

Correlative analyses of the expression levels of PIAS3, p-SHP2, SOCS1 and SOCS3 with STAT3 activation in human astrocytomas

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Abstract. The importance of signal transducer and activator of transcription 3 (STAT3) signaling in the growth and survival of glioblastoma cells has been well documented, while the reasons leading to STAT3 activation remains to be elucidated. Suppressors of cytokine signaling (SOCS) 1 and SOCS3, SH2 domain-containing phosphatase (SHP2) and protein inhibitors of activated STAT3 (PIAS3) are known to inhibit STAT3 signal transduction, while their expression statuses in the four grades of astrocytomas and relevance with STAT3 activation remain to be described. The present study aimed to address these issues by tissue microarray-based immunohistochemical profiling the expression levels of phosphorylated (p)-STAT3, SOCS1, SOCS3, PIAS3 and p-SHP2. The results revealed that p-STAT3 nuclear translocation was rarely observed in non-cancerous brain tissues and its frequencies were increased in a tumor grade-associated manner (65.2, 77.1, 81.8 and 85.7% for grade I-IV, respectively). PIAS3, p-SHP2, SOCS1 and SOCS3 were expressed in higher levels (++ and +++) in 63.6, 90, 87.5 and 81.8% of tumor surrounding brain tissues, which reduced to 13.1, 47.8, 33.3 and 50% in grade I, 11.4, 65.7, 58.3 and 77.1% in grade II, 9.1, 63.6, 38.1 and 31.8% in grade III and 7.1, 66.7, 30.8 and 7.1% in grade IV astrocytomas. The above results revealed that although the expression levels of SOCS1, SOCS3 and, in particular, p-SHP2, tend to decrease in the four types of astrocytomas, PIAS3 downregulation is more negatively correlated with STAT3 activation in the stepwise

progress of astrocytomas and would indicate an unfavorable outcome.

Introduction

Astrocytomas are the most common primary brain tumor types, which, according to the criteria of the World Health Organization (1), are classified to grade I (pilocytic), grade II (diffuse), grade III (anaplastic) and grade IV (glioblastoma multiforme; GBM) (2,3). GBM is the most common central nervous system primary malignancy, which accounts for 60-70% of all gliomas (4). It is known that GBM may develop *de novo* or as the consequence of stepwise progression of low-grade or anaplastic astrocytomas (5,6). Multiple cancer-associated factors are known to be involved in the formation and progression of astrocytomas (7-10), of which activated signal transducer and activator of transcription 3 (STAT3) signaling serves pivotal roles in promoting the growth and survival of GBMs by triggering multiple oncogenic signaling cascades (10-12). STAT3 signaling thus emerges as a key initiator and master regulator of malignant transformation of glial cells (13), and the central player in the maintenance and progression of glioblastomas (14-16). Therefore, it would be of clinical values to explore the underlying reason(s) leading to STAT3 activation in stepwise carcinogenesis of GBMs.

It has been recognized that STAT3 signaling transduction can be activated by numerous factors, including extracellular cytokines, growth factors, hormones and oncoproteins (17,18). On the other hand, the data obtained from human and mouse cell lines reveal that the phosphorylation of STAT3 can be negatively regulated in different manners by a group of suppressors, including protein inhibitors of activated stats (PIAS), suppressors of cytokine signaling proteins (SOCS) and SH2 containing tyrosine phosphatase (SHP1 and SHP2) cascades (19-24). For example, inhibition of PIAS3 results in enhanced proliferation of glioblastoma cells and PIAS3 overexpression inhibits STAT3 transcriptional activity (25). However, no comprehensive *in vivo* data has been available concerning the expression patterns of those STAT3 negative regulators and their relevance with STAT3 activation in different grades of astrocytomas. The present study aims to address the aforementioned issues.

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Materials and methods

Glioblastoma specimens and tissue-microarray construction.

The archived paraffin tissue blocks of 105 cases of astrocytomas surgical specimens were kindly provided by the doctors at the Department of Clinical Pathology, Anshan Central Hospital (Anshan, China). Prior to experiments, hematoxylin and eosin staining was performed on the sections of those tissue blocks for morphological re-examination. The representative tumor and, where possible, tumor surrounding non-cancerous regions in each of the tissue blocks were determined and marked during the re-examination. These marked samples were used for glioblastoma tissue microarray construction, as previously described (26).

Antibodies and their working concentration. The antibodies used for immunohistochemical staining are as follows: Rabbit anti-human p-STAT3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; cat. no. sc-135649; 1:200), rabbit anti-human PIAS3 polyclonal antibody (Biomart Technology, Inc., St. Louis Park, MN, USA; cat. no. BS1467; 1:200), rabbit anti-human SOCS1 polyclonal antibody (Santa Cruz Biotechnology, Inc.; cat. no. SC9021; 1:180), rabbit anti-human SOCS3 polyclonal antibody (Santa Cruz Biotechnology, Inc.; cat. no. sc-9023; 1:180), rabbit anti-human p-SHP2 polyclonal antibody (Sangon Biotech Co., Ltd., Shanghai, China; cat. no. D155149; 1:150). A DAB Detection kit (streptavidin-biotin; ZSGB-BIO, Beijing, China; cat. no. SP-9000) was used for protein detection.

Immunohistochemical staining. The astrocytomas tissue microarrays in the densities of 56 tissue spots/cm² were constructed and subsequently sectioned in series. The 7- μ m thick tissue sections were respectively used for p-STAT3, SOCS1, SOCS3, PIAS3 and p-SHP2 oriented immunohistochemical staining, as described previously (27). Briefly, the tissue sections were washed with PBS and incubated in non-immune animal serum working solution blocking buffer for 20 min at 37°C. The primary antibody was applied to the tissue sections overnight at 4°C. Following three washes with PBS, the tissue sections were incubated with a goat anti-rabbit biotin-labeling generic secondary antibody (DAB Detection kit) for 20 min at 37°C. Horseradish peroxidase-labeled streptavidin solution was applied (1:500) to the slides for 20 min at 37°C. 3,3'-Diaminobenzidine (DAB)-staining was detected using a DAB Detection kit, according to the manufacturer's protocol. The array sections without primary antibody incubation were used as background controls. Based on the labeling density, two independent researchers, in a blinded manner, evaluated the staining results and scored them as negative (-), weak (+), moderate (++) or strong positive (+++) (28).

Statistical analysis. Non-parametric Mann-Whitney tests were applied to analyze the expression differences between different grade astrocytoma tissues and non-cancerous brain samples surrounding the tumor. The data were statistically analyzed by Spearman rank and bivariate correlation using SPSS 17.0 software. $P < 0.05$ was considered to indicate a statistically significant difference.

Table I. Correlation of p-STAT3 nuclear translocation with the expression of STAT3 negative regulator in different grade astrocytoma tissues.

p-STAT3	PIAS3 ^a				SOCS1 ^b				SOCS3 ^b				p-SHP2 ^a				p-SHP2 ^b			
	-	+	++	+++	-	+	++	+++	-	+	++	+++	-	+	++	+++	-	+	++	+++
-	6	9	1	-0.298	0.018	2	6	8	-0.009	0.944	4	6	6	0.058	0.652	9	5	2	-0.002	0.986
+	13	8	3			1	12	11			2	8	14			16	3	5		
++	16	4	1			1	10	10			0	12	9			12	5	4		
+++	1	0	0			0	1	0			1	0	0			1	0	0		
																	1	4	10	1
																	1	8	15	0
																	1	7	13	0
																	1	0	0	0

^aNuclear labeling; ^bcytoplasmic labeling; p-, phosphorylated-; STAT3, signal transducer and activator of transcription 3; PIAS3, protein inhibitor of activated STAT3; SOCS, suppressor of cytokine signaling; SHP, SH2 domain-containing phosphatase.

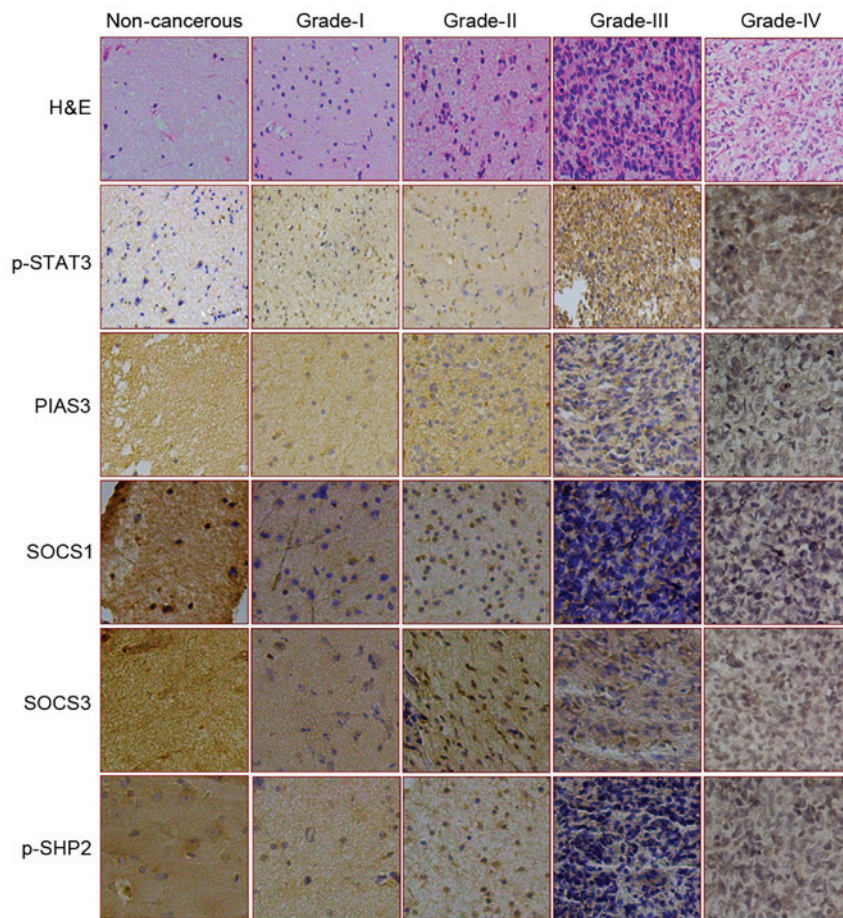


Figure 1. Expression and intracellular distribution patterns of p-STAT3 and its four negative regulators in various brain tissue samples. The expression and distribution of p-STAT3, PIAS3 and p-SHP2 were assessed by immunostaining in non-cancerous brain tissue and four grades of astrocytomas. H&E staining was also used to look at the cells (magnification, x100). H&E, hematoxylin and eosin staining; p-, phosphorylated-; STAT3, signal transducer and activator of transcription 3; PIAS3, protein inhibitor of activated STAT3; SOCS, suppressor of cytokine signaling; SHP, SH2 domain-containing phosphatase.

Results and Discussion

According to the classification criteria of World Health Organization (1), the astrocytoma specimens are classified as grade I (pilocytic), grade II (diffuse), grade III (anaplastic) or grade IV (GBM) (2,5). In the case of GBMs, they may arise primarily (*de novo*) or are transformed from the lower-grade astrocytomas (29). The primary and secondary GBMs can be classified by several factors including the patient age (5). It has been demonstrated that primary GBMs usually occurs in patients aged >50 years, while the secondary glioblastomas are more common among younger patients (30). Of the 94 surgical astrocytomas specimens used in the present study, 23 cases were grouped into grade I (pilocytic), 35 cases to grade II (diffuse), 22 to grade III (anaplastic) and 14 to GBMs. According to the clinical records, 5/14 GBM patients are >50 years and, therefore, can be considered as the primary GBMs and the remaining 9 cases as the secondary tumor types. The representative portions of the tumor, as well as tumor surrounding non-cancerous brain tissues of the above specimens, were sampled from the tissue blocks for tissue microarrays construction, as previously described (31). The prepared tissue microarrays in the density of 56 tissue spots/microarray were used for immunohistochemical examination.

A body of evidence demonstrates that STAT3 activation is positively correlated with astrocytomas progression (25,32,33) and is critical for the growth and survival of glioblastoma cells since p-STAT3 proteins trigger or upregulate its downstream gene expression following translocation to the nucleus (34). The results of immunohistochemical staining (Figs. 1 and 2) revealed that p-STAT3 nuclear translocation was rarely observed in non-cancerous brain tissues (0/11; 0%), while the frequencies were increased to 65.2% (15/23) in grade I, 77.1% (27/35) in grade II, 81.8% (18/22) in grade III and 85.7% (12/14) in grade IV astrocytomas. p-STAT3 nuclear translocation was observed in all of five GBMs (100%) from patients >50 years and 7/9 GBMs (77.8%) from patients >50 years. Statistical analyses revealed the following: i) The frequencies of p-STAT3 nuclear translocation were significantly increased in the four subtypes of astrocytomas compared with that of the non-cancerous brain tissues and ii) that the incidences of p-STAT3 nuclear translocation are closely correlated with tumor grading [Spearman rank and bivariate correlation ($r_s=0.207$, $P=0.045$)]. These results further demonstrated the potential promoting effects of STAT3 signaling in the stepwise progress of astrocytomas and *de novo* formation of GBMs. Further investigation of the underlying reasons leading to the disordered STAT3 activation is of potential prognostic and therapeutic value in the management of astrocytomas.

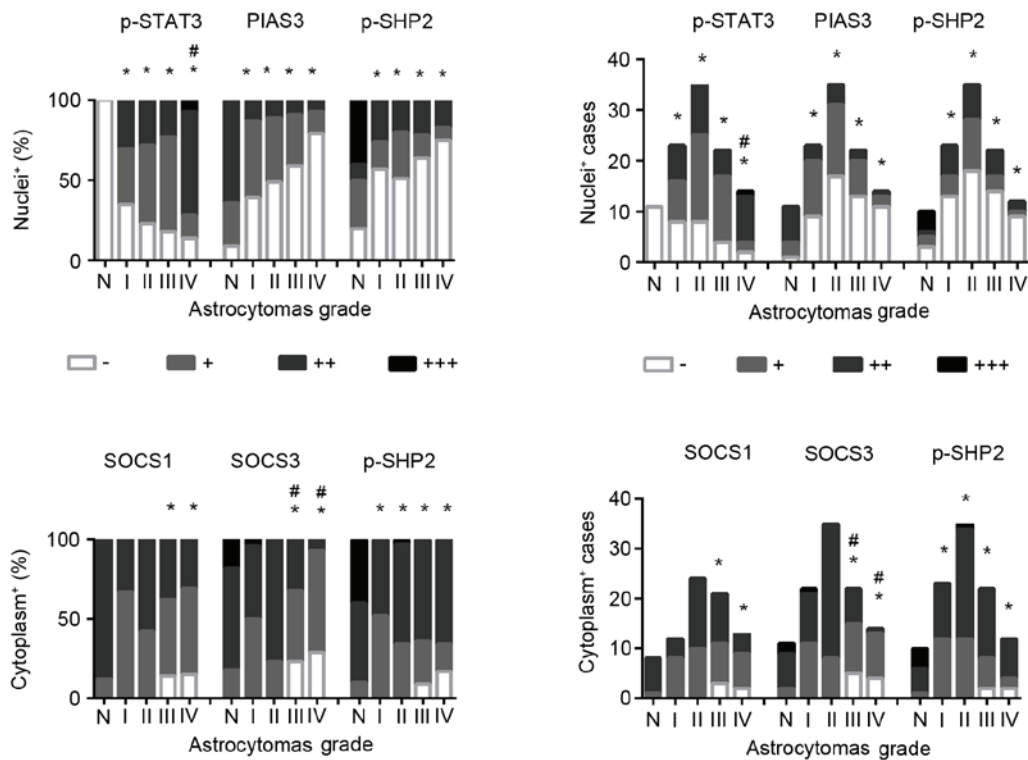


Figure 2. Fractionation of p-STAT3 nuclear translocation and the expression levels of the four STAT3 negative regulators in various brain tissue samples. The presence of p-STAT3, PIAS3 and p-SHP2 in the nuclei and cytoplasm was assessed in the N tissue and four grades (I, II, III and IV) of astrocytomas (*P<0.05 compared with N; #P<0.05 compared with different grades of astrocytomas tissues). p-, phosphorylated-; STAT3, signal transducer and activator of transcription 3; PIAS3, protein inhibitor of activated STAT3; SOCS, suppressor of cytokine signaling; SHP, SH2 domain-containing phosphatase.

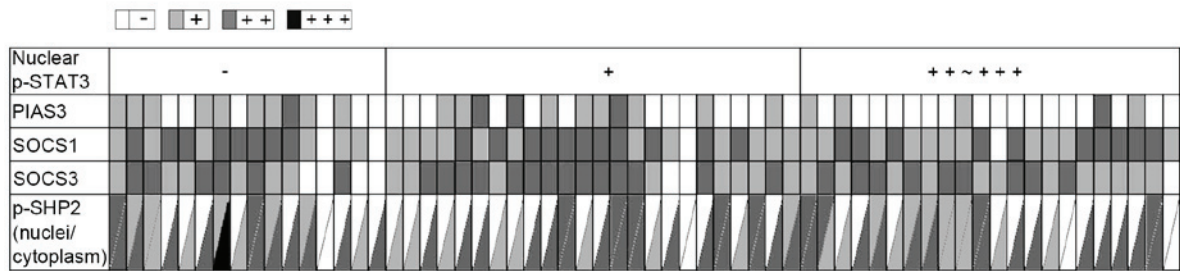


Figure 3. Relevance of p-STAT3 nuclear localization with the expression levels of PIAS3, SOCS1, SOCS3 and p-SHP2 in astrocytoma tissues. The staining results were scored as negative (-), weak (+), moderate (++) or strong positive (+++), and are indicated as white, light gray, medium grey and black, respectively. Each small grid represents an array point and each vertical represents different antibody expression in the same array point. p-, phosphorylated-; STAT3, signal transducer and activator of transcription 3; PIAS3, protein inhibitor of activated STAT3; SOCS, suppressor of cytokine signaling; SHP, SH2 domain-containing phosphatase.

It has been recognized that PIAS3 functions as a negative regulator of STAT-3 signaling by interfering with the interaction between p-STAT3 and its target genes (19). In agreement with the above notion, the present *in vitro* data revealed upregulated expression and increased nuclear translocation of PIAS3 in resveratrol-suppressed glioblastoma cells, accompanied by STAT3 inactivation (35). However, no data concerning the status of PIAS3 in different grades of astrocytomas has been thus far available, although the activated STAT3 signaling has been frequently observed in astrocytomas (15). As shown in Fig. 2, higher levels (++ and +++) of PIAS3 expression were observed in 63.6% (7/11) of tumor surrounding brain tissues, which is reduced to 13.1% in grade I (3/23), 11.4% in grade II (4/5), 9.1% in grade III (2/22) and 7.1% in grade IV (1/14)

astrocytomas. Accordingly, distinct PIAS3 nuclear labeling is observed in the non-cancerous, but not in the majority (54%) of tumor tissues (Figs. 1-3). Statistical analyses revealed significant differences of PIAS3 detection rates between the four subtypes of astrocytomas and the non-cancerous brain tissues, and the negative correlation of PIAS3 expression with astrocytomas formation (P<0.05). Furthermore, the expression of PIAS3 was negatively-correlated with STAT3 nuclear translocation ($r_s=-0.298$; P=0.018; Table I). These results together with our aforementioned *in vitro* findings indicated that PIAS3 may serve negative roles in regulating STAT3 signaling in glioblastoma cells *in vitro* and *in vivo*. Alternatively, PIAS3 downregulation in the four subtypes of astrocytomas may results in STAT3 activation or indirectly

enhance the biological effects of the upstream STAT3 activators (15). In this context, the decreased expression of PIAS3 and the lack of PIAS3 nuclear translocation is an unfavorable prognostic factor of astrocytomas.

SOCS1 and SOCS3 are the predominant members of the SOCS protein family, which work in a classic negative feedback loop to attenuate STAT3 activity by suppressive binding with phosphorylated JAK and/or facilitating ubiquitination of JAK in the cytoplasm (21). SOCS1 and SOCS3 expression in GBMs can be epigenetically regulated in the form of hypermethylation in CpG island (36,37). For instance, the methylation of SOCS3 appears to be involved in the pathogenesis of GBMs and in the resistance of GBMs to conventional anticancer drugs (38). However, the correlation of SOCS1 and SOCS3 downregulation with STAT3 activation in human astrocytomas remains to be reported. The present IHC results revealed that SOCS1 and SOCS3 are expressed in higher levels (++ and +++) in the non-cancerous specimens assessed, while their levels are decreased (+ or -) in the astrocytomas tissues (Figs. 1-3). Statistical analyses demonstrated that SOCS3 ($r_s = -0.400$; $P = 0.000$), rather than SOCS1 downregulation ($r_s = -0.160$; $P = 0.187$), is negatively correlated with the tumor grading (Fig. 2). Nevertheless, neither SOCS1 nor SOCS3 expression pattern is statistically correlated with p-STAT3 nuclear translocation ($r_s = -0.009$, $P = 0.944$; $r_s = -0.058$, $P = 0.652$). It has been reported that SOCS3 inactivation by promoter hypermethylation is mutually exclusive to EGFR activation in glioblastomas and preferentially promotes glioma cell invasion through the activation of STAT3 and FAK (39). Therefore, it would be possible that the epigenetically downregulated SOCS3 and SOCS1 may confer on GBM cells more aggressive biological behaviors, although the relevance of their downregulation with STAT3 activation cannot be totally ruled out at present stage.

SHP2 is a non-receptor type protein tyrosine phosphatase (40) and its phosphorylated form (p-SHP2) downregulates STAT3 activation by dephosphorylating active STAT3 complexes both in the cytoplasm and in the nucleus (41). The statuses of SHP2 and their relevance with STAT3 signaling in GBMs have been reported with differing opinions (42,43). It was revealed that SHP2-mediated antagonism of STAT3 phosphorylation prevails in the promotion of GBM cell death in response to EGFR and c-MET co-inhibition (42), while SHP2 can promote glioblastoma cell growth by suppression of cellular senescence (43). The present immunohistochemical results using a p-SHP2 specific antibody revealed that cytoplasmic p-SHP2 staining (++ and +++) was observed in all of the non-cancerous specimens examined, of which 8 cases (8/10; 80%) were found with p-SHP2 nuclear translocation (Figs. 1-3). In the case of astrocytomas, the detection rates of cytoplasmic p-SHP2 are not changed distinctly, but the frequencies of nuclear p-SHP2 detection are remarkably decreased in the tumor tissues, in particular in grade III (36.4%, 8/22) and grade IV (25%, 3/12) (Figs. 1 and 3). However, the statistical analyses revealed no correlation of nuclear translocation ($r_s = -0.106$, $P = 0.315$) and cytoplasmic staining ($r_s = 0.065$, $P = 0.536$) of p-SHP2 with astrocytomas grading and p-STAT3 nuclear translocation (nuclei, $r_s = -0.002$ and $P = 0.986$; cytoplasm, $r_s = -0.124$ and $P = 0.337$; Fig. 2; Table I). Although the present findings may implicate that the

reductive tendencies of p-SHP2 level and nuclear translocation may be favorable for astrocytomas formation presumably via preventing cell death (43) and/or reinforcing STAT3 activation caused by STAT3 activator overexpression and PIAS3 reduction (25).

In conclusion, SOCS1, SOCS3, PIAS3 and p-SHP2 expression patterns and the frequencies of phosphorylated STAT3/p-STAT3 nuclear translocation in non-cancerous brain tissues and the four grades of astrocytomas were profiled by tissue microarray-based immunohistochemical staining. The results revealed that p-STAT3 nuclear translocation is progressively common as the tumor grades increase. By contrast, the expression levels of SOCS1, SOCS3, PIAS3 and p-SHP2 tended to decrease as the tumor progressed. Statistical analyses revealed that downregulation of PIAS3 is more correlated with p-STAT3 nuclear translocation compared with other STAT3 negative regulators. As a result of the importance of STAT3 activation for the growth and survival of glioblastoma cells, the decreased expression of PIAS3 can be regarded as an unfavorable prognostic factor of astrocytomas patients. SOCS1, SOCS3 and p-SHP2 downregulation, and p-SHP2 nuclear translocation in astrocytomas tissues must have certain biological implications and it would be of value to further investigate.

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