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SERUM GFAP AS A DIAGNOSTIC & PROGNOSTIC MARKER FOR GLIOBLASTOMA MULTIFORME

Neurosurgery

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ABSTRACT

Glial fibrillary acidic protein (GFAP) as a diagnostic and prognostic serum marker for glioblastoma multiforme (GBM) could improve both diagnosis and clinical management of brain tumour patients. To evaluate whether the serum concentration of GFAP may indicate glioblastoma multiforme (GBM) in patients with single supratentorial space-occupying lesions, we prospectively examined 27 consecutive patients with histologically proven GBM, World Health Organization (WHO) grade IV, 6 patients with anaplastic astrocytoma (WHO grade III), 9 patients with anaplastic oligodendroglioma(III), 11 patients with diffuse astrocytoma (WHO grade II), 4 patients with a single cerebral metastasis and 7 healthy controls. Serum was taken from the patients before & after tumour resection. Serum GFAP levels were determined using a commercially available ELISA test and 25 were detectable in out of the 27 GBM patients. The levels were significantly elevated compared with those of the non-GBM tumour patients and healthy controls. Non-GBM tumour patients and all healthy subjects showed zero serum GFAP levels. There was a significant correlation between serum GFAP level and GBM detection and also between the survival period and post operative GFAP levels. A serum GFAP level of >0.11ng/ml was 78% sensitive and 98% specific for the diagnosis of GBM in patients with a single supratentorial mass lesion in this series. Post resection serum levels had a mean of 0.197ng/ml & levels >1.2 ng/ml were associated with survival period of less than one year. Therefore, it can be concluded that serum GFAP constitutes a diagnostic & prognostic biomarker for GBM.

KEYWORDS

Glioblastoma multiforme, Serum GFAP, Tissue GFAP

Introduction:

Glioblastoma multiforme (GBM; WHO grade IV tumour) is the most malignant form of cerebral glioma and accounts for ~50% of such tumour.[1,2] It can present as a high-grade lesion from the onset (primary or de novo GBM)[3] or can evolve from a lower-grade precursor lesion, such as WHO grade II astrocytoma and WHO grade III anaplastic astrocytoma (secondary or progressive GBM). Using brain imaging, the differential diagnosis of GBM includes metastases, abscesses or sometimes even immune mediated demyelinating mass lesions.[4] Although brain imaging and clinical characteristics may suggest the diagnosis of a GBM, histopathological analysis of the tumour tissue is mandatory for a definite diagnosis. Despite recent progress in the combined use of surgical resection, radiation and chemotherapy, the prognosis of GBM is still poor, with a median survival time of 12.1–14.6 months after diagnosis.[5] Glial fibrillary acidic protein (GFAP), first described in 1971 by Eng et al., is a member of the cytoskeletal protein family and is widely expressed in astroglial cells and in neural stem cells.[6,7] It is also expressed in astroglial tumours, such as astrocytoma and GBM.[8,9] We hypothesized that GFAP may be released from astroglial tumours, especially malignant gliomas, into the serum and could therefore be used as a diagnostic biomarker for GBM. On the basis of this hypothesis, we studied GFAP levels in the serum of GBM patients and various control groups. We have followed the WHO 2016 classification of brain tumours.

Methods:

Patient Inclusion:

This is a prospective study conducted between January 2016 and December 2016 at the Institute of Neurosurgery, Rajiv Gandhi Government General Hospital and Madras medical college, Chennai, India. Inclusion criteria were (I) Patients with newly diagnosed single space-occupying supratentorial lesion detected by MRI. (II) Seven healthy subjects without intracerebral lesions were also included as controls. Exclusion criteria were (I) history of traumatic brain injury (II) history of ischemic stroke or intracerebral hemorrhage. (III) Patients who had already undergone invasive diagnostic or therapeutic interventions due to the cerebral lesions were not considered.

Totally 104 patient were selected. Of the 104 patients, 27 patients were GBM, 4 were pilocytic astrocytoma, 9 were oligodendroglioma, 6 were anaplastic astrocytoma, 11 were diffuse astrocytoma, 2 were ganglioglioma. (Total glial tumour – 59). Among 37 non glial tumour patients, 3 were ependymoma, 6 were tuberculoma, 3 were central neurocytoma , 10 were meningioma, 2 were pituitary macroadenoma, 4 were brain secondaries, 2 were Craniopharyngioma, 2 were esthesioneuroblastoma , one was epidermoid, one was melanocytoma, one was choroid plexus papilloma, one was arachnoid cyst , one was fungal granuloma and one was brain abscess. For control, seven normal healthy individuals were added in this study.

Survival rate and tumor recurrence:

Regular clinical and radiological follow up examinations were performed up to week 78 or death. Survival rate at week 78 was defined as the primary endpoint of the study. According to the response assessment in neuro-oncology (RANO)-criteria, tumor recurrence was defined as a clinical deterioration in combination with concomitant changes in T1-weighted (enhancement of the contrast agent of more than 25%) and T2-weighted (increasing area of the hyperintense signal) images compatible with tumor recurrence. Progression-free survival rate at week 78 was defined as the secondary endpoint of the study.

Blood sampling and GFAP estimation:

First blood sample was taken from each subject at the time of admission, prior to any treatment e.g. cranial surgery, radiation therapy or chemotherapy. Second sample was taken on 10th postoperative day. Samples were transferred into an Eppendorf tube. Within 2 hours after blood draw, samples were centrifuged at 10000 rpm at 4 min and supernatants were immediately frozen and stored at -20°C to -70°C until the levels of GFAP were assessed. GFAP is known to be stable in whole blood for several days at 4°C and in frozen samples, stable for several months to years. Freezing and thawing was found not to influence GFAP concentrations.

Serum GFAP levels were determined blind to the clinical data on the corresponding patients using a biotin-labelled antibody based sandwich enzyme immunoassay for the quantitative measurement of human GFAP. GFAP-ELISA test conditions and quantification were as described by the manufacturer (Mybiosource). In short, samples, quality controls and calibrators were diluted 1:3 prior to analysis. Duplicates of 100 µl samples and calibrators were then pipetted into the ELISA wells and incubated for 2 hours at room temperature,

followed by 1 hr incubation with a biotin-labelled Anti-GFAP-antibody solution and 1 h of incubation with a Streptavidin-HRP conjugate. Between each step, 4 cycles of thorough washing (400 ml washing solution per well) were performed. Finally, the plate was incubated for approximately 20 min at room temperature under avoidance of direct sunlight with the substrate solution of the kit. The colour development was stopped by adding the commercially available stopping solution of the kit. The absorbance was measured by reading the ELISA plate at 450 nm. The detection limit of the test, defined as the concentration of the mean of the absorbance of blanks (calibrator diluent) plus 3 SD of the absorbance of the blanks ($A_{blank}+3\times SD_{blank}$), was measured and calculated as 0.018ng/ml to 20ng/ml. All values below this detection limit (0.018ng/ml) were defined as 0 ng/ml.

GFAP analysis in tumour tissue:
GFAP expression after GFAP immunohistochemical staining of the tumour specimen was determined by two independent observers who were blinded to GFAP serum levels. GFAP expression was assessed in non-necrotic tumour areas of five separate microscopic fields of view under a magnification of 40×, and was classified as the mean of the percentage of GFAP immunohistochemical positive tumour cells. GFAP expression was ranked as: <25, 25–50, 51–75 and >75% GFAP positive tumour cells. Furthermore, on the assumption that the assessed GFAP expression of non-necrotic tumour areas is comparable to those in necrosis, the product of GFAP expression and tumour necrosis volume was determined as a measure for the amount of necrotic GFAP positive cells in GBM.

Volumetric measurements:
The tumour volume of each patient as well as the necrotic area within the GBM were estimated from preoperative, post-contrast T1-weighted magnetic resonance images (MRI) using a modified ellipsoid volume equation: (A×B×C)/2, with A, B and C representing the three dimensions of the tumour and the three dimensions of the necrotic area, detectable as hypointense, non contrast enhancing area within the GBM.[10] In addition, T2-weighted images and FLAIR-weighted images were used to verify the measurements and to further differentiate tumour from perifocal oedema. The MRI scans had been performed not more than 14 days before serum sampling.

Results:
In GBM patients, we found a mean preop serum GFAP level of 0.31ng/ml (range: 0.13–3.21ng/ml; n = 27 Standard deviation(SD): 0.64ng/ml). This was significantly higher than in the non-GBM tumour patients (median: 0; range 0–0.022ng/ml; n = 70) or in the normal subjects (median: 0; range 0–0ng/ml; n = 7) (P<0.001 respectively). Pearson correlation value preop GFAP level showed 0.813 which is statistically significant . All normal subjects showed zero serum GFAP levels (detection limit: 0.018ng/ml). Six non-GBM tumour patients with serum GFAP levels of 0.022ng/ml to 0.067ng/ml. The remaining non-GBM tumour patients showed serum GFAP levels below the detection limit. Furthermore, in 2 GBM patients, serum GFAP was not detectable (<0.018ng/ml).

Tumour volume significantly correlated with tumour necrosis volume in GBM patients (Spearman Rho, CC = 0.78; 95% confidence interval, 0.65–0.91; P<0.0001). Furthermore, there was a significant correlation between tumour volume and serum GFAP level as well as between tumour necrosis volume and serum GFAP in GBM patients (Spearman Rho, CC = 0.51; 95% confidence interval, 0.4–0.63; P<0.001 and CC = 0.49; 95% confidence interval, 0.2–0.72; P = 0.004, respectively).

Post operative mean GFAP level in GBM is 0.197 ng/ml (range 0 – 2.8ng/ml, n=27)(P <0.001).

Patients were followed up to 78 weeks. 2 patients died within 2 months due to non neurosurgical complications. As per Pearson correlation value is -0.413 showed survival period is inversely proportional to the value of GFAP. Irrespective of tumour size if GFAP is elevated above 1.2ng/ml in the post resection period, longevity is decreased. Median survival period 14months after post operative standard chemo and radiotherapy. Range is 14±2 months.

CHART -1

Age distribution					
Age		Frequency	Percent	Valid Percent	Cumulative Percent
	<20	11	10.6	10.6	10.6
	21- 40	35	33.7	33.7	44.2
	41 - 60	26	25.0	25.0	69.2
	61>	32	30.8	30.8	100.0
	Total	104	100.0	100.0	

FIGURE 1

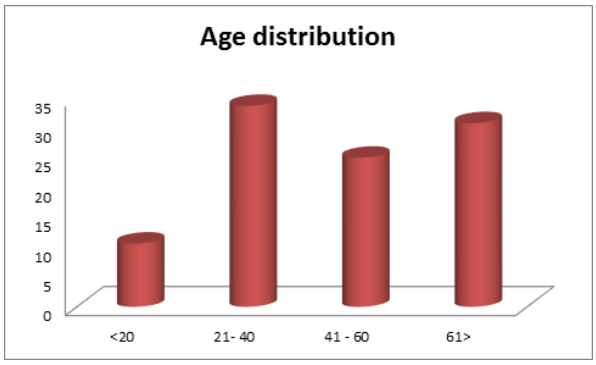


CHART 2

Sex					
Sex		Frequency	Percent	Valid Percent	Cumulative Percent
	Male	79	76.0	76.0	76.0
	Female	25	24.0	24.0	100.0
	Total	104	100.0	100.0	

FIGURE 2



CHART 3

Diagnosis					
		Frequency	Percent	Valid Percent	Cumulative Percent
	GBM	27	26.0	26.0	26.0
	NON-GBM	77	74.0	74.0	100.0
	Total	104	100.0	100.0	

Figure 3

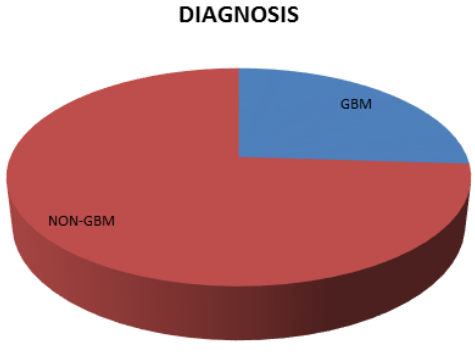


CHART 4
T- test

	N	Mean	Std. Deviation	P-Value t-test
Serum GFAP (Pre op)	104	0.31130	.640891	0.001
Serum GFAP (Post op)	104	0.19702	.404710	

CHART 5

Correlations			
		Diagnosis	Serum GFAP (Pre op)
Diagnosis	Pearson Correlation	1	0.813
	Sig. (2-tailed)		.000

	N	104	104
Serum GFAP (Pre op)	Pearson Correlation	0.813	1
	Sig. (2-tailed)	0.000	
	N	104	104

CHART 6

Correlations			
		Serum GFAP (Post op)	Pt status
Serum GFAP (Pre op)	Pearson Correlation	1	-0.494
	Sig. (2-tailed)		0.000
	N	104	104
Pt status	Pearson Correlation	-0.494	1
	Sig. (2-tailed)	0.000	
	N	104	104

The ROC analysis cut-off point of 0.11 ng/ml of serum GFAP afforded a sensitivity of 78% and a specificity of 98% for the differentiation of GBM patients from non- GBM tumour patients or healthy controls (area under the curve, 0.791; P<0.001). The positive and negative predictive values were 1.0 and 0.82, respectively.

Discussion:

Our study shows that GFAP is detectable in the serum of many patients with GBM (WHO grade IV tumour). The serum GFAP levels of these patients were significantly higher than those of patients with WHO grade II or III astrocytomas or of patients with brain metastases. Furthermore, serum GFAP levels were correlated with the GBM volume and tumour necrosis volume measured from MRI scans. In this sample, a serum GFAP level above 0.11 ng/ml provided a sensitivity of 78% and a specificity of 98% for the diagnosis of GBM. Serum GFAP therefore appears to be a promising new diagnostic biomarker. Elevated GFAP levels also occur after head trauma, intracerebral haemorrhage and ischaemic stroke,[11,12,13] but these conditions were excluded from this study.

GFAP is highly specific for cells with astrocytic differentiation and is widely used as a reliable marker in the immunohistochemical diagnosis and differentiation of brain tumours.[14] Whereas GFAP is consistently expressed, at least to some extent, in all gliomas including glioblastoma, it is usually not found in meningiomas, medulloblastomas and brain metastasis.[15,16] In our series, all glioblastomas, WHO grade II and III astrocytomas as well as oligoastroglial and oligodendroglial tumours, showed to some extent immunoreactivity for GFAP, whereas negative in cerebral metastases,menigiomas and normal individuals. Comparable to previous reports, the most uniform staining was seen in well-differentiated grade II astrocytomas, whereas GBM patients showed a strong variability in GFAP expression and distribution. Although GFAP is immunohistochemically evident in low and high-grade glioma, it remains unclear why serum GFAP mainly indicates GBM. However, several pathophysiological mechanisms or their combination may explain increased GFAP levels in serum of GBM patients, including: (i) GFAP expression, (ii) tumour necrosis and/or (iii) disruption of the blood–brain-barrier (BBB).

As described previously, a high variability of GFAP expression in GBM tumour tissue was also observed in this study. However, GFAP expression was not directly correlated with GFAP serum levels.[16] GFAP expression is believed to indicate the differentiation status of astrocytes. Some GBM, which show high cell proliferation, contain more undifferentiated astrocytes and do not express GFAP extensively. Others express GFAP at the same amounts as anaplastic astrocytomas,[17] or even demonstrate increased expression of all GFAP isoforms.[18] A decrease in GFAP expression has been associated with growth and malignancy of glial tumours[19] and was more prominent in high-grade than in low-grade gliomas.[20] In vitro, a reduced expression of GFAP in some glioblastoma cell lines was also associated with more aggressive and invasive potentials.[21,22,23] While some factors like TGF-alpha lead to decreased GFAP expression,[22,23] multiple other factors including glial growth factor (GGF)[24] and glial maturation factor (GMF)[25] were proven to influence and upregulate GFAP expression in vitro.[26,27]

Immunohistochemically, GFAP was not detectable in cerebral metastases. However, all glial tumours expressed immunohistochemically GFAP. Patients with astrocytoma grade III revealed GFAP expression of >50% and those with astrocytoma grade II of <25%. Oligodendroglial and oligoastroglial tumours expressed low amounts of GFAP (<25%). GFAP expression varied little within each non- GBM tumour group. However, GBM tumour tissue samples showed a strong variability in GFAP expression ranging from <25% in some patients to almost 100% in others. Although, there was no direct correlation between GFAP expression and serum GFAP levels (P = 0.3), it was evident, that GBM patients without tumour necrosis had, independent of their GFAP expression, serum GFAP levels below the detection limit. Whereas, those GBM patients with similar necrosis volumes and differing GFAP expressions showed with increasing GFAP expression a corresponding increase in serum GFAP level. The product of GFAP expression and tumour necrosis volume as a measure for the amount of necrotic GFAP positive cells in GBM patients was strongly correlated with GFAP serum levels

Tumour necrosis volume therefore appears to influence the sensitivity of GFAP detection in GBM patients. Tumour necrosis volume was further correlated with tumour volume. Tumour necrosis, which is absent in low-grade glioma and present in GBM was significantly correlated with serum GFAP levels. Tumour necrosis might therefore explain elevated GFAP serum levels in patients with voluminous GBMs.

GFAP has a relatively high molecular weight of 52 kDa,[27] which limits its transit through the BBB under physiological conditions. Similar to acute head trauma, intracerebral haemorrhage or brain infarction, [28] elevated serum GFAP concentrations in GBM patients are likely to reflect, both, GFAP leakage from glial cells (e.g. tumour necrosis) and a disruption of the BBB. In fact, both are among the diagnostic criteria defining GBM.

In this study, the ciruculating levels of GFAP after tumour resection were found to be higher in patients with GBM compared with non GBM and healthy controls. In cases of GBM, high postoperative serum GFAP levels were associated with worse prognosis.

Conclusion:

This study showed that serum GFAP is a very good diagnostic and prognostic biomarker for GBM. Post operative GFAP levels were closely associated with prognosis of GBM. High post operative GFAP levels were associated with worse prognosis. Thus, GFAP is a useful prognostic indicator in GBM patients.

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