lines and primary gliomas as well non-tumour brain specimens were purified and used as templates for BSP and PCR (MSP) after bisulphite modification. As shown in Fig. 1B–F and Table 2, the methylation of *PDCD4* 5′CpG island was detected in both *PDCD4*$^-$ cell lines (U251 and U87) and 14 specimens of *PDCD4* mRNA$^-$ primary gliomas whereas no methylation was found in non-tumour brain tissues. Among methylated glioma samples, 5 (nos. 3, 7, 17, 19, 21) showed complete methylation (M: + and U: −) and the

Table 2 Expression and methylation status of *PDCD4* in cell lines and tissues

		mRNA	Methylation	Grade
Cell lines	U251	−	+	
	U87	−	+	
Non-tumour brain tissues	1	++++	−	
	2	++++	−	
	3	++++	−	
Gliomas	1	++++	−	IV
	2	−	+	IV
	3	−	+	IV
	4	+	−	IV
	5	+	−	III
	6	+	−	III
	7	−	+	III
	8	+++	−	III
	9	−	+	IV
	10	+	−	III
	11	++++	−	IV
	12	++	−	IV
	13	+	−	III
	14	+	−	IV
	15	−	+	IV
	16	+	−	IV
	17	−	+	III
	18	+	−	II
	19	−	+	II
	20	+++	−	IV
	21	−	+	IV
	22	++	−	III
	23	+	−	IV
	24	+	−	III
	25	−	+	III
	26	−	+	III
	27	−	+	IV
	28	−	+	III
	29	−	+	IV
	30	−	+	III

Expression and methylation status of PDCD4 in cell lines and tissues. The pathological grades were assigned according to the new 2007 WHO criteria for gliomas. The PDCD4 mRNA levels were determined by semi-quantitative RT-PCR, and normalized against the levels of β-actin in the same sample: −, not detectable; +, 0.01 to 0.49; ++, 0.5 to 0.99; +++, 1.0 to 1.99; ++++, ≥2.00. Methylation was detected by methylation-specific PCR: +, methylated; −, unmethylated.

rest (nos. 2, 9, 15, 25, 26, 27, 28, 29 and 30) as well as two cell lines, showed incomplete methylation (M: + and U: +). Both complete and incomplete methylations of 5′CpG island were able to cause the silencing of *PDCD4* at mRNA level.

5′CpG island methylation is associated with loss of *PDCD4* expression at mRNA level in the glioma cell lines and primary glioma tissues

We next examined the relationship between *PDCD4* 5′CpG island methylation and *PDCD4* gene silencing at mRNA level. Normal brain tissues and several *PDCD4*-expressing gliomas showed no methylation of *PDCD4* 5′CpG island. In contrast, glioma cell lines and all of the *PDCD4*$^-$ glioma tissues showed clear *PDCD4* 5′CpG island methylation (Table 2). The correlation between 5′CpG island methylation and *PDCD4* gene silencing at mRNA level was statistically significant (Pearson's correlation coefficient is -1.000, $P < 0.0001$). These results indicated that CpG island methylation is significantly associated with the loss of *PDCD4* expression at mRNA levels.

Blocking methylation in glioma cells effectively restores *PDCD4* expression

To determine whether CpG island methylation of the *PDCD4* 5′CpG island causes the loss of *PDCD4* expression, glioma cell lines which had the *PDCD4* 5′CpG island methylation (U251 and U87) were exposed to increasing concentrations of the DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine. After 48 hrs treatment, the methyltransferase inhibitor had no effect on the viability of the glioma cells (data not shown) but significantly decreased the methylation (Fig. 2A). To further confirm the status of demethylation, the PCR products were also sequenced after the treatment of high concentration 5-aza-2′-deoxycytidine. The methylated sites disappeared in both glioma cell lines (data not shown). Remarkably, blocking methylation resulted in a substantial restoration of *PDCD4* expression at both mRNA (Fig. 2B) and protein levels (Fig. 2C and D). Of note is that although two bands appeared in the PDCD4 PCR products of treated U251 cell line, only the upper band was *PDCD4* specific as confirmed by sequencing. These results indicated that *PDCD4* silencing at mRNA level in glioma cells was predominantly caused by 5′CpG island methylation.

Restoration of *PDCD4* expression in glioma cells inhibited their proliferation, induced their apoptosis and prevented their colony formation

The frequent loss of *PDCD4* expression in glioma cells suggests that *PDCD4* may inhibit the proliferation of gliomas. To determine the effect of *PDCD4* on the growth and survival of glioma cells, a

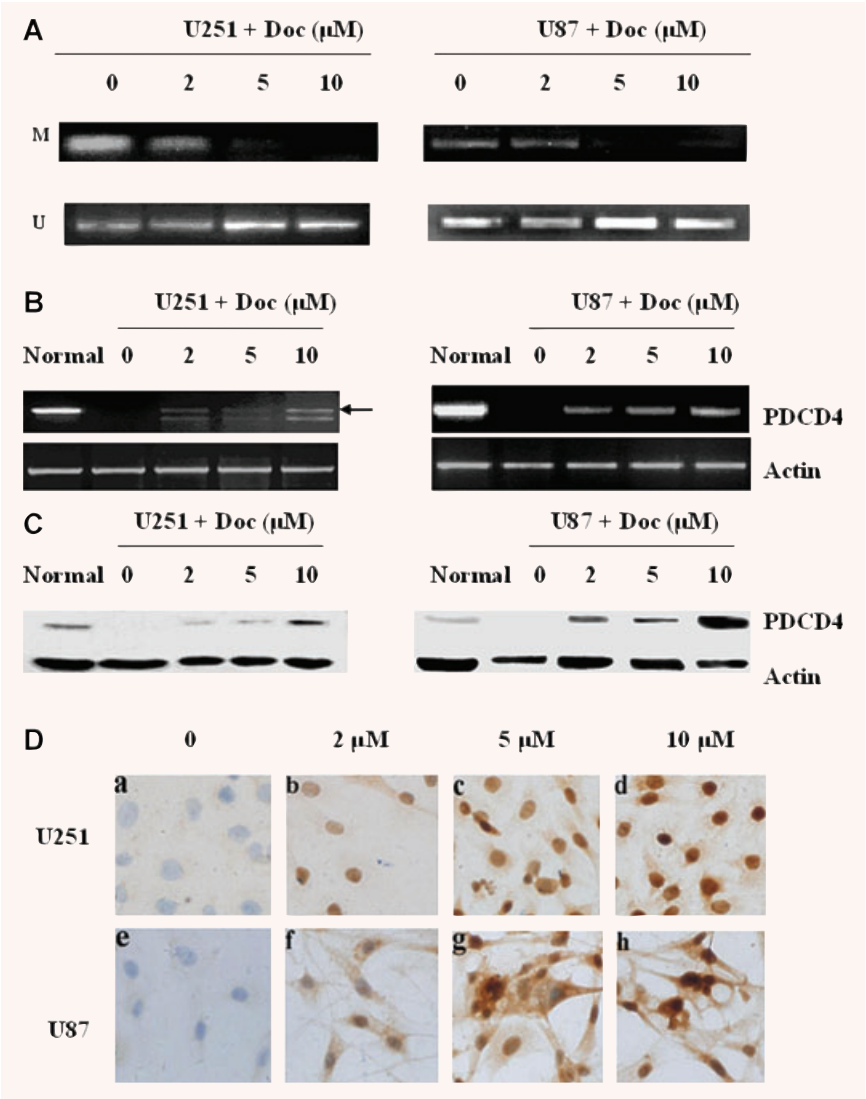

Fig. 2 Demethylation of *PDCD4* gene restored the *PDCD4* expression in glioma cells. Glioma cells (U251 and U87) were cultured with 0, 2, 5, or 10 μM 5-aza-2′-deoxycytidine (Doc) for 48 hrs. De-methylation status of the *PDCD4* CpG island was detected by methylation-specific PCR; M, methylated; U, unmethylated (**A**). The expression of *PDCD4* at 48 hrs was examined by RT-PCR (**B**), Western blot (**C**) and immunocytochemistry (**D**). Normal brain tissue (normal) not treated with Doc was used as a control.

recombinant pDsRed plasmid carrying the full-length *PDCD4* cDNA was transfected into *PDCD4*$^-$ U251 and U87 glioma cells. Forty-eight hours later, both the fluorescent marker and *PDCD4* were detected in the glioma cells (Fig. 3A and B). Importantly, *PDCD4* expression in these cells significantly inhibited their growth, compared with untransfected control and mock-transfected groups (Fig. 3C and D). Cell cycle analysis further revealed that PDCD4 expression significantly increased the number of cells in the S-phase of the cell cycles (38.47% ± 1.39 in mock *versus* 50.83% ± 2.27 in the PDCD4 group) (Fig. 3E). Furthermore, annexin V-FITC/PI analysis clearly showed that PDCD4-transfected glioma cells underwent more apoptosis than the controls (4.96% ± 1.04 in mock *versus* 11.62% ± 0.95 in the PDCD4 group) (Fig. 3F).

Glioma cells, unlike normal glia cells, have the tendency to form colonies in the culture (Fig. 4). Remarkably, *PDCD4*-transfection significantly diminished the capacity of these cells to form colonies. Specifically, compared with the mock control, the expression of PDCD4 caused 86.47% ± 1.10 and 32.68% ± 0.34 reductions in the number of colonies formed by U251 cells and U87 cells, respectively ($P < 0.01$). Thus, *PDCD4* expression reduces the growth and colony forming capacity of glioma cells.

PDCD4 expression in human primary glioma tissues improved the prognosis of patients with high-grade gliomas

The above results showed that restoration of *PDCD4* expression inhibited proliferation and prevented colony formation of cell lines *in vitro*. To determine whether *PDCD4* expression in primary

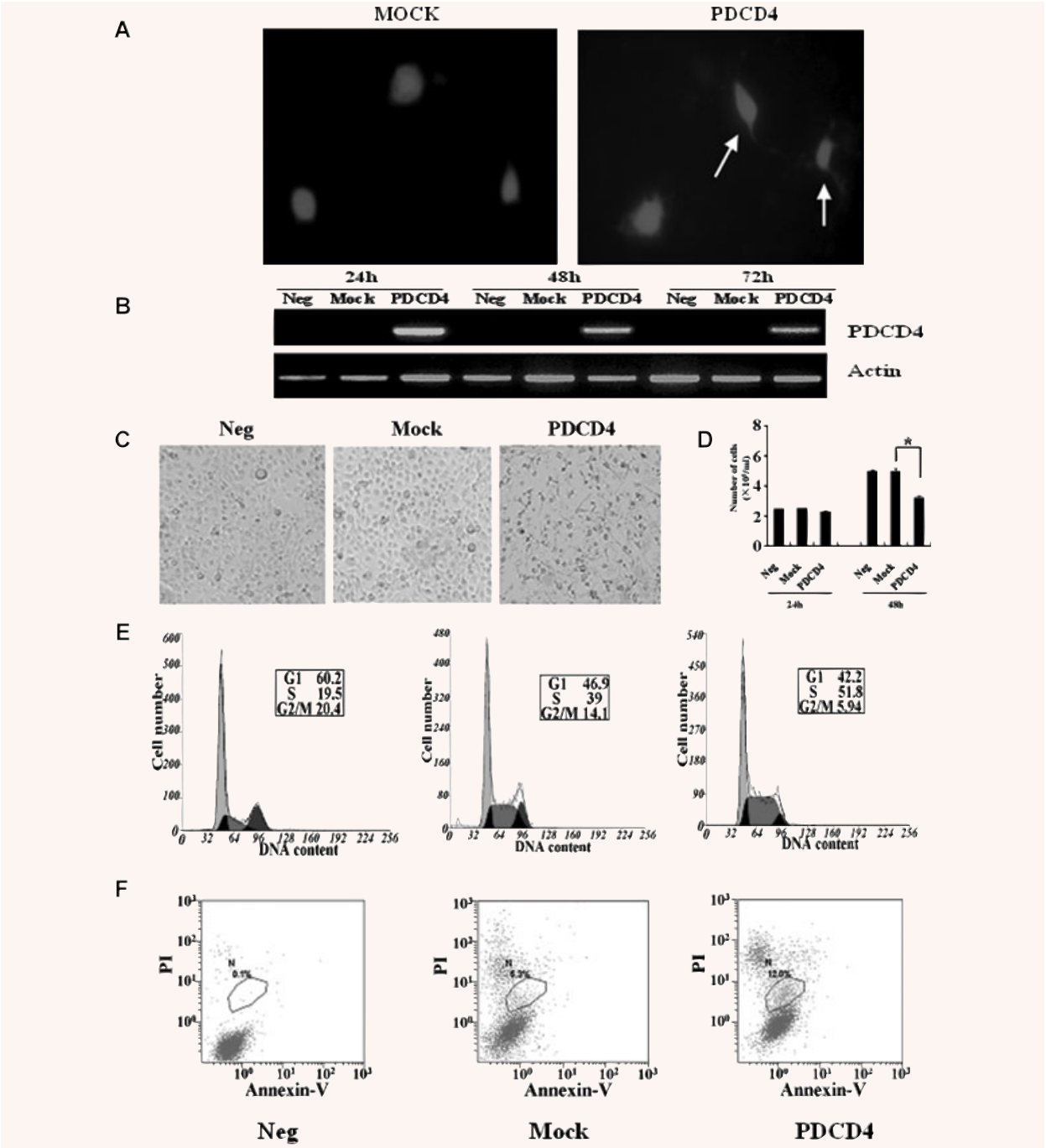

Fig. 3 Re-constitution of *PDCD4*⁻ glioma cells with *PDCD4* inhibited their growth and induced their apoptosis. (**A**) After transient transfection with *PDCD4* for 48 hrs, the U251 cells were examined by fluorescent microscopy. Transfected cells express the red fluorescent protein, DsRed2, which are shown in red. (**B**) *PDCD4* expression in U251 glioma cells was examined by RT-PCR after transient transfection (24, 48 and 72 hrs) with *PDCD4* plasmids (PDCD4), or empty vector (Mock). Untransfected *PDCD4*⁻ glioma cells were used as a control. (**C**) The morphology of U251 cells with or without *PDCD4* expression. (**D**) Total number of U251 cells per millilitre of culture medium 24 and 48 hrs after the transfection. *, $P < 0.05$. Data shown are representative of three independent experiments. **(E)** Cell cycle analysis. (**F**) Apoptosis analysis. Data shown are representative of three independent experiments.

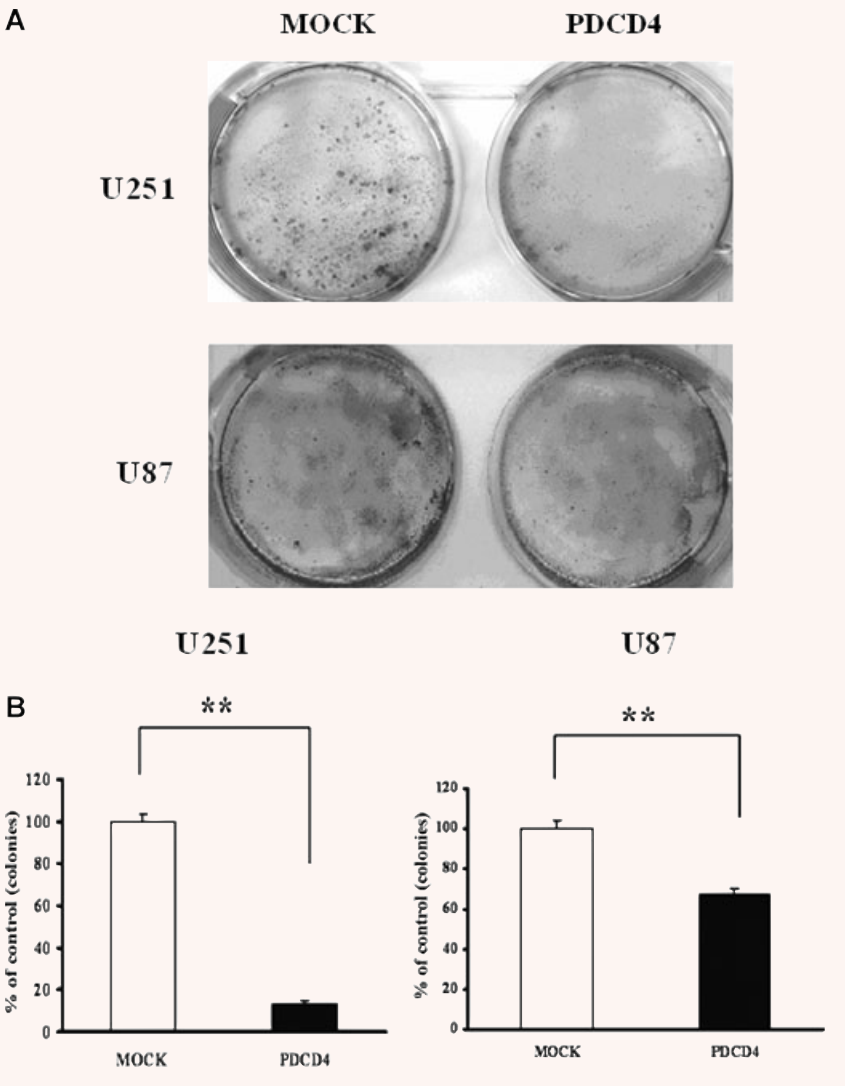

Fig. 4 Re-constitution of *PDCD4*$^-$ glioma cells with *PDCD4* reduced their colony-forming capacity. The U251 and U87 glioma cells were transfected with *PDCD4* plasmids or empty vector (Mock) and selected with G418 for 2–3 weeks. The number of colonies formed on the plates was counted. Data shown are representative of three independent experiments. **, $P < 0.01$.

gliomas also affects tumorigenesis, we examined firstly the *PDCD4* expression in paraffin-embedded glioma tissues of an additional 54 patients by immunohistochemistry [4]. We found that the vast majority of these tissues (45 of 54) expressed no detectable *PDCD4* protein whereas the other nine glioma tissues showed decreased *PDCD4* expression compared with normal brain tissues adjacent to the tumour, which showed strong *PDCD4* expression. Collectively, 81% (68 of 84) of glioma tissues we examined lacked detectable *PDCD4* protein expression (Table 1), indicating that loss of *PDCD4* expression is a frequent event in primary gliomas. Consistent with this view, Jansen *et al.* reported that all of five tumour cell lines derived from central nerve system lacked *PDCD4* expression [17].

Furthermore, to assess the association of *PDCD4* expression with patient survival, the survival data from 84 patients with gliomas (33 low grade and 51 high grade) were generated by a 3-year follow-up. We analysed the relationship between *PDCD4* expression at protein level and clinicopathological characteristics in primary gliomas. The results showed that no significant correlation between *PDCD4* expression and age, histological type or pathological grade existed (Table 1). The expression of *PDCD4* was neither significantly associated with the survival rate of patients with a low grade of gliomas ($n = 33$) (data not shown). However, among 51 patients with high-grade gliomas, the expression of *PDCD4* significantly correlated with the long-term survival rate of patients. The survival rate of patients with *PDCD4*-expressing gliomas was significantly higher than that of patients with *PDCD4*$^-$ gliomas ($P = 0.0013$) (Fig. 5). To examine whether the *PDCD4* silencing was an independent unfavourable prognostic factor for patients, we performed a multivariant Cox regression

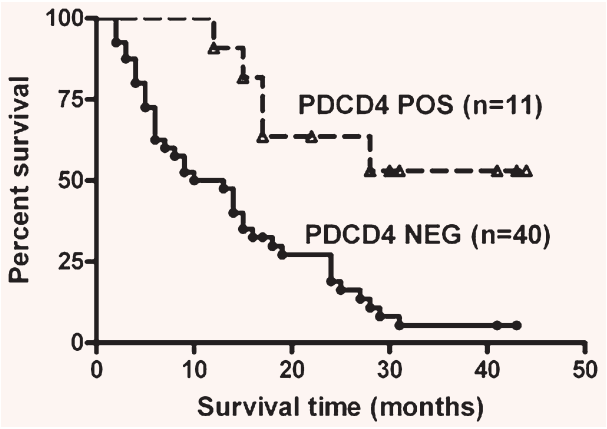

Fig. 5 Prognostic value of *PDCD4* expression for patients with gliomas. The survival times of 51 patients with high-grade gliomas were analysed using the Kaplan–Meier method. The difference in survival time between patients with $PDCD4^{+}$ gliomas and patients with $PDCD4^{-}$ gliomas is statistically significant ($P < 0.05$) as determined by the log-rank test.

analysis, including gender, age, histological types, grade and *PDCD4* expression (Supporting Table S1). The level of *PDCD4* expression could significantly predict the patient outcome independent of other clinicopathological variables for disease-specific survival (relative risk, 0.063; 95% confidence interval, 0.014–0.276, $P < 0.0001$). Thus, *PDCD4* expression in primary gliomas can serve as an important factor for prognosis of high-grade gliomas. In addition, there appeared to be a tendency for *PDCD4* expression to be associated with gender (see Table 1). The expression of *PDCD4* in gliomas from male patients was significantly higher than that from female patients ($P = 0.0245$).

Discussion

In the present study, we showed that the tumour suppressor *PDCD4* was frequently silenced in primary gliomas and glioma cell lines, and that this silencing may be caused by 5′ CpG island methylation. By analysing a large cohort of patients with gliomas, we further concluded that *PDCD4* expression might serve as a prognostic factor in patients with high-grade gliomas. Although our current study focused only on gliomas, the findings reported here may also apply to other tumours as well. It has been reported that *PDCD4* expression showed a progressive decrease in several human tumour cell lines [17, 30]. Furthermore, loss or reduction of *PDCD4* expression was detected in human primary tumour tissues including lung cancer [18], hepatocellular carcinomas [19] and pancreatic cancer [20]. However, to date, the mechanism of *PDCD4* silence in tumours is unclear.

Hypermethylation in 5′CpG island has been found in many tumours, which is often associated with the inactivation of cancer-related genes such as *p16* [31, 32], *PTEN* [33] and *EMP3* [34]. In the present study, we provide evidence to demonstrate, for the first time, that 5′CpG island methylation contributes to *PDCD4* silencing at mRNA levels in gliomas. Non-tumour brain tissues and gliomas with *PDCD4* expression showed undetectable methylation of *PDCD4* 5′CpG island. In contrast, both glioma cell lines and all of glioma tissues without *PDCD4* mRNA expression showed clear *PDCD4* 5′CpG island methylation (Fig. 1 and Table 2). *PDCD4* gene silencing at mRNA levels was significantly associated with 5′CpG island methylation. Importantly, this silencing could be reversed by treating glioma cells with the methyltransferase inhibitor, 5-aza-2′-deoxycytidine (Fig. 2). Consistent with this view, Fan *et al.* reported that DNA methyltransferase 1 knockdown induced re-expression of many genes including *PDCD4* by demethylation of methylated CpG in hepatocellular carcinoma cell line SMMC-7721 [35]. These data demonstrate that 5′CpG island methylation of *PDCD4* is significantly associated with the silencing of gene expression in gliomas at mRNA levels. Is the methylation of the 5′CpG island of *PDCD4* also responsible for gene silencing in other tumours? Our preliminary studies indicate that methylation of PDCD4 5′CpG island was detected in the ovarian cancers with no *PDCD4* expression (data not shown). The study by Fan *et al.* also suggests that methylation of the *PDCD4* may exit in human hepatocellular carcinoma [35]. However, methylation status of *PDCD4* 5′CpG island in other cancer cells remains to be determined.

Our results indicate that methylation of *PDCD4* 5′CpG island only involves in silencing of *PDCD4* mRNA but do not affect the expression of *PDCD4* protein. Dorrello *et al.* find that degradation of *PDCD4* protein in response to stimulation, such as mitogens, may decrease levels of *PDCD4* protein [22]. Some recent reports showed that *MicroRNA-21* (*miR-21*) may post-transcriptionally down-regulate *PDCD4* expression in human tumours, such as colorectal cancer and breast cancer [21, 36]. It has been known that *microRNA* (*miR-21*) is also overexpressed in gliomas, suggesting that *microRNA* (*miR-21*) may influence *PDCD4* protein levels in gliomas. However, further investigation should be done in future.

We demonstrated that restoration of *PDCD4* expression in glioma cell lines inhibited their proliferation and prevented their colony formation *in vitro*. To clarify whether *PDCD4* expression in primary gliomas also affects tumorigenesis, we divided patients with gliomas into two groups: low-grade group (grades I and II) and high-grade group (grade III and IV) according to the WHO classification system, and then analysed the association of *PDCD4* expression (detected by immunohistochemistry) with clinicopathological characteristics and patient survival over a period of 3 years. Our data indicated that *PDCD4* expression was associated with long-term survival of patients with high-grade gliomas and was an independent favourable prognostic factor for patients while it had no relationship with grade or stage in gliomas. This result is not entirely consistent with a report about human primary lung cancer in which down-regulation of *PDCD4* protein was significantly associated with the grade and stage of the cancer [18].

These results indicate that the role of *PDCD4* in carcinogenesis may be tissue specific. The mechanisms through which *PDCD4* expression affects tumorigenesis may vary in different tumours. *PDCD4* might induce apoptosis of human breast cancer cell line T-47D [30], human hepatocellular carcinoma-derived cell line Huh7 [19] *in vitro* and enhance the apoptosis of murine lung cancer cells and control their cell cycles in K-ras null mice or AP-1 luciferase reporter mice [37, 38]. We found that reintroduction of PDCD4 in glioma cell lines could also induce their apoptosis and block cell cycle progression (see Fig. 3E and F). However, *PDCD4* expression does not affect cell cycle or apoptosis in colon carcinoma cells [39]. Other researchers demonstrated that overexpression of PDCD4 could enhance the sensitivity of renal cancer cell lines to geldanamycin [17]. Our studies showed that overexpression of *PDCD4* could enhance the sensitivity of ovary cancer-derived cell lines to cisplatin (data not shown). It suggested that *PDCD4* might prolong the survival of patients through enhancing the sensitivity of tumour cells to chemotherapy. In addition, our analysis of the 5′CpG island of *PDCD4* using genomatix promoter database (http://www.genomatix.de/products/index.html), indicates that *PDCD4* may be an important target for other tumour suppressor genes. Many genes including some oncogenes or tumour suppressor genes, such as *p53* could bind to 5′CpG island of *PDCD4*. Therefore, these genes might inhibit the cell proliferation by down-regulating or up-regulating expression of *PDCD4*. So the different status of other related genes among various tumours may affect the effect of *PDCD4*.

Unexpectedly, our data indicate that *PDCD4* expression was significantly higher in gliomas of male patients than in that of female (see Table 1), suggesting that male hormones might up-regulate the *PDCD4* expression. The previous report demonstrated that sex hormones and/or genetic differences between males and females may play a role in the pathogenesis of gliomas [40]. We noticed that *sex-determining region Y* (*SRY*), a transcription factor that initiates male sex determination, could bind to the regulatory region of *PDCD4* 5′CpG island. It suggests that the male hormones may up-regulate PDCD4 expression by *SRY*. However, the mechanisms by which that male hormone regulates *PDCD4* expression remained to be clarified.

In summary, the frequent inactivation of *PDCD4* at mRNA level in gliomas was significantly associated with 5′CpG island methylation. The restoration of *PDCD4* expression in glioma cell lines inhibited their proliferation and prevented their colony formation. Furthermore, *PDCD4* expression in primary glioma tissues is associated with better prognosis of patients with high-grade gliomas. Therefore, *PDCD4* reactivation might be an effective new strategy for the treatment of gliomas.

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