GFAP EXPRESSION IN NEUROGLIAL TUMOURS- IMMUNOHISTOCHEMICAL CONFIRMATION FOR DIAGNOSIS AND GRADING

Amit V. Varma[1], Garima Gupta[2], Jagdish Gupta[3], Sapan Gupta[4]

[1]*Professor and HOD, Department of Pathology, SAIMS, Indore, Madhya Pradesh, India.*
[2]*Assistant Professor, Department of Pathology, Amaltas Institute of Medical Sciences, Bangar, Dewas, Madhya Pradesh, India.*
[3]*Consultant Radiologist, Department of Radiology, Amaltas Institute of Medical Sciences, Bangar, Dewas, Madhya Pradesh, India.*
[4]*Senior Resident, Department of Paediatrics, Columbia Asia Hospital, Bengaluru, Karnataka, India.*

ABSTRACT

BACKGROUND
GFAP is a reliable marker for differentiating glial tumours from non-glial tumours. In addition, the percentage and intensity of staining helps in grading of glial tumours.

The aim of this study was to determine the distribution of Glial Fibrillary Acidic Protein (GFAP) in neuroglial tumours and its correlation with histologic grading.

MATERIALS AND METHODS
Representative formalin-fixed paraffin-embedded blocks were selected for 51 cases of CNS tumours. 3 micrometer thick sections were cut from each block and sections were taken on poly-L-lysine coated slides. Immunostaining was done by Streptavidin-biotin immunoperoxidase technique (LSAB) using antibody to Glial Fibrillary Acidic Protein. Descriptive statistics was used to show characteristics of collected data. On application of Kruskal-Wallis test, a significant inverse correlation was found between grade of tumour and total average GFAP staining score with statistically significant $p < 0.002$.
Study Design- Cross-Sectional Study.

RESULTS
In the current study, all glial tumours were stained positive for GFAP. The average score of Grade 1 tumours was 6.7, Grade 2 tumours was 6.6, Grade 3 tumours was 5.75 and that of Grade 4 tumours was 5.2.

CONCLUSION
GFAP is a reliable marker to differentiate between glial and non-glial tumours, and a high degree of malignancy is associated with reduced cellular differentiation as shown by reduced GFAP staining. Therefore, GFAP IHC is complimentary to the histological diagnosis and grading of brain tumours.

KEY WORDS
Glial Fibrillary Acidic Protein Astrocytoma, Ependymoma, Glioblastoma, Oligodendroglioma.

BACKGROUND
Gliomas encompass astrocytoma, glioblastoma, ependymoma, oligodendroglioma and their various subtypes and combinations. Gliomas contain Glial Fibrillary Acidic Protein (GFAP) and lack collagen, reticulin and fibronectin in their parenchyma, distinguishing them from non-glial neoplasms. However, oligodendroglioma cells are more variable in their glial fibrillary acidic protein (GFAP) expression.[1]

GFAP, first described by Amico Bignami[2] is an intracytoplasmic intermediate filament protein specific for the cytoskeleton of glial cells.[3]

Its immunoreactivity in the mature CNS is restricted to glial filaments within protoplasmic astrocytes in gray matter, fibrous astrocytes in white matter, radial glia in the cerebellum (Bergmann glia) and subependymal astrocytes adjacent to the cerebral ventricles.

At the surface of the brain, GFAP immunoreactivity is especially concentrated in astrocytes, which form the glia limitans. The greatest contribution of GFAP has been in the elucidation of the cellular constituencies of neoplasms previously considered of enigmatic origin.[4-6]

In astrocytes and other glial cells, it is expressed in the cytoplasm, while in astrocytomas it is expressed in the cellular processes rather than the cytoplasm. Anti-GFAP stains astrocytes, ependymal cells and corresponding tumours and studies have shown usefulness of GFAP in distinguishing neoplasms of astrocytic origin and in differentiating gliomas from metastatic lesions in the brain.[7,8]

Clinical needs like knowing the effectiveness of chemotherapy for gliomas with an oligodendroglial component, especially malignant gliomas have increased the pathologist's role in recognising these tumours.[1,7,9,10]

Astrocytomas are among the most fibrillar of CNS neoplasms and nearly always contain GFAP.[11] The gemistocytic variant shows strong immunoreactivity in cytoplasm and Rosenthal fibres. Fibrillary astrocytes show
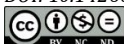
perinuclear rim of positivity. Oligodendrogliomas: Expression of GFAP is usually absent in tumour cytoplasm, but there may be overlapping of GFAP-positive fibrillary neuropil background. Glioblastoma multiforme (GBM): The vast majority express GFAP, but this antigen may occasionally be lacking.[12]

There are two types of glioma specimens- First, the tumour itself with cellular density exceeding that of surrounding brain. Second, is the brain tissue infiltrated by the margin of glioma. Immunohistochemical stains for brain neuroanatomic components are very useful in identifying this brain tissue. Further, from the glioma itself, neoplastic glia in CNS parenchyma are difficult to distinguish from gliosis. GFAP can help identify gliosis by showing excess cytoplasmic GFAP and regular spacing between cells in gliosis.[1]

Hence, assessment of GFAP status is an important step in diagnostic confirmation of neuroepithelial tumours and also for assessing the degree of differentiation. There is literature available on GFAP staining of glial tumours, but very few studies have been conducted on correlation with grading of these tumours on GFAP immunostaining.

MATERIALS AND METHODS

The study design was cross-sectional. Forty cases of glial tumours and 11 cases of non-glial tumours were included in the study group. Serial 3 mm thick sections were cut from representative paraffin-embedded tissue blocks and taken on poly-L-lysine coated slides. Deparaffinization was done as per standard protocol. Then the slides were rehydrated in decreasing concentration of alcohol. Antigen retrieval of all slides was done, after which each slide was treated with methanol containing 4% hydrogen peroxidase for 30 minutes followed by placement in 0.05 M-Tris - HCL buffer pH 7.4 for 10 minutes. Sections were then covered with primary antibody in the specified dilutions and incubated for 45 minutes in a humid chamber at room temperature. (Primary Antibody-GFAP was obtained from (BioGenex, USA) in dilution 1: 200 and used in dilution of 1: 200) This was followed by incubation at room temperature for 45 minutes after covering with biotinylated secondary antibody of anti-mouse antiglobulins in Phosphate Buffer Saline (PBS) containing carrier protein and Sodium Azide (15 mmol/L) large volume (universal BioGenex kit). Horseradish Peroxidase (HRP) conjugated Streptavidin was used to cover the slides at room temperature and incubated for 30 minutes. After rinsing, slides were covered with substrate chromogen solution and incubated at room temperature for minutes till development of optimum brown colour peroxidase product. Counter staining was done with Harris Haematoxylin.

Each batch of slides was immunostained with appropriate positive controls of sections from non-neoplastic brain tissue. For negative control, sections from medulloblastoma/ meningioma were used.[7,13,14] Scoring of GFAP expression was done semi-quantitatively for both cell number and staining intensity.[15]

Number of Positive Cells			Intensity of Staining
Nil	-	0	0
<5%	-	1	1
5-25%	-	2	2
25-75%	-	3	3
75-100%	-	4	

Total score 0 - 2 = negative. ≥ 3 = positive.

Statistical Analysis

Data were analysed using SPSS version 16. Descriptive statistics was used to show characteristics of collected data. Kruskal-Wallis test was used to calculate average score between GFAP staining, which was statistically significant at $p < 0.002$.

RESULTS

| Type of Tumour	No. of Cases	Result for Staining
Glial tumours	40	Positive
Non-glial tumours	11	Negative
Table 1. GFAP Staining Distribution		

Staining Pattern

In the current study, low-grade astrocytomas (Grade 1 and 2) showed intense GFAP staining mostly in the fibrillary processes, whereas the grade 3 astrocytomas showed cytoplasmic staining. Grade 4 tumours (GBM) showed less positivity. Pilocytic astrocytomas showed staining, mostly in the Rosenthal fibres. Ependymomas including myxopapillary ependymomas also showed positive staining, mainly in ependymal rosettes. Oligodendrogliomas also showed positivity for GFAP, but in ring-like pattern around nucleus.

In the current study, all glial tumours stained positive for GFAP. Also scoring was done according to percentage of cells stained and intensity of staining amongst various grades of glial tumours.
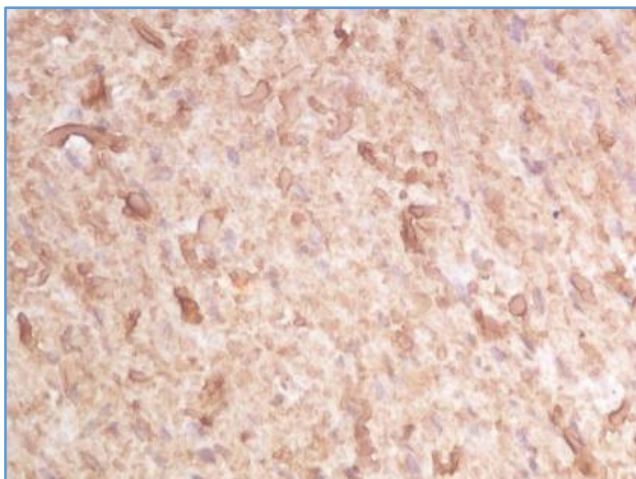
| WHO Grade of CNS Tumours	No. of Cases	Median and IQR
I	10	6 (7)
II	11	6 (6)
III	4	5 (1.75)
IV	15	5 (3.2)
Table 2. GFAP Scores in Glial Tumours		
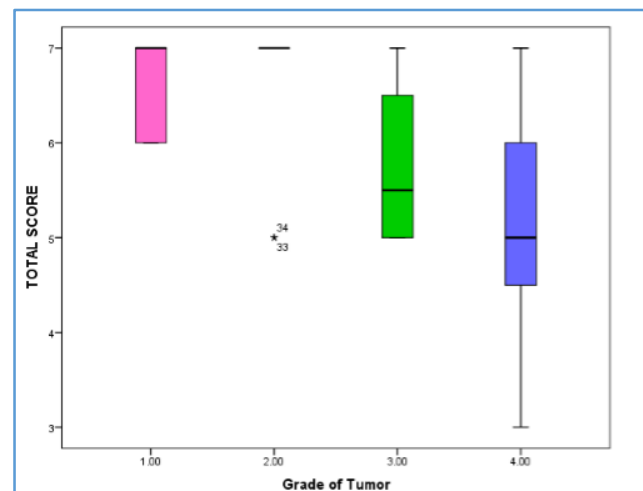
*Figure 1. Kruskal-Wallis Representation of GFAP Staining Score*

The average score of Grade 1 tumours was 6.7, Grade 2 tumours was 6.6, Grade 3 tumours was 5.75 and that of Grade 4 tumours was 5.2.
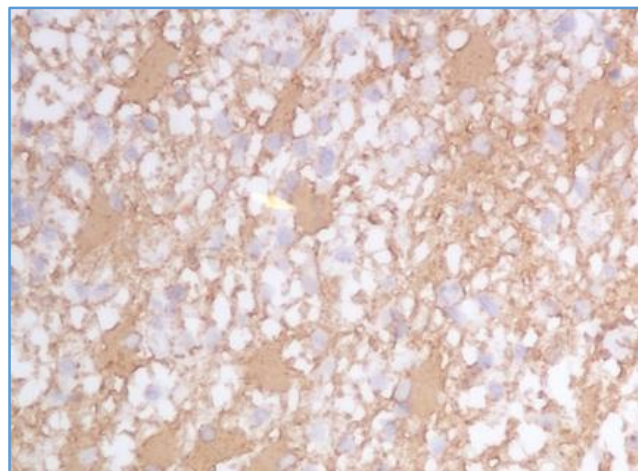

Figure 2. Pilocytic Astrocytoma- Positive GFAP Stain demonstrated by Rosenthal Fibres and EGB (40x)
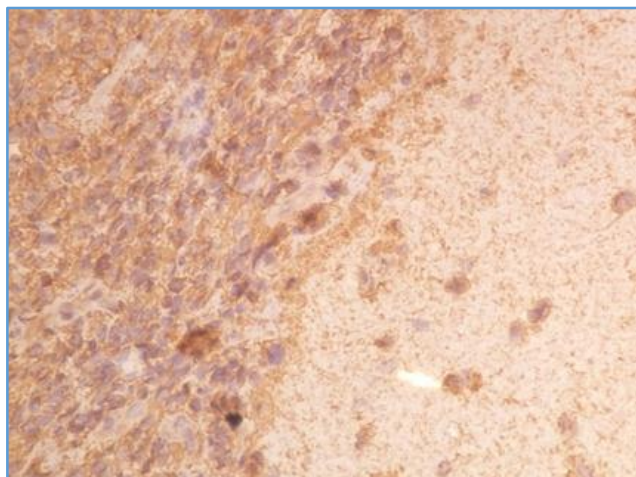
Figure 3. IHC: Grade 3 Astrocytoma: Microphotograph showing Positive GFAP Staining in Neoplastic Astrocytes and Junction Normal Brain Tissue (40x)
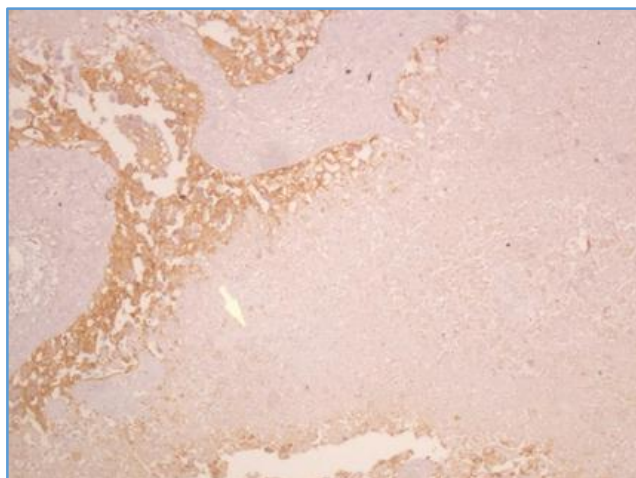
Figure 4. IHC: Glioblastoma Multiforme: Microphotograph showing Low Staining Intensity of Tumour Cells (10x)

Figure 5. IHC Microphotograph (40x) of Ganglioglioma showing Positive GFAP Staining in the Neoplastic Ganglion Cell Cytoplasm and Fibrillary Processes

DISCUSSION

The histological diagnosis of a brain tumour is not always straight forward, and the pathologist faces diagnostic dilemmas because of overlap of morphologic features, divergent differentiation within a tumour and non-neoplastic lesions sometimes mimicking tumours. Hence, application of immunohistochemical markers has become imperative for an exact diagnosis, subtyping and grading.

The distribution of GFAP in gliomas has been extensively studied in recent years.[16,17]

In the current study, we applied glial fibrillary acidic protein antibody on 51 cases to establish the diagnosis of glial tumours, their grading and distinguishing them from non-glial tumours. The various grades of astrocytomas showed variable degrees of staining in terms of cellular percentage and intensity. Non-glial tumours were found to be negative. Venugopal Madabhushi et al (2015) applied in their study GFAP antibody on few confusing cases of glioblastoma, which confirmed their diagnosis. In their study, one case of ganglioglioma was proved by GFAP application, in which ganglion cell like astrocytes were differentiated from neoplastic neurons by positive expression for the antibody.[18]

Most studies have found that tumours deriving from cells with gliofibrillogenetic capacity are GFAP-positive. Also, an inverse relationship between the degree of anaplasia, that is rapid proliferation, invasiveness and growth, and the number of cells staining positive has been found.[19-24]

David Schiffer et al (1986) found in their study that in Astrocytoma cases, positive reaction for GFAP was found in typical fibrillary areas with low cell density and predominance of protoplasmic and gemistocytic aspects. Whereas areas with anaplasia showed most of the cells to be GFAP negative. In Glioblastomas, the proliferative area was GFAP negative, in invasive cortical areas small hyperchromatic cells were negative.[25]

Wahda Al Nuaimy et al (2009) found that GFAP expression was highest in glioma cases (P value < 0.05). Most of those cases were astrocytomas followed by few cases of oligodendrogliomas which showed positive expression, as were all the cases of ependymoma and oligoastrocytoma. The intensity and pattern of staining varied in each type of glioma.

They found a significant inverse relation between both total score and proportional score of GFAP and the grade of glioma with p value < 0.05. In their study medulloblastomas, medulloepithelioma and meningiomas were negative for GFAP.[26]

Jones et al (1982) also found positive GFAP expression in 88.3% of their neuroepithelial tumours.[27] Gullotta et al (1985) found in their study, 96% of glial tumours to be GFAP positive.[28]

The results of our study have shown that only glial tumours stained positive for GFAP, whereas the non-glial tumours were negative. Also, among the glial tumours, the low- grade ones showed a higher overall GFAP staining score, whereas the score was low in most grade 4 tumours. There was an inverse correlation between GFAP intensity and the grade of gliomas with statistically significant p value (P < 0.002).

The staining pattern in low-grade astrocytomas was in fibrillary processes, whereas in Grade 3 tumours there was more of cytoplasmic staining. Pilocytic astrocytomas showed positivity in Rosenthal fibres. Oligodendrogliomas showed positivity, mainly in perinuclear area in only a ring-like pattern. Ependymal tumours showed positivity in rosettes. These findings are in concordance with those found by other studies by Wahda M et al, Gross JR et al, Duffy PE et al, Vyberg M et al, Ulrika W et al and Manuel E et al.[26, 29-32]

But studies by Hannah C Chueng et al, Jossef Zameccuk et al and Tajika et al did not show any significant relation.[33-35]

Author	Year	No. of Cases	Country	GFAP Relation to Tumour Grade
Tascos N A et al[6]	1982	131	USA	Inverse
Tajika T et al[35]	1986	91	Japan	No relation
Bian X W[36]	1992	243	China	Inverse
Reyaz N et al	2005	50	Pakistan	Direct
WM Al-Nauimy[37]	2010	56	Iraq	Inverse
Current study	2015	51	India	Inverse
Table 3. Findings from Other Parts of the World				

CONCLUSION

GFAP staining applied on glial tumours showed positivity in all specimens, whereas the non-glial tumours staining turned out to be negative. Amongst the glial tumours, all four grades of tumours were taken in different numbers and a semi-quantitative staining for both cell number and staining intensity was done. The results revealed an inverse relation between the grade of tumour and staining score.

Hence, it can be concluded that GFAP is a reliable marker to differentiate between glial and non-glial tumours, and a high degree of malignancy is associated with reduced cellular differentiation as shown by reduced GFAP staining. Therefore, GFAP IHC is complimentary to the histological diagnosis and grading of brain tumours.

Glial fibrillary acidic protein is now an established immunohistochemical marker for diagnosis of glial tumours. Numerous studies have been conducted in various parts of the world to show the correlation. However, there are sparse studies to show the correlation between intensity of staining and grading of tumours. In the current study, the average score of Grade 1 tumours was 6.7, whereas that of Grade 4 tumours was 5.2. Kruskal- Wallis test was the statistical method applied, which showed an inverse correlation between grade of tumour and GFAP staining score.

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