

Original Article

High-level of STAT4 protein expression interrelates with the deterioration and proliferation index of glioma: an immunohistochemical study

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Abstract: Objective: Overexpression of signal transducers and activators of transcription 4 (STAT4) has been reported in various types of malignancies. However, its abnormal expression and clinicopathological contribution in glioma remain virtually unknown. The objective of the current study was to explore the clinicopathological significance of STAT4 and its effect on deterioration and proliferation index (PI) in glioma. Materials and methods: A total of 140 glioma patients and 16 cases of normal brain tissues were recruited, and the expression of STAT4 was detected by using immunohistochemical staining. The PI was calculated from Ki-67 staining. Furthermore, the relationship between STAT4 and clinical parameters including PI was analyzed. Results: The expression level of STAT4 in gliomas was significantly higher than that in normal brain tissues ($P = 0.004$). Moreover, a notable correlation was found between STAT4 expression and the tumor pathological grade ($r = 0.240$, $P = 0.003$). There was also a positive correlation between STAT4 and PI ($r = 0.323$, $P < 0.05$). Conclusion: Up-regulation of STAT4 in glioma indicates aggressive tumor behavior and predicts a worse clinical outcome. STAT4 might be a promising novel prognostic biomarker for glioma patients.

Keywords: STAT4, glioma, immunohistochemistry, deterioration, proliferation index

Introduction

Malignant glioma, is the most frequent primary brain tumor in adults, and also one of the most fatal and difficultly treated solid tumors, due to the highly invasive and neurologically destructive capacity [1, 2]. Despite relatively rapid development of multimodal therapeutics for patients afflicted by glioma, the disease prognosis has not been optimistically improved, and the median survival for glioma patients still remains less than 1.5 years [3]. Due to the particularity and complicated pathogenesis in most series of gliomas, the clinical molecular mechanism underlying the oncogene and the progression of glioma still remains poorly clarified. Thus, it is of great value to discover the etiology and to explore effective diagnostic and prognostic markers as well as novel therapeutic strategies for the disease [4].

Signal transducers and activators of transcription (STAT) family regulate the entire hemato-

poietic process and influence interactions between tumor cells and their immune microenvironment through induction or suppression of specific cytokines and growth factors. Signal transducer and activator of transcription 4 (STAT4), locating on chromosome 2q32, is a member of the STAT family of transcription factors. STAT3 and STAT5 have been extensively shown to play crucial roles in tumor cell proliferation [5, 6]. In contrast, studies on STAT4 have been rarely reported. Up to date, there has been no direct evidence on the expression of STAT4 in human glioma tissues, except that a mouse model was applied to study the influence of STAT4 through interleukin 4 channel to depress the function of glioma immune cells [7]. Beside, Ki-67 is a commonly used marker of cell proliferation and has been studied widely in almost all malignancies. Due to the fact that Ki-67 is expressed only by cells actively engaged in mitosis cycle, high level of Ki-67 is considered to have a remarkable correlation of biological aggressiveness. The current study aimed to

detect the expression of STAT4 in different glioma samples by immunohistochemistry and to elucidate its association with the tumor deterioration and proliferative status of glioma patients.

Materials and methods

Tissue samples

A total of 140 cases of FFPE gliomas and 16 normal brain tissues were enrolled in the present study. The age of the glioma patients ranged from 7 to 75 years, with a mean age of 41 years. All samples include 42 female patients and 98 cases of males. Clinicopathological information was provided from medical records. In present study, all cases were classified into four groups for differentiation. Among them, 23 cases were pilocytic astrocytomas (WHO grade I) and 44 cases were grade II, including 21 cases of fibrillary astrocytomas, 16 cases of serous astrocytoma, and 7 cases of oligodendrogliomas. Grade III included 41 cases of anaplastic astrocytomas and 9 cases of anaplastic oligodendrogliomas, while 8 cases of glioblastomas and 15 cases of glioblastomas were contained in the grade IV. The 16 normal tissues were obtained from decompressive resections of traumatic brain injuries. The age of the normal controls ranged from 23 to 59 years, with a mean age of 44 years. All cases were initial tumor ectomies without treatment and randomly selected in the First Affiliated Hospital of Guangxi Medical University, China, between January 2008 and April 2013. The study protocol was approved by the Ethical Committee of the First Affiliated Hospital of Guangxi Medical University. Written informed consent was obtained from the patients and clinicians for the usage of the samples for research. All samples were reviewed and diagnosed by two independent pathologists.

Immunohistochemistry

Sections were deparaffinized in xylene and hydrated through a graded series of ethanol and then rehydrated and subjected to antigen retrieval by microwaving. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 min at room temperature. Antigen retrieval of the sections was achieved in a multifunctional microwave histoprocessor at 100°C by microwave heating of the samples on slides in 0.01 mol/L of pH 6.0 citrate buffer for 24 min: Mouse monoclonal antibody STAT4

(PL68, sc-101160, Santa Cruz Biotechnology Inc., CA, USA, 1:300 dilution). Sections were stained with a secondary horseradish peroxidase-tagged antibody labeled with anti-rabbit polymers (Cat. No. D-3004, Shanghai Long Island Biotec. CO., LTD). Finally, positive staining was visualized with diaminobenzidine and cell nuclei were counterstained with haematoxylin. Immunohistochemistry was performed as previously described with the monoclonal antibodies Ki-67 (Beijing Zhongshan Jinqiao Inc., Beijing, China). The positive signals of STAT4 locate in the cytoplasm. Negative (-), weakly positive (+), moderately positive (++) and strongly positive (+++) were determined according to the immunodetection of stain intensity and amounts of positive cells by two pathologists (YD and GC), who discussed each case until they reached a consensus. All of (+), (++) and (+++) were considered as positive expression [8]. The positive signal of Ki-67 is distributed in the nuclei. The proliferation index (PI) of Ki-67 was calculated with the formula (number of positive cells/total number of the cells × 100%) by counting at least 10 representative visions of high magnification (40 × 40).

Statistical analysis

SPSS20.0 (Munich, Germany) was used for statistical analysis. Mann-Whitney U test and Kruskal-Wallis H test were performed to analyze the relationship between STAT4 expression and clinicopathological parameters. Values were presented as the mean ± standard deviation (SD) for Ki-67 PI. The median of PI was used as cutoff value to divide the patients as high or low expression groups, because the median is not affected by outliers. One-Way Analysis of Variance (ANOVA) test and Student's *t*-test were used to analyze significance between groups. Bivariate correlations between two independent variables were analyzed by calculating the Spearman's correlation coefficients. Moreover, ROC curve was performed to analyze the predictive value of STAT4 at differentiation and proliferation. Statistical significance was determined at a *P* < 0.05 level.

Results

STAT4 expression in glioma normal and tumor tissues

STAT4 was found to be highly expressed in the glioma tissues (56.4%, 79/140), whereas it was less expressed in the normal controls

STAT4 in glioma

Table 1. Relationship between STAT4 and Ki-67 expression and clinicopathological features

Parameters	Total (n)	Expression of STAT4 n (%)		Z	P	Ki-67 relevant expression (2-ΔCq)	t	P
		Negative	Positive			Mean ± SD		
Tissue								
Normal tissue	16	13 (81.2%)	3 (18.8%)	-2.850	0.004	0.6062±0.9518	-11.244	< 0.001
Glioma	140	61 (43.6%)	79 (56.4%)			10.5786±10.1096		
Gender								
Female	42	22 (52.4%)	20 (47.6%)	-1.371	0.170	10.2595±10.3102	-0.244	0.808
Male	98	39 (39.8%)	59 (60.2%)			10.7153±10.0729		
Age (median)								
≤ 42	72	28 (38.9%)	44 (61.1%)	-1.146	0.252	11.1278±10.6194	0.660	0.510
> 42	68	33 (48.5%)	35 (51.5%)			9.9971±9.5846		
Grade								
I	23	14 (60.9%)	9 (39.1%)	13.983 ^a	0.003	2.0000±2.5045	8.642 ^b	< 0.001
II	44	25 (56.8%)	19 (43.2%)			2.0568±1.4609		
III	45	17 (37.8%)	28 (62.2%)			16.6778±8.0801		
IV	28	5 (17.9%)	23 (82.1%)			21.2143±7.1355		

a. Kruskal-Wallis H test was performed. b. One-way analysis of variance (ANOVA) test was used.

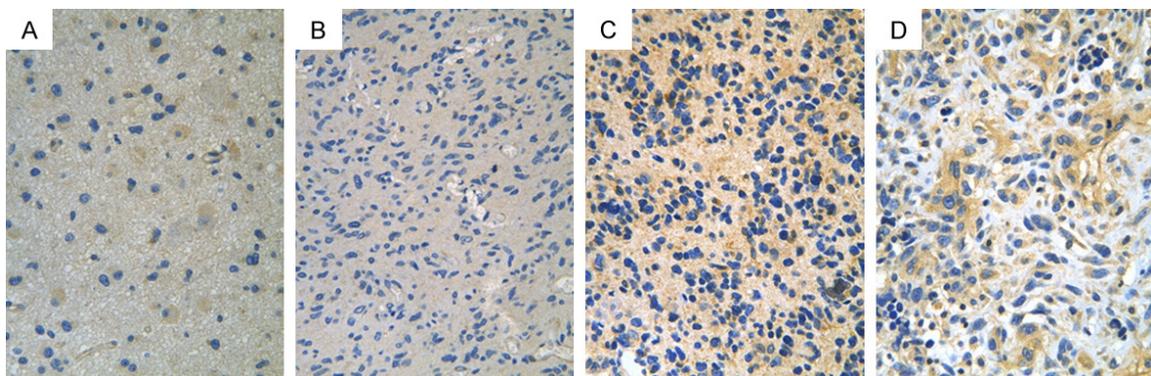


Figure 1. Relationship between STAT4 expression and the grade of glioma. (A) grade I, (B) grade II, (C) grade III, and (D) grade IV (immunohistochemistry × 400).

(18.8%, 3/16, $P = 0.004$, **Table 1; Figures 1, 2A**). In addition, ROC curve was performed to prove the diagnostic value of STAT4 in glioma. The area under curve (AUC) of STAT4 was 0.688 (95% CI: 0.562-0.815, $P = 0.014$). In terms of the differentiation of glioma, the STAT4 expression was associated with histologic grade which in higher grade glioma has a higher positive rate ($t = 13.983$, $P = 0.003$, **Table 1; Figure 2B**). STAT4 expression was weak (39.1%, 9/23) in incipient glioma (grade I). The positive ratio of STAT4 expression was moderate (43.2%, 19/25) in the group of grade II, significantly lower than the cases of grade III (62.2%, 28/45). The strong expression (82.1%, 23/28) was only observed in advanced glioma (grade

IV, **Figure 1**). However, no relative relationship was observed between STAT4 expression and gender or age (both $P > 0.05$).

Ki-67 expression in glioma normal and tumor tissues

In comparison with STAT4 expression of glioma tissue, there were similar expression patterns and trends in Ki-67 expression. The PIs of Ki-67 was significantly higher in glioma tissues as compared to the normal brain tissues ($P < 0.001$, **Table 1; Figure 3**). Furthermore, ROC curve were used to prove the diagnostic value of PI in glioma. The area under curve (AUC) of PI was 0.895 (95% CI: 0.826-0.964, $P < 0.001$,

STAT4 in glioma

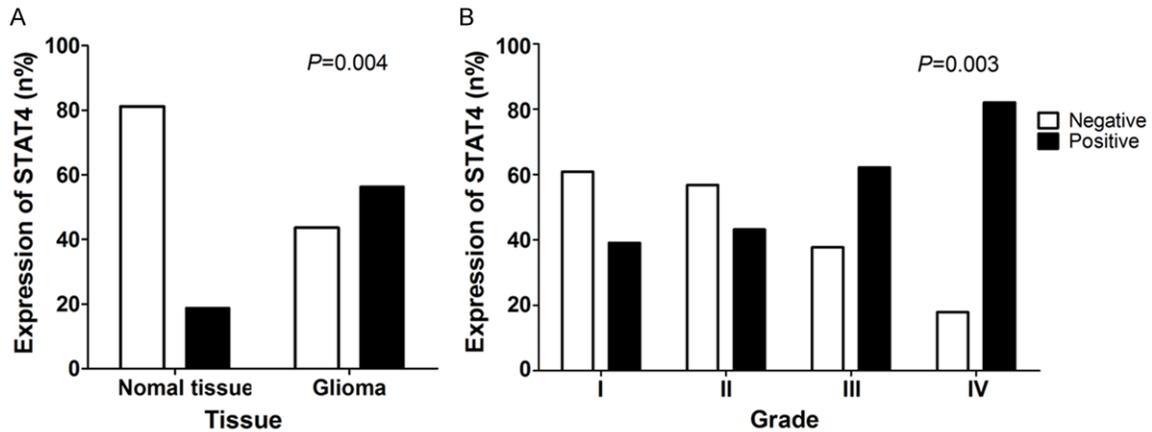


Figure 2. Expression of STAT4 in normal tissue and glioma. A. Expression of STAT4 in normal tissue and glioma; B. Relationship between STAT4 expression and the grade of glioma.

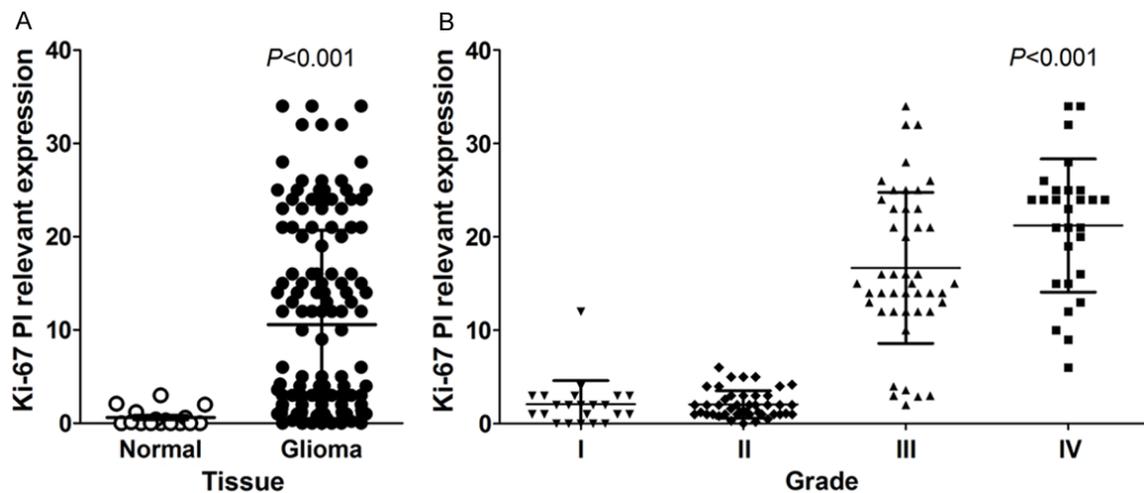


Figure 3. Ki-67 PI in normal tissue and glioma. A. Ki-67 PI in normal tissue and glioma; B. Relationship between Ki-67 PI and the grade of glioma.

Figure 4. PIs of Ki-67, similar as STAT4, increased with the development of pathology grade ($P < 0.001$, **Table 1**). Nevertheless, no statistically significant correlation was found between Ki-67 expression and age or gender (both $P > 0.05$).

Relationship between STAT4 and PIs of Ki-67

Both of the STAT4 and PIs of Ki-67 were significantly higher in the glioma tissues as compared to the normal brain tissues. Both STAT4 expression and PIs increased with the growth of pathology grade (**Table 2**). Simultaneously, further analysis by Spearman correlation test showed a significant positive relationship between STAT4 and PIs ($r = 0.261$, $P = 0.001$,

Figure 5). As the statistical analysis showed, when the original data of PI was divided into two groups base on its median value, the expression of STAT4 and Ki-67 PI in 140 case glioma tissues also showed an obvious difference ($r = 0.277$, $P = 0.001$, **Figure 5**). Spearman analyze suggested a desirable correlation coefficient between STAT4 and PI ($r = 0.277$, $P < 0.001$). Additionally, STAT4 would have a more effective and efficient diagnosis value for glioma when it was combined with PI proved by ROC curve. The AUC of confederative markers of both STAT4 and PI was 0.767 (95% CI: 0.647-0.887, $P < 0.001$) to predict pathological changes of glioma, remarkably higher than STAT4 itself alone.

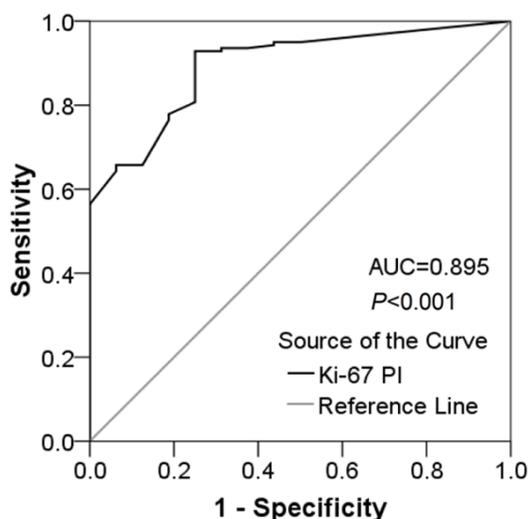


Figure 4. ROC curve of Ki-67 PI to distinguish glioma from brain tissues. The area under curve (AUC) of Ki-67 PI was 0.895 (95% CI: 0.826-0.964, $P < 0.001$).

Discussion

To date, few molecular signatures of glioma have been validated and widely accepted as predictive indicators in clinical practice. Since more accurate prognostic predictors and more efficacious therapies for glioma patients are required, it is extremely necessary to explore novel molecular signatures that can precisely predict the clinical outcome and response to the treatment of this disease with reliable clinicopathological significance. In the current study, we demonstrated the expression condition of STAT4 in glioma tissues. Furthermore, we investigated the relationship between STAT4 level and clinicopathological parameters including tumor differentiation and proliferative status. Our results suggest that STAT4 may act as an oncogene factor in glioma and could be a potential biomarker for the diagnosis and prognosis for glioma patients.

The existing findings describe STAT4 as a member of a growing list of genes that bind multiple sites in the genome to promote interferon (IFN) signaling, interleukin (IL)-12-dependent activation of immune cells and polarization of CD4⁺ T cells to IFN- γ -producing Th1 cells, among others. Previously, several lines of evidence have reported that STAT4 were abnormally expressed in HCC, breast cancer, gastric cancer, non-Hodgkin lymphoma, colon and rectal cancer [9-13]. The result of Junichi et al. [7] showed

that STAT4 influenced the function of glioma immune cells in mouse model via interleukin 4 activation. Nevertheless, no direct report on STAT4 expression in glioma was published and the role of STAT4 in human glioma tissues remained unclarified. In the present study, concerning the expression of STAT4 on glioma tissues, we found significantly higher expression of STAT4 protein in glioma FFPE tissues than that in the normal brain, which was similar to the status of STAT4 in other malignancies. The results, together with literatures, support the agreement that STAT4 plays a role as an oncogene in various malignant tumors, including gliomas.

Next, we studied the correlation between STAT4 expression and glioma differentiation, which was evaluated by histological features. In the current study, the expression patterns of STAT4 were divided into two sections that include a negative group and the positive one. No relative relationship was found between STAT4 and age or gender. But there was a positive correlation between STAT4 expression and the tumor grade ($r = 13.983$, $P = 0.003$), in agreement with other malignancies. An aforementioned finding reveals that high-level STAT4 expression might be a novel molecular alteration involved in glioma progression and STAT4 might serve as a useful biomarker to monitor the clinical course of patients with glioma.

Ki-67 is a commonly used marker of cell proliferation and has been reported extensively in almost all malignancies [14]. Because Ki-67 is expressed only by cells actively engaged during the DNA synthesis phase of the cell cycle (G1, S, G2, and mitosis) but is absent from resting cells (G0), positive immunostaining is considered a good correlate of biological proliferation [8]. Great deals of studies have identified Ki-67 as an independent prognostic marker of disease oncogene, progression and disease-specific survival. To explore if STAT4 expression was associated with tumor growth, we detected Ki-67, a biomarker of proliferation, in glioma tissue with different clinicopathological features. As shown in **Table 1**, Ki-67 PI showed a significant correlation with the tissues and grades of glioma but not with age or gender. Simultaneously, positive correlation could be observed between the STAT4 expression and Ki-67 PI (**Table 2; Figure 4**), that is, in the higher actively

STAT4 in glioma

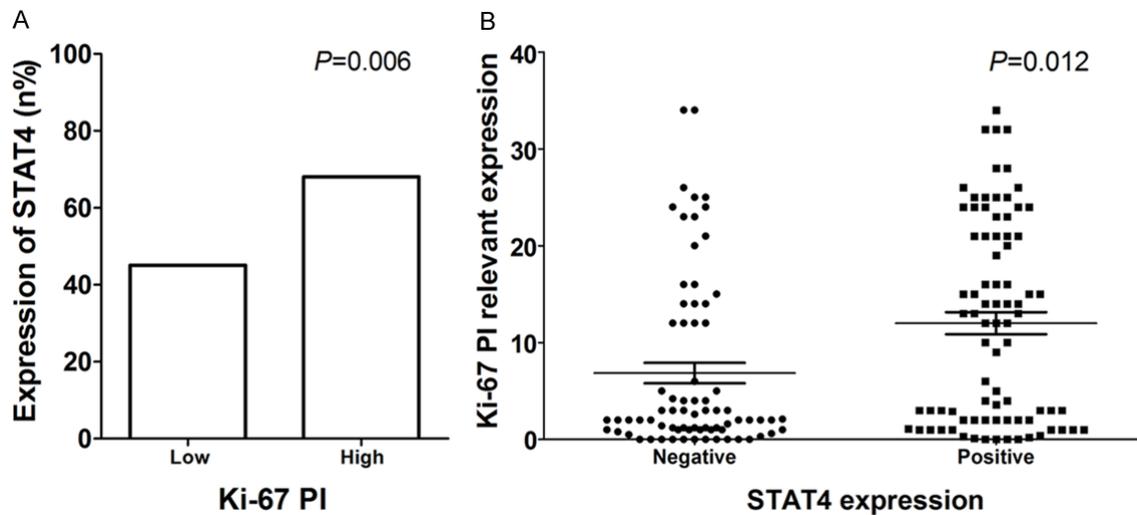


Figure 5. Relationship between STAT4 and Ki-67 PI in glioma. A. The differences between STAT4 and Ki-67 PI based on the median value of Ki-67 PI. B. Relationship between STAT4 expression and Ki-67 PI.

Table 2. Relationship between STAT4 and Ki-67 LI

Parameter	n	Expression of STAT4 n (%)		t	P	Parameter	n	Ki-67 relevant expression (2-ΔCq)		t	P
		Negative	Positive					Mean ± SD			
Ki-67 (median)						STAT4					
Low	71	39 (54.9%)	32 (45.1%)	-2.739	0.006	Low	61	8.1623±9.4689		-2.533	0.012
High	69	22 (31.9%)	47 (68.1%)			High	79	12.4443±10.2505			

proliferated gliomas, the positive expression of STAT4 showed stronger expression, which indicates that STAT4 is closely related to the proliferation of glioma cells. Moreover, Wubetu et al. [9] found that STAT4 was correlated with the growth of HCC cells, which provide sidelong evidence to validate the ability of STAT4 to enhance the tumor proliferation.

Conclusion

In brief, the current findings confirm the role of STAT4 acting as an oncogene during the tumorigenesis and deterioration of human glioma. STAT4 expression in FFPE samples might be a prognostic biomarker for the differentiation and proliferative status of glioma cells. However, more evidence should be obtained with more in-depth and larger scale studies to further identify the contribution and molecular mechanism of STAT4 in glioma.

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Disclosure of conflict of interest

None.

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