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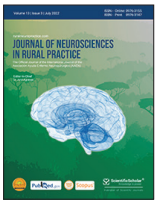

Original Article

Expression and association of vascular endothelial growth factor, vascular endothelial growth factor receptor, and phosphorylated signal transducer and activator of transcription factor 3 in malignant gliomas

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ABSTRACT

Objectives: Angiogenesis is one of the main characteristic features of malignant gliomas. Phosphorylated signal transducer and activator of transcription factor 3 (pSTAT3) is not only involved in glioma cell proliferation, anti-apoptosis, and immunosuppression but also plays a key role in cell migration and invasion. Constitutively, activated pSTAT3 induces expression of vascular endothelial growth factor (VEGF) and its receptor VEGFR, leading to endothelial cell proliferation and abnormal microvascular formation causing peritumoral edema (PTE). PTE is one of the significant contributors to mortality in malignant gliomas. Therefore, understanding the molecular mechanism involved in the evolution of gliomas is necessary. This study was to assess the level of expression of pSTAT3, VEGF, and VEGFR in malignant gliomas and analyze the extent of PTE and the extent of expression of one or more of these markers.

Materials and Methods: This study included 84 patients categorized as per the World Health Organization classification of central nervous system tumors into grade IV, III, and II gliomas to investigate the expression of pSTAT3, VEGF, and VEGFR by immunohistochemistry. Furthermore, the presence or absence of PTE was determined using magnetic resonance imaging/computed tomography scans in these patients.

Results: The association between the markers (pSTAT3, VEGFR, and VEGF) and the extent of PTE in these patients was statistically significant ($P < 0.05$).

Conclusion: The pSTAT3, VEGF-R, and VEGF signaling pathways could contribute to peritumoral edema and might be a regulatory mechanism during PTE formation during tumorigenesis and progression.

Keywords: Malignant gliomas, Phosphorylated signal transducer and activator of transcription factor 3, Vascular endothelial growth factor, Vascular endothelial growth factor receptor

INTRODUCTION

Gliomas are the most common brain tumors, attributing to 80% of cases and 30% of malignant tumors. According to the World Health Organization (WHO) classification, there are four grades; grade I is predominantly benign, and grades II–IV are malignant gliomas.[1] Lower-grade gliomas (WHO grade I) have a better prognosis than higher-grade gliomas (II, III, and IV). Of these, glioblastoma (GBM) is the most common and most lethal. Primary GBM arises *de novo*, while secondary GBM arises from the lower-grade gliomas. Primary and secondary GBMs cannot be differentiated histologically but differ in their genetic and epigenetic profiles.[2] Magnetic resonance imaging (MRI) helps visualize changes in the morphological characters and directly reflects biochemical changes in the tumor itself and surrounding tissue. Despite aggressive treatment, the median survival rate in these patients is <15 months.[3] The major challenge in treating gliomas is the extent of surgical resection and resistance to chemoradiotherapy. Since gliomas have a distinctive character of infiltrating adjacent brain parenchyma, thus compromising complete surgical removal of the tumor. Despite the incurability and resistance to chemoradiotherapy, the identification of proper drivers of infiltration of gliomas is needed for anti-invasive therapy. The

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recent identification of the signal transducer and activator of the transcription factor 3 (STAT3) pathway, a molecular hub for signal transduction in gliomas, may be considered as potential therapeutic target.[4] STAT3 is generally absent in normal brain tissue. However, it gets activated by growth factor receptors at the tyrosine residues and gets phosphorylated, leading to altered normal cell functions. Janus Kinases (JAK1 and JAK2) are the primary upstream mediators of STAT3 activation.[5] STAT3 is involved in glioma cell proliferation and anti-apoptotic activity, aiding in invasion and infiltration. STAT3 also regulates vascular endothelial growth factor (VEGF), resulting in neoangiogenesis,[6] a prerequisite for tumor growth. Hypoxia stimulates VEGF and gets upregulated by various oncogenes and proto-oncogenes.[7] Peritumoral edema (PTE) is the characteristic feature of malignant gliomas. VEGF plays a pivotal role in the PTE of gliomas by down-regulating the tight junction proteins like occludin, leading to the formation of cleft and fenestra between the endothelial cells, leading to increased vascular permeability.[8] PTE is an important focus, where tumor cells migrate to the adjacent normal brain parenchyma creating a chance of recurrence.[9] At diagnosis, these tumors show heterogeneous contrast enhancement with PTE on MRI. Increased mortality is reported in these patients due to PTE, indicating a poor prognosis. To address this issue, we analyzed the extent of PTE and its association with phosphorylated signal transducer and activator of transcription factor 3 (pSTAT3), VEGF, and VEGFR expression in malignant gliomas.

MATERIALS AND METHODS

This retrospective study was conducted at Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, a tertiary care center. The study is conducted after clearance from the Ethics Committee of the institute.

Eighty-four patients were operated on for tumor resection in the Department of Neurosurgery and diagnosed as gliomas in the Department of Pathology, JIPMER, fulfilling the WHO criteria/grade for astrocytoma/oligodendroglioma/GBM grades II, III, and IV were included in the study. Grade l gliomas and patients who received neoadjuvant chemotherapy, recurrent cases, and ependymomas were excluded from the study.

Clinical and radiological examination details were obtained from patient record archives. Pre-operative MRI/computed tomography (CT) data were collected for every patient. All MRI scans were evaluated by a radiologist who was blinded to pathological diagnosis. A region of very bright T2-W signal and low T1-W signal without enhancement around the tumor was determined as PTE.

Grading of the extent of PTE was measured using MRI scans as explained by Carlson *et al.*[10]

- Grade 1 – Maximum distance between the edema's outer edge and the nearest tumor margin point <2 cm
- Grade 2 – Edema extending ≥2 cm from the tumor margin in axial T2-W images.

In the event of the unavailability of an MRI scan, the hypodensity seen around the tumor in a CT scan was used to grade the edema.

Edema shape was classified as follows:

- Roundish – Shape is regular/round, and
- Irregular – Shape tends to be irregular such as radial or finger-like, as shown in [Figure 1].

Immunohistochemistry (IHC)

IHC was performed on the freshly cut formalin fixed paraffin embedded tissue (4 μm). The following primary antibodies were used pSTAT3 (phospho Y705) rabbit monoclonal antibody (1:100 dilution) Abcam; VEGFR (ab39256) rabbit monoclonal antibody, (1:200 dilution) Abcam; and VEGF rabbit monoclonal antibody (1:200 dilution), thermo scientific. Normal kidney and colon adenocarcinoma were used as external control and endothelial cell nucleus was taken as internal control for pSTAT3, whereas breast and placenta tissue were positive controls for VEGFR and VEGF, respectively. In addition, endothelial cell cytoplasm was taken as an internal control for VEGFR and VEGF.

The semi-quantitative scoring system used for grading the expression of pSTAT3, VEGF, and VEGFR is as follows:

0: Negative; +1: < 20% of positive cells (weak); +2: 20–50% of positive cells (Moderate); +3: >50% of positive cells (strong)

Statistical analysis

The resultant data were analyzed using SPSS 19.0 statistical software. The distribution of categorical variables such as

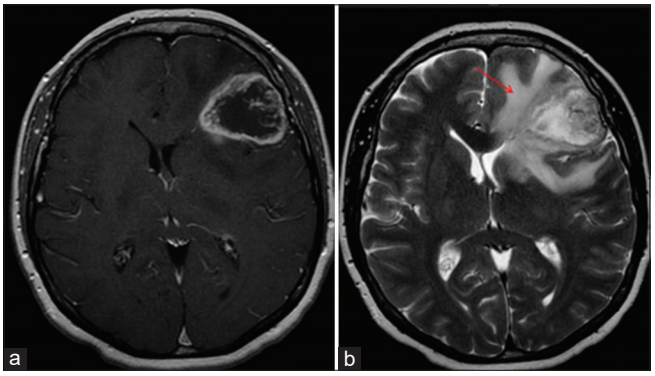

Figure 1: Contrast-enhanced magnetic resonance imaging shows a tumor with perilesional edema (a) well-circumscribed lesion with enhancement representing edema regular in shape, size <2 cm. (b) tumor with necrosis and edema irregular in shape with >2 cm in size (indicated by arrow).

gender, peritumoral edema, and tumor grade was expressed in frequency percentage. The distribution of continuous variables such as age and expression of VEGF, VEGFR, and pSTAT3 were represented in terms of mean with standard deviation. The comparison of the above-stated continuous variables with PTE and tumor grade was made using Fisher's exact test. The relationship between IHC markers and PTE was carried out using correlation analysis. The independent factors associated with the level of IHC markers were carried out using multiple linear regression analysis. All statistical analyses were carried out at a 5% significance level with $P < 0.05$ considered statistically significant.

RESULTS

In the present study, 84 patients were enrolled per inclusion and exclusion criteria. These included 51 cases of Grade IV (GBMs), 16 cases of Grade III (anaplastic astrocytoma and oligodendroglioma), and 14 cases of Grade II (astrocytoma and oligodendroglioma). Of the included 84 patients, 52 were male and 32 were female, with an M: F ratio equal to 1.6:1. Mean age of the patient was 41.3 ± 13.3 years. Most patients presented with symptoms of seizures 32 (38%) as a chief complaint, followed by altered sensorium 18 (21%), diplopia, and slurred speech. Headache and vomiting indicated increased intracranial tension in 11 (13.09%) patients.

Peritumoral edema was reported in 63/84 (75%) patients. Of these, PTE was grade 1 (<2 cm) in 33 (52.3%) and grade 2 (≥2 cm) in 30 (47.6%) patients. The edema was round or regular in shape in 34 (53.9%) and irregular or finger-like in 29 (46.0%) cases.

The study demonstrated a positive association of immunohistochemical expression of VEGF, VEGFR, and pSTAT3 across the various grades of diffuse gliomas [Figure 2]. The same is shown in [Tables 1-3]. Significant correlations in the expression of pSTAT3, VEGF, and VEGFR were seen in GBM, anaplastic astrocytoma, and anaplastic oligodendroglioma, while, in diffuse astrocytoma and oligodendrogliomas, this correlation was insignificant. The positive expression of the above markers was also significantly associated with the presence of PTE, along with its grades ($P < 0.05$). The expression and corelation are represented in [Table 4].

DISCUSSION

The outcomes associated with gliomas are very poor, despite aggressive treatment. In the present study, we evaluated the

Table 1: Expression of VEGF across grades of tumor.

Diagnosis	Negative (%)	Weak (<10% of tumor cells positive) (%)	Moderate (10–50% of tumor cells positive) (%)	Strong (>50% of tumor cells positive) (%)	Total	*P*-value
Grade II	2 (11.5)	4 (23.5)	6 (35.2)	5 (29.4)	17	<0.02
Grade III	2 (12.5)	4 (25.5)	4 (25)	6 (37.5)	16	
Grade IV	1 (1.9)	4 (7.8)	23 (45)	23 (45)	51	
Total	5 (5.9)	12 (14.2)	33 (39.2)	34 (40.4)	84	

VEGF: Vascular endothelial growth factor

Table 2: Expression of VEGFR across grades of tumor.

Diagnosis	Negative (%)	Weak (<10% of tumor cells positive) (%)	Moderate (10–50% of tumor cells positive) (%)	Strong (>50% of tumor cells positive) (%)	Total	*P*-value
Grade II	5 (29.4)	2 (11.7)	4 (23.5)	6 (35.2)	17	<0.03
Grade III	0 (0)	1 (6.2)	5 (31.2)	11 (68.7)	16	
Grade IV	1 (1.9)	8 (15.6)	18 (35)	24 (47)	51	
Total	6 (7.1)	11 (13.09)	27 (32.1)	41 (48.8)	84	

VEGFR: Vascular endothelial growth factor receptor

Table 3: Expression of pSTAT3 across grades of tumor.

Diagnosis	Negative (%)	Weak (<10% of tumor cells positive) (%)	Moderate (10–50% of tumor cells positive) (%)	Strong (>50% of tumor cells positive) (%)	Total	*P*-value
Grade II	9 (52.9)	2 (11.7)	3 (17.6)	3 (17.6)	17	<0.02
Grade III	2 (12.5)	2 (12.5)	6 (37.5)	6 (37.5)	16	
Grade IV	2 (3.9)	14 (27.4)	17 (33.3)	18 (35.4)	51	
Total	12 (14.2)	18 (21.4)	26 (30.9)	27 (32.1)	84	

pSTAT3: phosphorylated signal transducer and activator of transcription factor 3

Table 4: Relationship between PTE and PTE shape with VEGF, VEGFR, and pSTAT3 expression.

S. No.	IHC marker	No PTE *n*=21 (%)	PTE Grade 1 (<2 cm) *n*=33 (%)	PTE Grade 2 (>2 cm) *n*=30 (%)	*P*<0.05	PTE shape regular *n*=34 (%)	PTE shape irregular *n*=29 (%)	*P*<0.05
1.	VEGF							
	0	8/21 (66.)	2/33 (6.0)	0/30 (0)		2/34 (5.8)	0/29 (0)	
	1	4/21 (33)	2/33 (6.0)	5/30 (16.7)	<0.02	2/34 (5.8)	5/29 (17.2)	0.159
	2	6/21 (50)	17/33 (51)	9/30 (30)		13/34 (38.2)	12/29 (41.3)	
	3	3/21 (25)	12/33 (36.3)	16/30 (53.3)		17/34 (50)	12/29 (41.3)	
2.	VEGFR							
	0	5/21 (41.6)	2/33 (6.06)	0/30 (0)		2/34 (5.8)	0/29 (0)	
	1	4/21 (33.3)	4/33 (12.1)	4/30 (13.3)	<0.01	5/34 (14.7)	3/29 (10.3)	0.147
	2	8/21 (66.6)	16/33 (48.4)	7/30 (23.3)		13/34 (38.2)	10/29 (34.4)	
	3	4/21 (33.3)	11/33 (33.3)	20/30 (6.7)		14/34 (41.1)	16/29 (55.2)	
3.	pSTAT3							
	0	11/21 (52.3)	1/33 (3.0)	2/30 (6.6)		1/34 (2.9)	2/29 (6.8)	
	1	5/21 (41.6)	11/33 (33.3)	5/30 (16.6)	<0.02	10/34 (29.4)	7/29 (24.1)	0.228
	2	3/21 (25)	15/33 (45)	8/30 (26.6)		13/34 (38.2)	9/29 (31.0)	
	3	2/21 (16.6)	6/33 (18.1)	15/30 (50)		10/34 (29.4)	11/29 (37.9)	

IHC: Immunohistochemistry, PTE: Peritumoral edema, VEGF: Vascular endothelial growth factor, VEGFR: Vascular endothelial growth factor receptor, pSTAT3: phosphorylated signal transducer and activator of transcription factor 3

expression of pSTAT3, VEGF, and VEGFR across tumor grades and correlated the expression of pSTAT3, VEGF, and VEGFR with PTE. The role of STAT3 is to maintain and proliferate malignant cells and also plays a role in the transition to more malignant subtypes.

A study by Wang *et al.*,[11] which included 84 cases of GBM, found pSTAT3 positivity in 20% (median) of cases and VEGF expression in 65% (median) of the WHO grade IV gliomas using IHC and found a statistically significant association of these proteins with PTE. In the present study, we detected the expression of pSTAT3, VEGFR, and VEGF, not only in the WHO grade IV but also in the WHO grade III and II tumors. Higher expression of these markers is found in higher grades of tumors.

A retrospective study by Susman *et al.* was done to evaluate the prognostic role of STAT 3, they reported an increase in pSTAT3 level which is associated with poor prognosis in GBM patients. With a median survival of 8.9 months in patients expressing more than 20% positivity in comparison with 13.7 months in patients expressing <20% positivity of STAT3. Hence, emphasizing the role of STAT3 as target for treatment.[12] In this study, patient survival was not studied and only the expression of STAT3 with tumor grade and its association with PTE was evaluated.

A study by Leventoux *et al.* claimed that high-density foci of grade II gliomas showed a high percentage of STAT3-positive cells, which indicated that the STAT3 pathway activated in these cells led to its malignant behavior.[13] A study by Wang *et al.* reported high expression in VEGF and PI3K levels in glioma cancer stem cells in grades III–IV compared to grade II cases using RT-qPCR.[14] Another study conducted by Carlson *et al.* proved that VEGF expression indicated poorer survival in patients with extensive edema with a concordance of 95%.[10] In the present study, VEGF expression was significantly associated with PTE, compromising survival. This might be due to the infiltration of tumor cells into the adjacent brain tissue.

Wu *et al.*[15] observed that PTE and necrosis shown by MRI scans were independent predictors of unfavorable prognosis in GBM (the WHO grade IV) patients. However, another study reported that VEGF-C and -D and their coreceptors VEGFR 2 and VEGFR 3 were overexpressed in the majority of GBMs. However, the IHC expression levels did not correlate with overall survival and isocitrate dehydrogenase status.[16] One study reported that VEGF stimulates GBM stem cells under both normoxic and hypoxic conditions in a dose-dependent manner, while VEGFR1 has a negative feedback effect on VEGFR2.[17]

Huang *et al.* found that both VEGF and VEGFR expression in various brain tumors differ and are not necessarily parallel. In our study, both are highly expressed in grade IV tumors.[18] Zhao *et al.* noticed pSTAT3 and VEGFR expression in small-cell lung carcinoma and lymph node metastasis through IHC.[19] Similar effects are also seen in head and neck squamous cell carcinoma, colon, breast, kidney, and endometrial cancers. The current study helps us understand the STAT3-VEGF signaling pathway in gliomas through the JAK-STAT pathway. pSTAT3, VEGF plays a pivotal role in apoptosis, migration, invasion, and neoangiogenesis, which are hallmarks of glioma aggressiveness. Thus, STAT3 knockdown renders future promise for effective chemoradiotherapy. The implication of our study is that many pathways have been reported in the

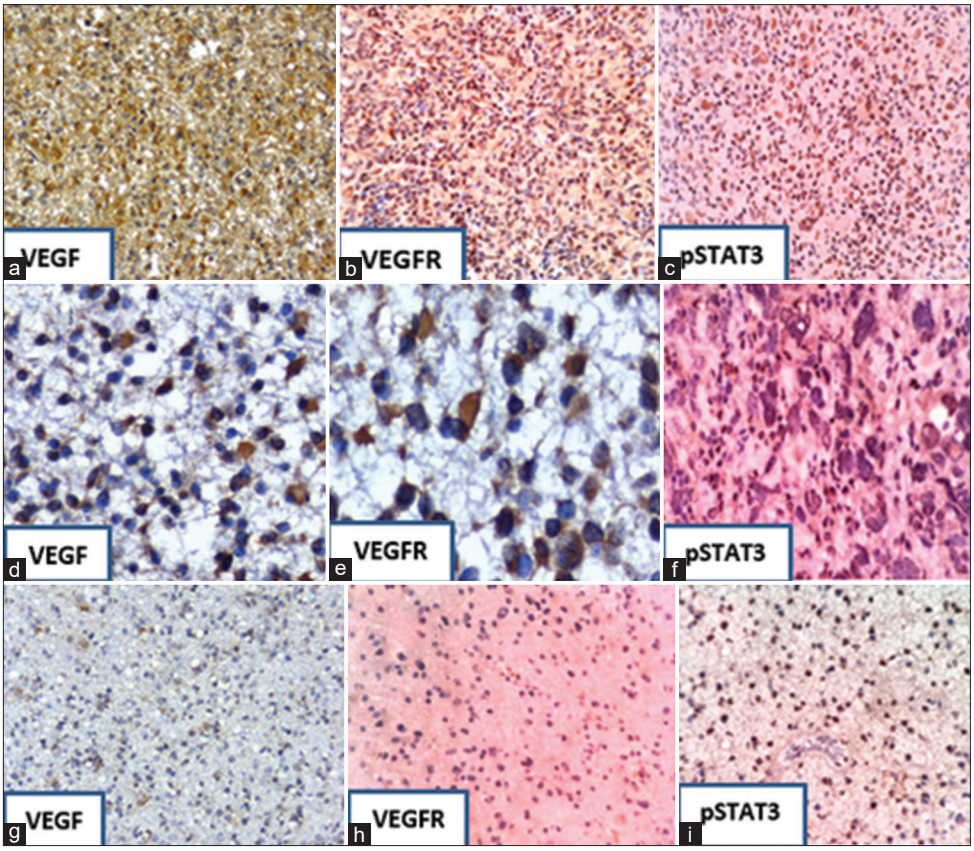

Figure 2: Immunohistochemical staining showing the expression of VEGF, VEFGR, and pSTAT3; Strong expression (a-c) in GBM (Grade IV) and from (d-f) moderate expression in anaplastic astrocytoma (Grade III). From (g-i), there is a weak expression of markers in diffuse astrocytoma (Grade II). Immunostaining is also seen in endothelial cells (f and i). Magnification ×200. VEGF: Vascular endothelial growth factor, VEGFR: Vascular endothelial growth factor receptor, and pSTAT3: Phosphorylated signal transducer and activator of transcription factor 3.

pathogenesis of malignant glioma. STAT3-VEGF signaling pathway is considered as the most important one due to the convergence of several pathways at p-STAT3 and also through modulation of genes implicated in cell proliferation, apoptosis, migration, invasion, and neoangiogenesis which are hallmarks of glioma aggressiveness. Hence, there is the intriguing possibility that p-STAT3 can be considered as a therapeutic target and increase the survival of these patients.

This study has a few limitations. Quantification of the molecular markers was not carried out in these samples. Furthermore, a follow-up survival analysis was not done to investigate the survival period in these patients.

CONCLUSION

This is the first study in the Indian population to confirm that PTE extent is positively associated with pSTAT3, VEGF, and VEGFR expression in high-grade gliomas. Our study provides evidence that the pSTAT3-VEGF-VEGFR signaling pathway might play a pivotal role in alleviating the PTE. Therefore, clinical approaches should consider including a STAT3 inhibitor in the therapeutic protocol to improve tumor response to chemotherapy and radiotherapy.

Declaration of patient consent

The Institutional Review Board (IRB) permission obtained for the study.

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Nil.

Conflicts of interest

There are no conflicts of interest.

Use of artificial intelligence (AI)-Assisted technology for manuscript preparation

The author(s) confirms that there was no use of Artificial Intelligence (AI)-Assisted Technology for assisting in the writing or editing of the manuscript and no images were manipulated using the AI.

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