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Original Article

PRDX-1 promotes cell proliferation and inhibits apoptosis of human gliomas via TNF-α/NF-κB pathway

Jin-Ge Liu[1], Ke-Bin Zhang[2], Jin-Liang Tang[1], Ya-Li Wang[1], Sheng-Qing Lv[3], Jian-Ping Xu[1]

Departments of [1]Pathology, [2]Central Laboratory, [3]Neurosurgery, Xinqiao Hospital, Third Military Medical University, Chongqing, China

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Abstract: Objective: To investigate the expression pattern of PRDX-1 in human glioma tissues and explore the role of PRDX-1 in regulating proliferation and apoptosis of glioma cells. Methods: The expressions of PRDX-1, TNF-α and NF-κB were detected by immunohistochemistry in 156 cases of human gliomas. The association of PRDX-1 with clinical parameters was also analyzed. The function of PRDX-1 was investigated by constructing PRDX-1 interference or over-expression U87MG cell lines. Flow cytometry was applied to analyze cell apoptosis. The growth ability of tumor cells was evaluated by colony formation assay. Western blot were applied to detect the protein expressions of PRDX-1, TNF-α and NF-κB. Results: PRDX-1 expression was positively correlated with tumor grade and Ki67 index. Suppression of PRDX-1 inhibited proliferation, colony-forming ability and promoted apoptosis of U87MG cells. PRDX-1 overexpression resulted in opposite effect compared to PRDX-1 knock down. In addition, the expression of TNF-α and NF-κB were positively correlated with PRDX-1 expression both in vitro and in human glioma specimen. Conclusion: PRDX-1 expressed higher in high grade gliomas than low grade ones, and promoted cell proliferation as well as inhibited apoptosis of human gliomas via TNF-α/NF-κB pathway.

Keywords: PRDX-1, glioma, proliferation, apoptosis, NF-κB

Introduction

Glioma is one of the most common primary brain tumors in adults. Glioblastoma (GBM), the most malignant form of gliomas, is characterized by high recurrence rate and mortality rate. Despite intensive therapeutic strategies, the median survival time of GBM patients has not improved obviously [1, 2]. It has been proposed that induction differentiation therapy might be an effective treatment for tumor [3, 4]. In previous studies, we found that peroxiredoxin1 (PRDX-1) expression was significantly down-regulated when human glioma cell lines U87MG was induced to differentiate [5]. It suggested that PRDX-1 may be a therapeutic target for glioma treatment.

PRDX-1, also named as proliferation-associated gene A (PAG-A), is a member of peroxidase family which has been reported to be closely related to cell proliferation and intracellular signal transduction [6, 7]. In addition, PRDX-1 played an important role in tumorigenesis of several malignant tumors, such as esophageal squamous cell carcinoma, pancreatic cancer and breast cancer [8-11]. Hansen JM et al. [12] found that PRDX-1 regulated the activation and nuclear transfer of NF-κB through TNF-α, which prompted that NF-κB and TNF-α are the downstream molecules of PRDX-1. In gliomas, PRDX-1 has been found to regulate the radio-and chemosensitivity [13-15]. However, the expression pattern and the effect on tumor growth of PRDX-1 in glioma need to be further clarified.

In this study, we detected PRDX-1 expression by immunohistochemistry and analyzed the relationship of PRDX-1 expression and the clinicopathologic characteristics of gliomas. We also investigated the effect of PRDX-1 on proliferation and apoptosis of glioma cells by interfering or overexpressing PRDX-1. In addition, TNF-α and NF-κB were detected in PRDX-1 shRNA cells, PRDX-1 overexpression cells and glioma specimens.

Table 1. The ShRNA sequences & cDNA Primer sequences of PRDX-1

Name	Primer sequences
shRNA1	F: 5'-GATCCGCTTTCAGTGATAGGGCAGAATGTGCTTTTCTGCCCTATCACTGAAAGCTTTTTTC-3'
	R: 5'-AATTGAAAAAAGCTTTCAGTGATAGGGCAGAAAAGCACATTCTGCCCTATCACTGAAAGCG-3'
shRNA2	F: 5'-GATCCGCCTGTCTGACTACAAAGGAAATGTGCTTTTTCCTTTGTAGTCAGACAGGTTTTTTC-3'
	R: 5'-AATTGAAAAAACCTGTCTGACTACAAAGGAAAAAGCACATTTCCTTTGTAGTCAGACAGGCG-3'
shRNA3	F: 5'-GATCCGAGATATCAGCCTGTCTGACTGTGCTTGTCAGACAGGCTGATATCTTTTTTTC-3'
	R: 5'-AATTGAAAAAAAGATATCAGCCTGTCTGACAAGCACAGTCAGACAGGCTGATATCTCG-3'
PRDX-1	F: 5'-AGTGAATTCATGTCTTCAGGAAATGCTAAAA-3'
	R: 5'-ATTAGCGGCCGCTCACTTCTGCTTGGAGAAATAT-3'

Table 2. The Primer sequences used for RT-PCR analysis

Genes	Primer sequences
PRDX-1	F: 5'-GCACCATTGCTCAGGATTATG-3'
	R: 5'-GCCAACAGGGAGGTCATTTAC-3'
GAPDH	F: 5'-GGTGTGAACCATGAGAAGTATGA-3'
	R: 5'-GAGTCCTTCCACGATACCAAG-3'

Materials and methods

Glioma specimens

This study was conducted complying with the principles of Helsinki Declaration. All human glioma specimens used in the experiments were approved by the ethics committees of the Third Military Medical University (TMMU). Human glioma samples were obtained from 156 patients who underwent surgery from January 2010 to December 2011. Pathological diagnosis was confirmed by 2 pathologists independently. All of the patients or their guardians provided written informed consent for the biological studies.

Cell lines

U87MG cells were kindly provided by Dr. Xiuwu Bian (Institute of Pathology, Southwest Hospital, Third Military Medical University, Chongqing, China). The cells were cultured in DMEM supplemented with 10% fetal calf serum (GIBCO™), 4 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 20 mM HEPES buffer. The cells were grown in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air.

Interference vector of PRDX-1

Three pairs of shRNA were designed base on PRDX-1 coding sequence (NM_002574.3) in Genebank (**Table 1**). The synthesis fragment double-stranded DNA was cloned into pHBLV-U6-ZsGreen-Puro, using a pair of BamHI and EcoRI linkers. Then the interference vector pHBLV-U6-ZsGreen-Puro-PRDX-1 and blank control vector pHBLV-U6-ZsGreen-Puro were transformed into DH5α E. coli cells. 150 μl bacterium solution (DH5α) was moved to agar culture-medium containing 100 mg/ml AMP and cultured at 37°C for 14 h. The positive clone was verified by sequencing in SangNi Technology Company, Shanghai.

Over-expression vector of PRDX-1

The amplification primers were designed base on PRDX-1 coding sequence (NM_002574.3) in Genebank (**Table 1**). The full length PRDX-1 digested by EcoRI and NotI was cloned into pHBLV-IRES-ZsGreen-PGK-puro. Then the over-expression vector and control vector were transformed into DH5α E. coli cells. 150 μl bacterium solution (DH5α) was moved to agar culture-medium containing 100 mg/ml AMP and cultured at 37°C for 14 h. The positive clone was verified by sequencing in SangNi Technology Company, Shanghai.

Packaging and infection of lentiviral vector

Interference or over-expression vector in lentiviral packaging system and assistant plasmids (pSPAX2, pMD2G3) were extracted by plasmid extraction kit (QIAGEN, USA). 293T cells were cultured at a density of 4×10^6 cells per well. PRDX-1 interference or over-expression vector were transfected into 293T cells with Lipofectamine kit when the cells reached 80% confluence. Liquid supernatant was collected in 48 h or 72 h, filtrated with 0.45 μm percolator and centrifuged in 72000 g/min at 4°C for 120 min. The sediment was re-suspended with 400 μl DMEM culture medium and infected to

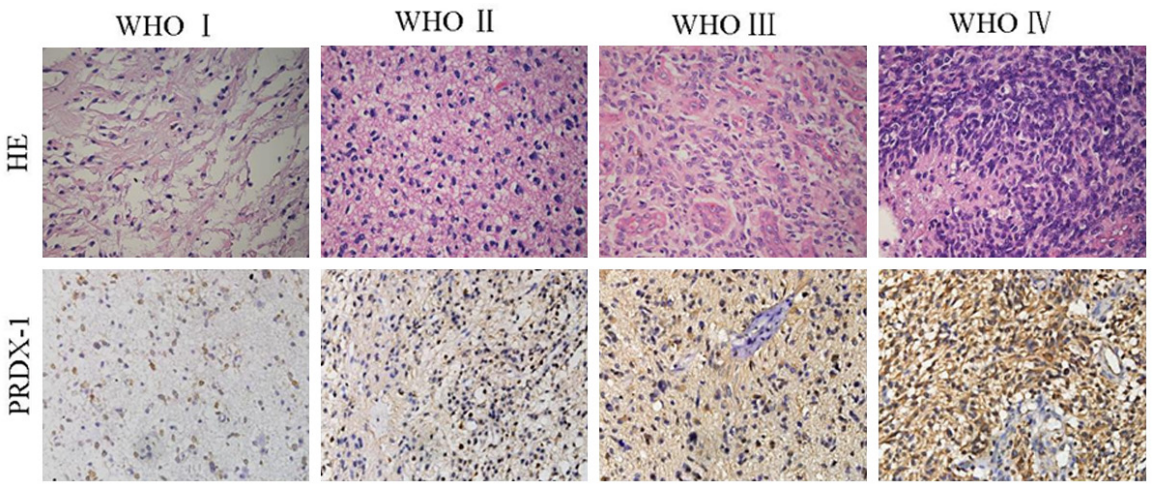

Figure 1. The expression of PRDX-1 in human glioma tissues. A. Representative images of H&E staining of gliomas with different WHO grade. B. Representative images of PRDX-1 immumohistochemical staining in glioma specimens.

U87MG cell lines (MOI=20) after counting. Forty-eight hours later, 2 μg/ml Puromycin was administered to select infected cells.

Real-time quantitative PCR (RT-qPCR)

The primers were designed based on the mRNA sequences of PRDX-1 and GAPDH in Genbank and synthesized in Shanghai Sangon Biotech (**Table 2**). Total RNA was extracted from U87MG cells with TRIZOL™ reagent (Beyotime, China) and was reverse-transcribed into cDNA with M-MLV reverse transcriptase according to the manufacturer's instructions. PRDX-1 expressions in interference/expression and control groups were detected by RT-PCR. All experiments were repeated in triplicate.

Western blot

Western blot was conducted as previously described [5]. The primary antibodies were as follows: anti-PRDX-1 (Abcam, USA), anti-TNF-α (Abcam, USA), anti-NF-κB (Abcam, USA) and anti-GAPDH (ComWin Biotech, China). All experiments were repeated in triplicate.

Proliferation and colony formation assay

U87MG cells at a density of 2000 cells per well were cultured in 96-well plates at 37°C in an atmosphere of 5% CO_2 and 95% air. The absorbance value in 450 nm wave length of each sample were detected in 1 d, 2 d, 3 d, 4 d and 5 d after 100 μl medium and 10 μl CCK-8 were added into each well. The cell growth curve was drawn with absorbance value as ordinate and time as abscissa. For colony formation assay, U87MG cells at a density of 500 cells per well were cultured in 96-well plates. The cells were fixed by paraformaldehyde and underwent Giemsa staining in 14 d. The colony formation efficiency was counted. All experiments were repeated in triplicate.

Apoptosis assay

U87MG cells transfected with pHBLV-PRDX-1 or pHBLV-PRDX-1-shRNA vectors were used to analyze cell apoptosis. 5 μl Annexin V-PE and 5 μl 7-AAD (BD, USA) were added into cell suspension containing 1×10^5 cells. The mixture was incubated in lucifuge at 37°C for 15 min and added with 400 μl 1×Binding Buffer. Then flow cytometry was applied to detect cell apoptosis. All experiments were repeated in triplicate.

Immunohistochemistry

The formalin-fixed, paraffin-embedded human glioma samples were used for immunohistochemistry. The primary antibodies were as follow: anti-PRDX-1 (Abcam, USA), anti-TNF-α (Santa Cruz, USA) and anti-NF-κB (Abcam, USA). All above proteins were detected by streptavidin-perosidase with colorectal carcinoma tissue sections as positive control. Isotype controls were used for the three primary antibodies as negative control. The immunohistochemical staining results were quantized in 12 immuno-

Table 3. The expression of PRDX-1 in human glioma

Parameter	n	Low expression	High Expression	Chi-square value	*P* value
Gender					
Male	89	26 (29.2%)	63 (70.8%)	0.104	0.858
Female	67	18 (26.9%)	49 (73.1%)		
Age (years)					
≥ 39.9	74	23 (31.1%)	51 (68.9%)	0.575	0.480
< 39.9	82	21 (25.6%)	61 (74.4%)		
WHO Grade					
I&II	92	36 (39.1%)	56 (60.9%)	13.218	0.000
III&IV	64	8 (12.5%)	56 (87.5%)		
Ki-67 Index					0.000
≥ 5%	109	16 (14.7%)	93 (85.3%)	32.688	
< 5%	47	28 (59.6%)	19 (40.4%)		
Predominan side					
L	84	26 (31.0%)	58 (69.0%)	0.678	0.477
R	72	18 (25.0%)	54 (75.0%)		
Predominant lobe					
Frontal	52	14 (26.9%)	38 (73.1%)	0.063	0.852
Other	104	30 (28.8%)	74 (71.2%)		

reactive score method, and the samples with scores lower than 4 were determined as low expression.

Statistical analysis

A statistical package SPSS19.0 was used for all analysis. $P < 0.05$ was considered as statistically significant. The Pearson χ^2 test was used to analyze the relationship between PRDX-1 expression and clinicopathological parameters. The unpaired two-group comparison was performed using Student's t-test. Multiple comparisons were made using one-way ANOVA.

Results

PRDX-1 expression is positively correlated with tumor grade in glioma tissues

In glioma tissues, PRDX-1 is mainly expressed in cytoplasm and nucleus of tumor cells. PRDX-1 expression was higher in high-grade gliomas than in low-grade ones (**Figure 1**). The levels of PRDX-1 expression were positively correlated with tumor grade and Ki67 index. However, there is no significant correlation between PRDX-1 expression and other clinicpathologic parameters such as age, gender, pathogenic site and pathogenic lobe (**Table 3**). These data indicated that PRDX-1 might play a part in glioma growth.

PRDX-1 promotes cell proliferation

We next investigated the effect of PRDX-1 on cell proliferation in vitro by PRDX-1 knockdown or overexpression. As expected, the expression level of PRDX-1 protein (**Figure 2A** upper) and mRNA (**Figure 2A** lower) increased obviously when U87MG cells was transfected with pHBLV-PRDX-1 vector ($P < 0.05$). In contrast, the expression of PRDX-1 protein (**Figure 2B** upper) and mRNA (**Figure 2B** lower) decreased when pHBLV-PRDX-1-shRNA vectors were transfected into U87MG cells ($P < 0.05$). The colony forming ability of U87MG cells was promoted by PRDX-1 overexpression as compared with control group and mock group (**Figure 2C** and **2D**). In contrast, the colony forming ability of U87MG cells whose PRDX-1 had been suppressed was significantly lower than control group and mock group (**Figure 2E** and **2F**). Furthermore, PRDX-1 overexpression promoted cell proliferation (**Figure 2G**), while PRDX-1 suppression inhibited cell proliferation significantly (**Figure 2H**).

PRDX-1 inhibits glioma cell apoptosis

We also analyzed the effect of PRDX-1 on cell apoptosis by flow cytometry. When PRDX-1 was over-expressed, cell apoptosis level was significantly lower than control group and mock group (**Figure 3A** and **3C**). As for U87MG cells whose PRDX-1 was suppressed, cell apoptosis was significantly higher than control group and mock group (**Figure 3B** and **3C**). These results suggested that PRDX-1 could block cell apoptosis of gliomas.

PRDX-1 induces the expression of TNF-α and NF-κB in gliomas

In PRDX-1 overexpression U87MG cells, the expression of TNF-α and NF-κB increased significantly compared with mock group and control group (**Figure 4A**). When PRDX-1 expression was inhibited, TNF-α and NF-κB decreased

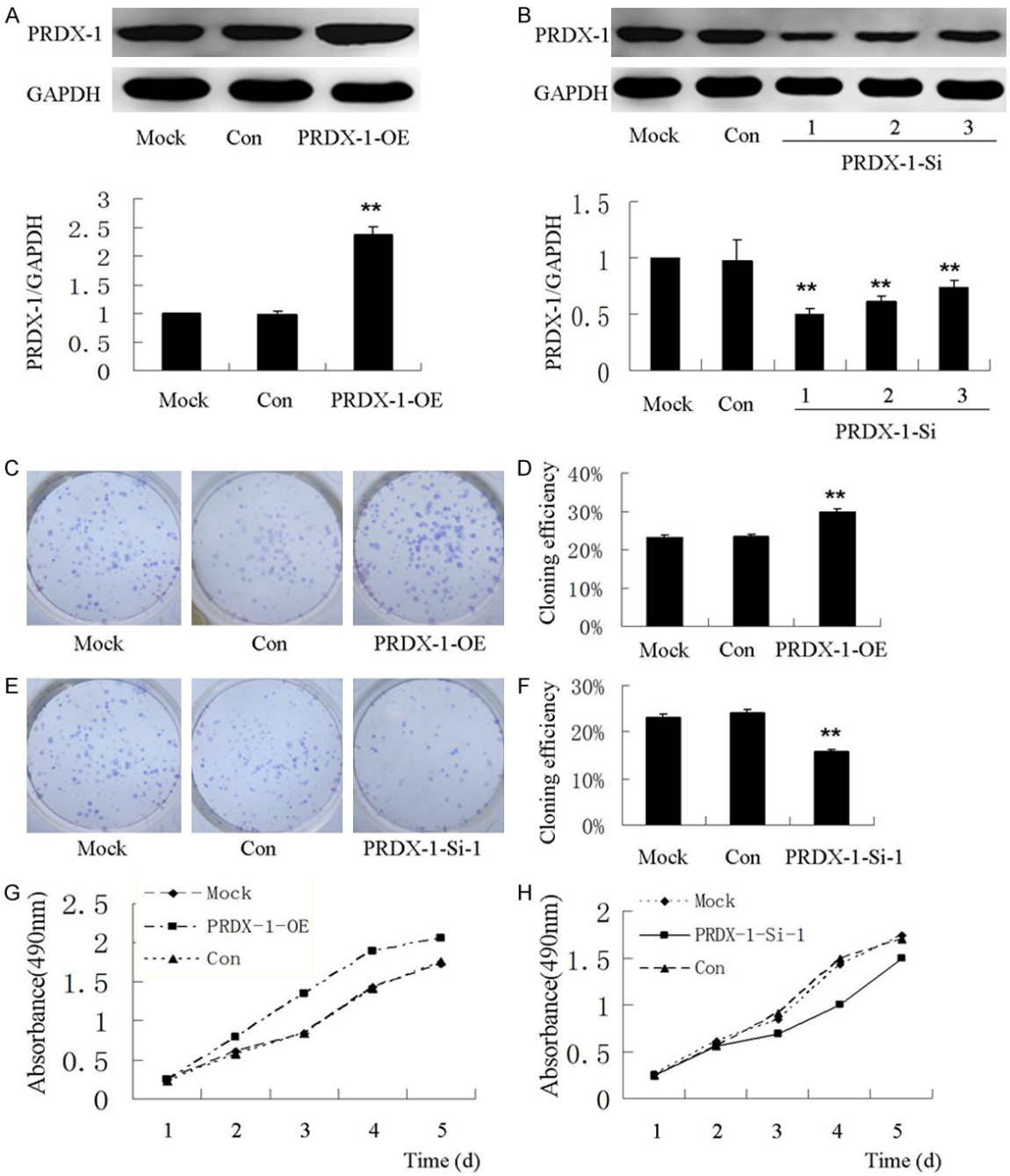

Figure 2. PRDX-1 promoted cell proliferation in vitro. A. PRDX-1 protein (upper) and mRNA (lower) expression of mock, control and PRDX-1 overexpression cells. B. PRDX-1 protein (upper) and mRNA (lower) expression of mock, control and PRDX-1 shRNA cells. C. Representative images of cloning formation assay of mock, control and PRDX-1 overexpression cells. D. Quantitative data of cloning formation assay of mock, control and PRDX-1 overexpression cells. E. Representative images of cloning formation assay of mock, control and PRDX-1 shRNA cells. F. Quantitative data of cloning formation assay of mock, control and PRDX-1 shRNA cells. G. Cell growth curve of mock, control and PRDX-1 shRNA cells. H. Cell growth curve of mock, control and PRDX-1 shRNA cells. **indicating $P < 0.01$ comparing with mock group.

(**Figure 4B**). We further detected the expression of TNF-α and NF-κB in human glioma specimens. As expected, the expression of TNF-α and NF-κB is higher in high-grade gliomas than low-grade ones (**Figure 5A**). Pearson correlation analysis showed that there is a positive

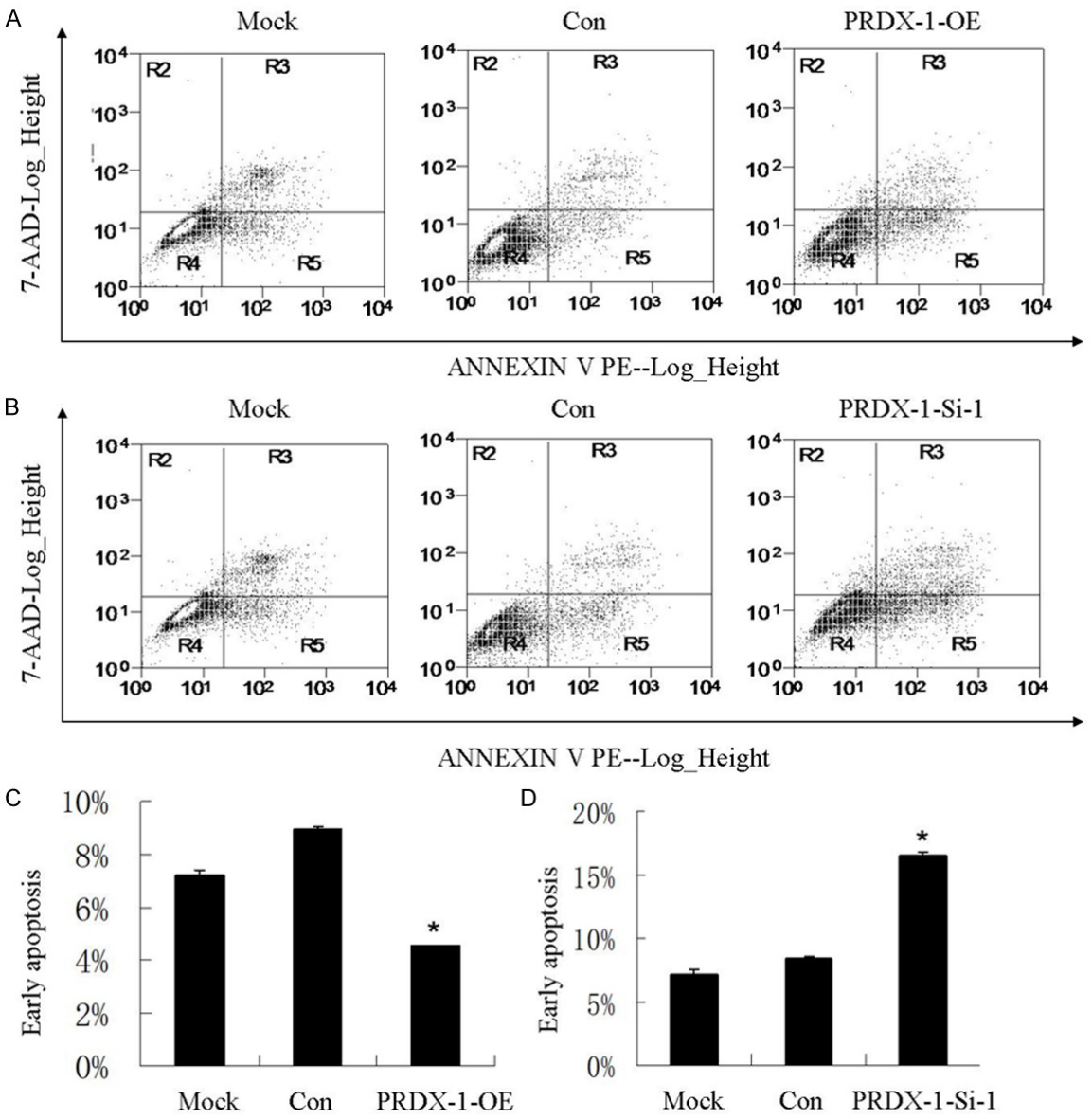

Figure 3. PRDX-1 inhibited cell apoptosis in vitro. A. Cell apoptosis of mock, control and PRDX-1 overexpression cells measured by flow cytometry. B. Cell apoptosis of mock, control and PRDX-1 overexpression cells measured by flow cytometry. C. Quantitative data of early apoptosis of mock, control and PRDX-1 overexpression cells. D. Quantitative data of early apoptosis of mock, control and PRDX-1 shRNA cells. *indicating $P < 0.05$ comparing with mock group.

correlation between PRDX-1 and TNF-α (**Figure 5B**). Furthermore, TNF-α expression was also positively related to NF-κB expression. These results indicated that PRDX-1 might promote tumor growth through inducing the downstream molecules, such as TNF-α and NF-κB in glioma cells.

Discussion

PRDX-1 is a member of 2-Cys PRDXs subclass which belongs to the peroxiredoxins protein family and locates in 1p34.1. It plays an important role in the elimination of ROS including H_2O_2, O_2- and ·OH-. Furthermore, PRDX-1 is reported to be involved in apoptosis and signal transduction [6, 7, 16]. In our previous study, we found that PRDX-1 expression decreased when cell proliferation was suppressed in U87MG [5]. It indicated that PRDX-1 might play a central role in cell proliferation and malignant progression of gliomas. In this study, we found that PRDX-1 expression was positively correlat-

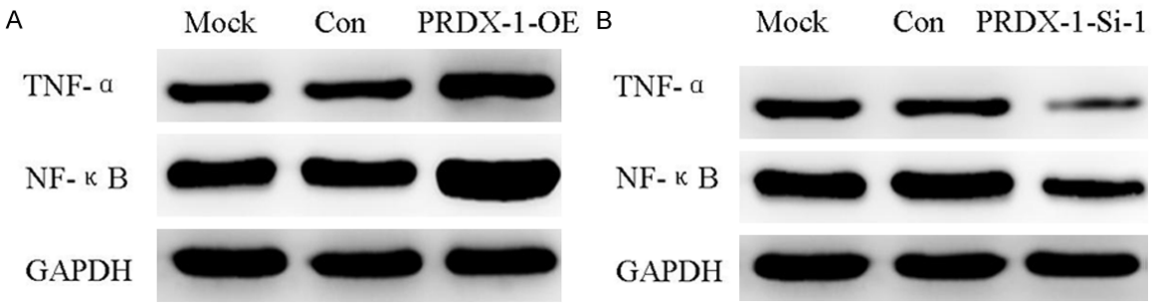

Figure 4. PRDX-1 induced the expression of TNF-α and NF-κB. A. The expression of TNF-α and NF-κB in mock, control and PRDX-1 overexpression cells. B. The expression of TNF-α and NF-κB in mock, control and PRDX-1 shRNA cells.

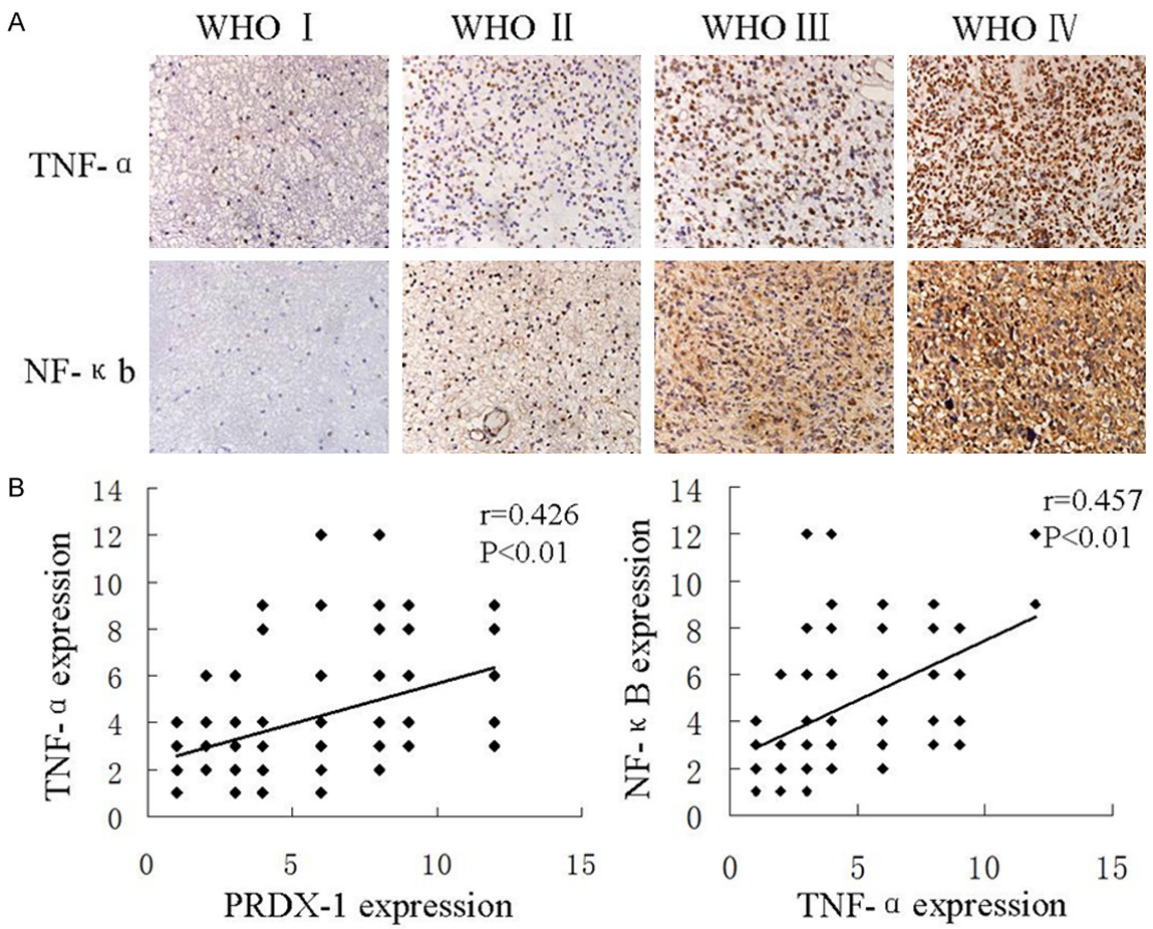

Figure 5. The expression of TNF-α and NF-κB in human glioma tissues. A. Representative images of TNF-α and NF-κB immumohistochemical staining in glioma specimens. B. Correlation of PRDX-1 and TNF-α in glioma specimens. B. Correlation of TNF-α and NF-κB in glioma specimens.

ed with Ki67 index and tumor grade in gliomas. When PRDX-1 was knocked down, cell proliferation was suppressed and cell apoptosis increased. In contrast, PRDX-1 overexpression promoted cell proliferation and inhibited cell apoptosis of glioma cells. In addition, PRDX-1 induced the expression of TNF-α and NF-κB in vitro and was positively correlated with their expression in human glioma specimens. Thus, we proposed that PRDX-1 promotes cell proliferation and tumor malignant progression via TNF-α/NF-κB pathway.

NF-κB regulates intracellular inflammatory mediators and cytokines in transcriptional level, which are involved in intracellular signal transduction, inflammatory response regulation and tumor genesis [17-20]. Excited IKK protein with kinase function increases and promotes serine residues of I-κB phosphorylation and degradation. When I-κB is inactivation, a large number of excited NF-κB enters into endonuclear because of dissociation of NF-κB/I-κB [21, 22]. Intracellular excited NF-κB regulates cell cycle, resists apoptosis, promotes invasiveness and angiogenesis in glioma [23, 24]. Our results indicated that the malignant progression of glioma is partly promoted by PRDX-1 via TNF-α/NF-κB pathway.

In recent years, PRDX-1 was reported to have closely relationship with multiple malignancies [8-14]. PRDX-1 expression was up-regulated in colorectal adenocarcinoma and PRDX-1 suppression inhibited tumor growth. This is consistent with our finding in gliomas. Thus, PRDX-1 might play a central role in tumor development and progression. Yanagawa et al. reported that PRDX-1 could promote tumor recurrence. Thus PRDX-1 may be a novel target for cancer screening, diagnosis and molecular targeting treatment.

At present, the prognosis of patients with malignant glioma was very poor. The average 5-year survival rate of glioblastoma (WHO IV) patients is less than 10% [25]. In this study, we found the relationship of PRDX-1 with glioma malignancy progression and illuminate the molecular mechanism. It may be helpful for induced differentiation therapy and predicting prognosis of patients with gliomas.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jian-Ping Xu, Department of Pathology, Xinqiao Hospital, Third Military Medical University, 183 Xinqiao Avenue, Shapingba, Chongqing, China. Tel: +86-13650554-256; Fax: +86-02368755306; E-mail: xujianping33@sina.com

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