

## Original Article

# Expression of PCAF in brain glioma and its molecular mechanism

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Received December 8, 2015; Accepted February 17, 2016; Epub March 1, 2016; Published March 15, 2016

**Abstract:** Brain glioma is one common primary intracranial malignant tumor with unfavorable prognosis. P300/CBP-associated factor (PCAF) belongs to acetyl transferase family and can catalyze acetylation of histones. Recent study has revealed the close correlation between PCAF and malignant tumors. This study thus aimed to investigate the expression and molecular biology implication of PCAF in brain glioma cells. A total of 50 brain glioma patients were recruited, along with 15 cases of normal brain tissues removed from internal decompression surgery after severe brain trauma as the control group. RT-PCR and immunohistochemistry (IHC) were employed to detect the expression of PCAF in glioma and normal brain tissues. RNA-interference combining with Western blotting was used to analyze the effect of PCAF on PTEN and p-Akt protein expression in brain glioma tissues. RT-PCR and IHC staining showed significantly elevated expression of PCAF in brain glioma tissues regarding mRNA or protein level ( $P < 0.05$ ). By RNA interference we found significant depression of p-Akt level and elevation of PTEN level inside cells with PCAF silencing ( $P < 0.05$ ). PCAF may influence the occurrence and progression of brain glioma via affecting PTEN and p-Akt expression.

**Keywords:** Brain glioma, PCAF, PTEN, p-Akt

## Introduction

As the most common primary malignant intracranial tumor, brain glioma has advanced malignancy, rapid growth and high heterogeneity. Glioma in human brain tissues displayed infiltrated diffusion growth pattern, causing difficulty in treatment, high recurrence rate and shorter survival span of patients [1]. Like other malignant tumors, brain glioma has unclear pathogenesis mechanism so far, although it is commonly believed that glioma was related with both susceptible genetic factors and environmental carcinogenic factors [2]. Currently multiple approaches including surgery, radio-/chemo-therapy and immune treatment have been applied and gained major progression in improving efficacy, but still cannot completely cure brain glioma [3, 4]. Therefore, the study of pathogenesis mechanism and biological activity of brain glioma is of critical importance for improving treatment efficacy and extending survival time.

P300/CBP-associated factor (PCAF) belongs to acetyl choline transferase family, and is also

named as lysine acetyltransferase 2B (KAT2B). PCAF can catalyze the acetylation of histone, thus acting as one important factor of epigenetics control for modulating biological behavior of cells [5]. Study has shown that PCAF could interact with P300/CBP or other sequence-specific transcription factors via auto-acetylation activity, to regulate transcriptional level of cells [6]. By molecular biology study of malignant tumors, PCAF expression level has been found to be closely related with the occurrence of various cancers including esophageal carcinoma, breast cancer and ovarian cancer [7, 8]. Other study also revealed the correlation between PCAF and brain glioma, but lacked intensive illustrations [9]. We thus studied the expression level of PCAF in brain glioma and possible molecular mechanisms.

## Materials and methods

### Research objects

A total of 50 brain glioma patients (30 males and 20 females, all were primary cases, aging between 28 and 64 years old, average age =

## PCAF expression in glioma

**Table 1.** Primer sequence for RT-PCR

Target gene		Primer sequence	Expected length
β-Tubulin	F	5'-TGTCCCGATGGCGAGTGTTT-3'	454 bp
	R	5'-CCTGTTGGCCATAGTACTGC-3'	
PCAF	F	5'-AGGCACCATCTCAACGAAGA-3'	166 bp
	R	5'-AAGACCGAGCGAAGCAATGT-3'	

**Table 2.** Nucleotide sequence for cell transfection

Name	Sequence
Anti-PCAF	5' AmCmCmUmGmUmGmUmGmUmGmAmGmGmAmAmUmU 3'
Scramble	5' AmUmAmCmCmUmGmUmGmAmGmUmGmAmUmUmGmGmA 3'

39.1±6.5 years) who were admitted in the department of neurosurgery in The First Affiliated Hospital of Jilin University from January 2012 to March 2015 were recruited in this study, along with 15 brain tissue samples (8 males and 7 females, aging between 31 and 67 years old, average age = 42.3±8.2 years) removed from internal decompression surgery after severe head trauma as the control group. All glioma patients had not received any radio-, chemo- or immune-therapy before admitting. The tumor lesion was confirmed by MRI examination. Based on brain glioma grade standard of WHO, there were 9, 11, 13 and 17 cases at grade I, grade II, grade III and grade IV. No statistically significance has been found regarding age or sex between control and experimental group. This study has been pre-approved by the ethical committee of The First Affiliated Hospital of Jilin University and has obtained written consents from all participants.

Brain tissues removed from the surgery were kept in liquid nitrogen. Some tissues were fixed in 10% formalin and were embedded in paraffin. Four consecutive slices were selected in each sample (5 μm thickness) for further histology examination and immunohistochemistry (IHC) staining.

### IHC staining

The expression of PCAF in brain glioma and normal tissues was determined by IHC staining. In brief, paraffin-based tissues sections were dewaxed in xylene, and were hydrated in gradient ethanol and distilled water. Endogenous peroxidase activity was quenched by 3% H<sub>2</sub>O<sub>2</sub>. After rinsing in distilled water, 5% BSA was used to block tissues under room temperature. PCAF-specific antibody solution was replenished

for 2-hour incubation at 37°C. Biotin-labelled secondary antibody was added for another 30-min incubation. Streptavidin with horseradish peroxidase labels was then added to incubate for 30 min. PBS rinsing was then performed to wash unreacted reagent. DAB substrate was used for 10-min staining, which was stopped by distilled water. Hematoxylin was

used to counter-stain sections for 2 min, followed by water rinsing, dehydration, and mounting. Under high-power microscope (400×), five tumor fields were randomly selected to count total cell number and positive cell number [10].

### Results deduction

PCAF was mainly expressed in cytoplasm. In IHC staining against PCAF, brown-yellow granules were deduced as positive staining. If more than 40% of total cells were positive for PCAF, the section was deduced as PCAF-positive [10].

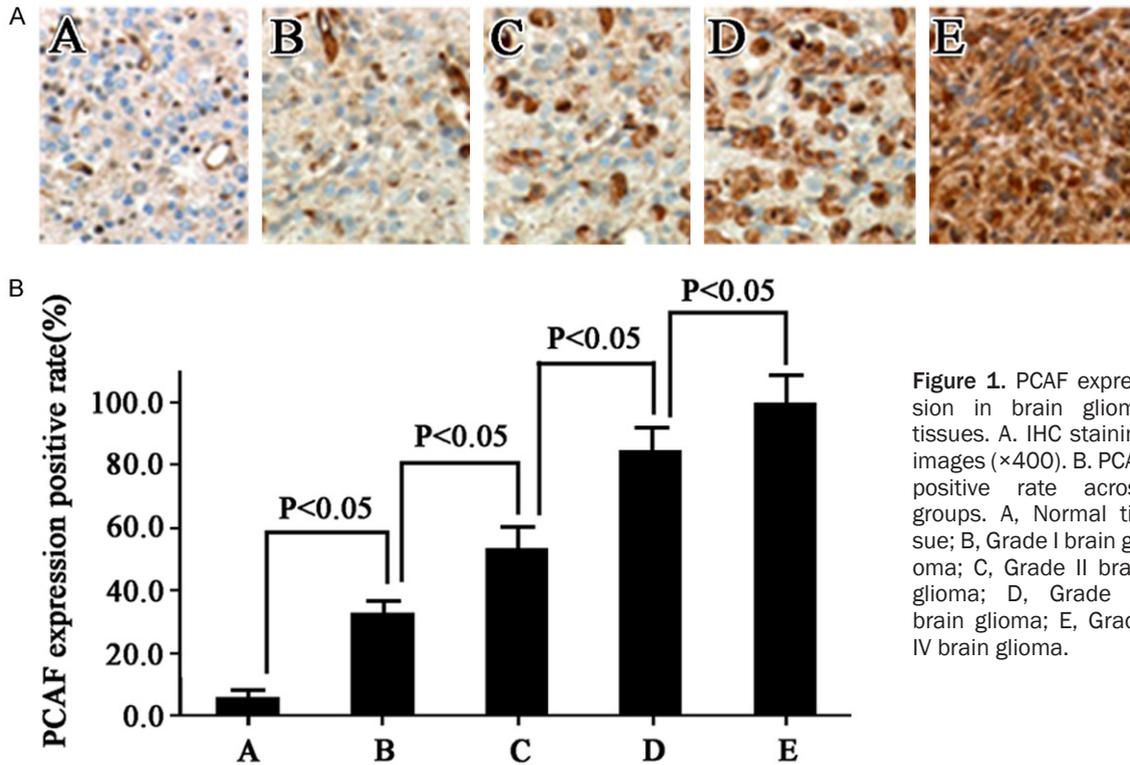
### RT-PCR

Based on mRNA sequence of PCAF (Genebank access No., NM\_003884), primers were designed for RT-PCR amplification (Table 1) using β-tubulin as the internal reference to quantify relative expression level of PCAF in all tissues. 20 mg fresh tissues were extracted for total RNA using quick RNA extraction kit (Aide Biomed., China). RT-PCR test kit (TianGen, China) was employed for amplification using normal brain tissues as the control. Reverse-transcription was firstly performed at 37°C for 2 hour. RT-PCR was then performed using cDNA as the template under the following conditions: 95°C denature for 5 min, followed by 30 cycles each containing 95°C denature for 1 min, 58°C annealing for 30 sec and 72°C elongation for 30 sec. PCR products were tested using 1% agarose gel electrophoresis. Gel imaging analyze was employed to determine the relative expression level of PCAF [11].

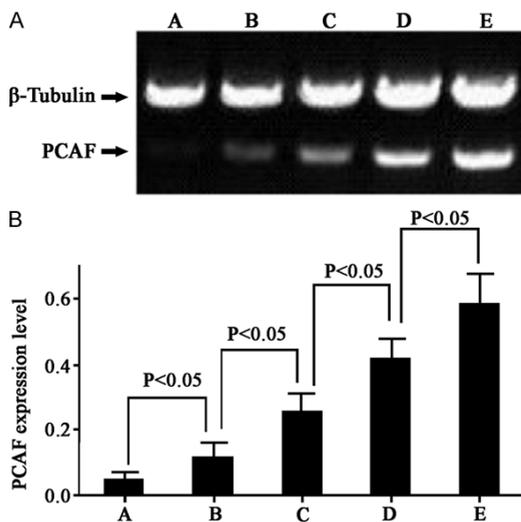
### RNA interference (RNAi)

Based on mRNA sequence of PCAF (Genebank access No., NM\_003884), RNA interference

## PCAF expression in glioma



**Figure 1.** PCAF expression in brain glioma tissues. A. IHC staining images ( $\times 400$ ). B. PCAF positive rate across groups. A, Normal tissue; B, Grade I brain glioma; C, Grade II brain glioma; D, Grade III brain glioma; E, Grade IV brain glioma.



**Figure 2.** Relative expression of PCAF. A. Gel electrophoresis bands of RT-PCR specific for PCAF. B. Relative expression level of PCAF. A, Normal tissue; B, Grade I brain glioma; C, Grade II brain glioma; D, Grade III brain glioma; E, Grade IV brain glioma.

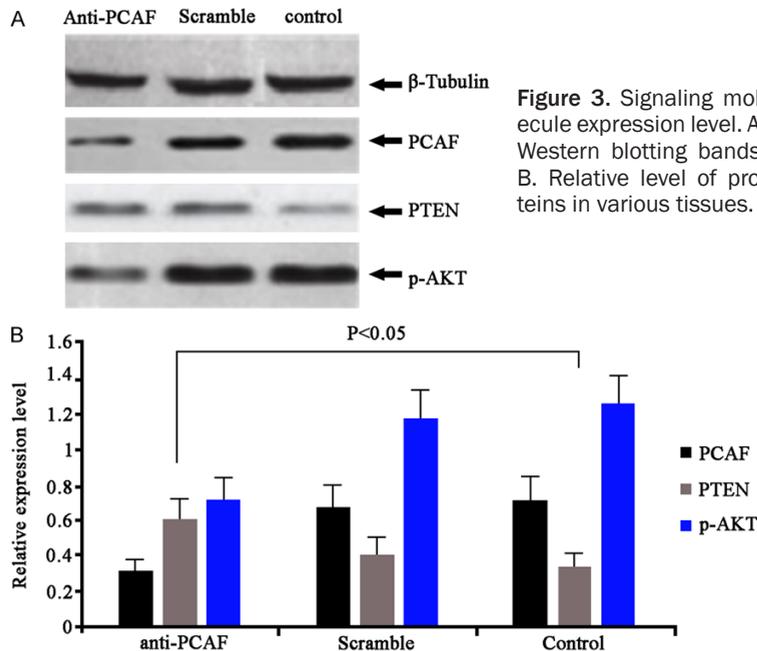
sequence was designed and was synthesized to obtain anti-PCAF sequence with controlled scramble sequence. All base pairs were modified by methylation and were synthesized by Sangon (China). Sequences were shown in

**Table 2.** Liposome INTERFERin transfection kit (Polyplus transfection, US) was used to transfect cells. Brain glioma cell line U251 was purchased from Cell Bank, Chinese Academy of Sciences. Cryopreserved cells were resuscitated until reaching log phase, and were digested in trypsin. After counting, cells were diluted in freshly prepared medium and were inoculated in 96-well plate. After 24-hour incubation, transfection was performed following manual instruction [10].

### Western blotting

U251 cells at late log-phase were mixed with 0.1 mL lysis buffer and were treated in homogenizer until complete resolving. After 13000 g centrifugation for 10 min, supernatants were extracted for protein electrophoresis and Western blotting. SDS-PAGE was employed to separate proteins using 15% separation gel plus 5% stacking gel. After electrophoresis, proteins were transferred to PVDF membrane, which was blocked in 5% defatted milk powder for 1 hour. Primary antibody (1:2000) including mouse anti-human PCAF, PTEN, pAkt and  $\beta$ -tubulin was added for overnight incubation at 4°C. TBST was used to rinse sections, followed by incubation in goat anti-mouse secondary

## PCAF expression in glioma



**Figure 3.** Signaling molecule expression level. A. Western blotting bands. B. Relative level of proteins in various tissues.

antibody (1:1 000) for 1 hour. Freshly made DAB substrate was used to develop the membrane for 10 min, followed by quenching in distilled water [12]. Blotting bands were captured and analyzed for integrated density value of each band for calculating relative expression level of PCAF using  $\beta$ -tubulin as the internal reference.

### Results

#### PCAF expression by IHC staining

IHC staining was performed in normal brain tissues and glioma tissues to analyze the expression of PCAF. When cell was positive for PCAF, brown-yellow granules may occur in cytoplasm (Figure 1A). As shown in our results, normal tissues had relatively lower staining intensity compared to brain glioma tissues, thus indicating elevation of PCAF in brain glioma tissues.

By random selection of five tumor fields with evenly distributed staining, total number of cells and positive cells were counted in Figure 1B. In normal brain tissues, positive rate of PCAF expression was only 6.7% (1/15), while such rate was elevated to 72% in glioma tissues. Regarding with histology stage, the positive rates for PCAF were 33.3% (3/9), 54.5% (6/11), 84.6% (11/13) and 100% (17/17) for stage I, II, III and IV tumors. These results sug-

gested elevation of PCAF in brain glioma cells. With advancement of tissue grade, the positive rate of PCAF was significantly elevated.

#### RT-PCR

To further investigate the mRNA level of PCAF in brain glioma tissues, we designed PCAF-specific primers in RT-PCR assay, whose products were analyzed by gel electrophoresis (Figure 2A). Results showed lowered PCAF mRNA level in normal brain tissues compared to glioma tissues. Using  $\beta$ -Tubulin as the internal reference, gel imaging system was employed to analyze relative level of mRNA by software (Figure 2B). With advanced

grade of tumors, the expression level of PCAF mRNA was gradually increased ( $P < 0.05$ ) as consistent with IHC results.

#### RNA interference

To further explore possible molecular mechanism of PCAF during pathogenesis and progression of brain glioma, we utilized RNA interference approach to knock-down the expression of PCAF in U251 brain glioma cells, followed by the quantification of critical proteins in PTEN/PI3K/Akt signaling pathway by Western blotting (Figure 3A). Using  $\beta$ -Tubulin as the internal reference, relative expression level of various proteins were normalized as shown in Figure 3B. After transfecting anti-PCAF oligonucleotide, PCAF expression level was significantly down-regulated compared to blank or negative control groups ( $P < 0.05$ ), suggesting the successful interfering of RNA. We also found significantly depressed p-Akt level and elevated PTEN level in those cells with PCAF-silencing ( $P < 0.05$ ).

#### Discussion

By IHC staining and RT-PCR, PCAF expression level in brain glioma tissues was significantly higher than that in normal brain tissues. With advanced grade of tumor, the expression of PCAF was gradually increased, thus indicating the possible correlation between PCAF over-

## PCAF expression in glioma

expression and brain glioma. To further illustrate the molecular mechanism underlying the effect of PCAF on brain glioma, the expression of PCAF, PTEN and p-Akt, all of which were critical molecules in biological activity, was studied in combined with RNA interference. Our results found significantly depressed p-Akt level ( $P < 0.05$ ) inside cells with lowering expression of PCAF in brain glioma cell U251, along with hither PTEN expression ( $P < 0.05$ ). In summary, we hypothesized that PCAF expression was closely correlated with brain glioma, and may affect tumor growth via modulating PTEN and p-Akt.

As one acetyl transferase, PCAF can catalyze acetylation of histones, for further modulating biological behavior of cells [13]. Study has shown the interaction between PCAF and various target proteins including Myc,  $\beta$ -catenin, HOXA10 and histones via auto-acetylation. Meanwhile, PCAF was found to be up-regulated in liver cancer and brain glioma cells, suggesting the enhancement of tumor proliferation [14]. Other study attributed the up-regulation of PCAF to intracellular miR expression level. Both DNA array and RT-PCR methods all found the down-regulation of miR-181 in brain glioma tissues [15, 16]. Other study up-regulated miR-181 expression level by cell transfection, and observed PCAF level in both mRNA and protein levels. Results showed no significant change of PCAF mRNA level when miR-181 was up-regulated. PCAF protein, however, was down-regulated, suggesting that miR-181 might suppress PCAF expression at mRNA level [17]. Based on these results, we can propose that brain glioma might elevate cellular PCAF expression level via down-regulating miR-181.

The proliferation of various malignant tumor cells was affected by PTEN/PI3K/Akt signal pathway, in which cellular PI3K can catalyze the phosphorylation of 3,4,5-triphosphate phosphatidylinositol, to further activate Akt by phosphorylation [18]. As one critical signal molecule for regulating cell proliferation and growth, p-Akt can stimulate cell growth and proliferation, in which PTEN can antagonize PI3K activity to maintain p-Akt within normal level [19]. Study has suggested the catalyze of Akt acetylation by PCAF, thus facilitating the phosphorylation of Akt at Thr<sup>308</sup> and Ser<sup>473</sup> sites for activation [20]. Other scholars found the potency of PCAF to catalyze acetylation of amino acid resi-

dues at Lys125 and Lys128, both of which were critical sites for PTEN to suppress its activity, further leading to over-activation of PTEN/PI3K/Akt signaling pathway and malfunction of cell proliferation [21].

Brain glioma is the most aggressive intracranial tumor in adults due to its rapid growth and heterogeneity. Although certain progresses have been obtained in treating against brain glioma, its 5-year survival time was still shorter than 10% [22]. Therefore it is necessary to further explore the cellular and molecular biological mechanism of brain glioma, in order to provide more evidences for novel treatment strategy. Currently little has been known regarding the effect of PCAF on pathogenesis and progression of brain glioma. This study investigated the expression profile of PCAF in brain glioma cells and its relationship with tumor, thus providing evidences for both basic study and clinical treatment of brain glioma.

### Disclosure of conflict of interest

None.

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