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RESEARCH

Expression Pattern and Prognostic Significance of IGFBP Isoforms in Anaplastic Astrocytoma

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Abstract The role of insulin- like growth factors and their regulatory proteins (IGFBP isoforms) in gliomas, particularly glioblastoma, has been a subject of active research in recent years. There is paucity of literature on their expression and impact on clinical outcome in anaplastic astrocytomas. To evaluate the expression patterns of IGFBP isoforms in anaplastic astrocytoma and correlate with clinical outcome, a retrospective study of 53 adult patients operated for supratentorial lobar anaplastic astrocytoma was performed. The protein expression of IGFBP isoforms (IGFBP-2, -3, -5 and -7), was studied by immunohistochemistry on all samples. The patients were followed up and outcome was documented. The median age at presentation in the present study was 35 years. The pattern of staining was intra cytoplasmic, homogenous and diffuse for IGFBP-2, -3 and -5 and granular for IGFBP-7. IGFBP-2 expression was significantly low in anaplastic astrocytoma as compared to other isoforms ($P<0.001$). IGFBP-3 expression was higher than the other isoforms. However, its' expression correlated with favorable overall survival and demonstrated a trend towards significance on univariate analysis. The present study is the first of its kind to describe comprehensively the pattern of expression of IGFBP isoforms (IGFBP-2, -3, -5 and -7) in anaplastic astrocytomas. IGFBP-2 and IGFBP-3 expression patterns and correlation to prognosis were distinct in anaplastic astrocytoma patients, contradictory to what has been reported in glioblastoma, thus giving further evidence that anaplastic astrocytomas are molecularly distinct from glioblastoma.

Keywords Anaplastic astrocytoma · Insulin-like growth factor binding protein · Immunohistochemistry · Prognosis

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Introduction

Malignant astrocytomas, which include the anaplastic astrocytoma (AA;WHO grade III) and glioblastoma (GBM;WHO grade IV), are the most common primary intra-axial brain tumors in adults [1, 2]. The current World Health Organization (WHO) guidelines distinguish their malignancy grades on the basis of histological features that also predict patient survival [3]. Glioblastoma is associated with a uniformly poor outcome, whereas survival varies considerably among patients with anaplastic astrocytoma [4, 5]. Prognostic stratification of these patients has been limited primarily to histological grading and clinical parameters, such as age and performance score [4]. However, these parameters do not fully account for the observed variation in survival [4, 5]. Recent studies have revealed numerous cytogenetic and molecular genetic alterations, involving multiple genetic events and a cascade of signaling pathways to play a key role in the pathogenesis of malignant astrocytomas and patient survival [6–10].

Insulin-like growth factor (IGF) signaling pathway has been implicated in the progression of glioma [11, 12]. Overexpression of Insulin-like growth factor binding protein (IGFBP)

isoforms has been well documented in gliomas with an emphasis on glioblastoma [13, 14]. A previous study by our group, showed the association of IGFBP-2, -3 and -5 expression with increasing grades of malignancy in astrocytomas and identified IGFBP-3 as a novel prognostic glioblastoma biomarker [15]. In view of the emerging data on the role of these isoforms in gliomas and their association with pathogenesis in glioblastoma, we analysed the expression of IGFBP isoforms (IGFBP-2, -3, -5 and -7) in patients with anaplastic astrocytoma and assessed their value as predictors of survival.

Materials and Methods

Patient and Tissue Samples

This study has been approved by the ethics committee of our institute and patient's consent was obtained. The study includes a retrospective cohort of adult patients with anaplastic astrocytoma (n=53) who underwent maximal safe resection of a supratentorial lobar tumor, at the National Institute of Mental Health And Neurosciences, Bangalore from January 2003 to December 2006. All patients had undergone microsurgical decompression with the aim of maximal safe resection. The extent of resection was assessed by reviewing the post operative CT scan images. The demographic data and clinical features of these patients were collected. Adjuvant therapy was administered to all patients.

The patients were followed up clinically and radiologically with MRI/CT imaging. The clinical status in the form of neurological deficits and Karnofsky's Performance Score (KPS) was documented regularly. The overall survival was defined as the duration between surgery and death of the patient due to disease. The clinical and radiological findings were considered to ascertain the tumor recurrence. Inclusion criteria for tumor recurrence were: (**1**) Radiological confirmation of appearance of the tumor at the primary site after total resection, or at a distant site; (**2**) Definite increase in the size of a residual lesion, even if clinically asymptomatic. Patients who developed symptomatic recurrence were advised to undergo re-surgery.

Histopathology and Immunohistochemistry (IHC)

Paraffin blocks of all cases (n=53), with a histological diagnosis of anaplastic astrocytoma were retrieved from the archives of the department of Neuropathology, NIMHANS. These cases were reviewed by the neuropathologist (VS), their histological features were examined by light microscopy using hematoxylin and eosin stain and diagnosis was re-confirmed. Tumor sections (4 μm) were collected on silane coated slides and IHC was performed to analyse the protein expression pattern of IGFBP isoforms (IGFBP-2, -3 -5 and -7). Tissue sections were stained using the polyclonal IGFBP antibodies (Santa Cruz Biotechnology, U.S.A) as follows: IGFBP-2 (C-18: sc-6001, dilution 1:100), IGFBP-3 (H-98: sc-9028, dilution 1:50), IGFBP-5 (H-100: sc-13093, dilution 1:25), IGFBP-7 (H-102: sc-13095, dilution 1:25). The antigen retrieval was performed by heat treatment of the deparaffinized sections in a microwave oven for 25–35 min at 700 W in citrate buffer (10 mM, pH 6.0). After the initial processing steps, sections were incubated overnight with primary antibody at room temperature. This was followed by incubation with the linked streptavidin-biotinylated secondary antibody (universal LSAB, DAKO, Denmark) for IGFBP-2 and with supersensitive non-biotin HRP detection system (QD440-XAK, Biogenex, U.S.A) for the other antibodies. 3, 3′-Diaminobenzidine (Sigma-Aldrich, St. Louis, U.S.A) was used as the chromogenic substrate. Glioblastoma tumors that had stained intensely positive for IGFBP-2, -3 and -5 by IHC in our earlier study [15] were used as positive controls. For IGFBP-7, human kidney section was used as positive control. A negative control slide in which the primary antibody was excluded was incorporated with each batch of staining. For comparison, normal brain samples from a portion of anterior temporal cortex resected from patients who underwent surgery for intractable epilepsy were assessed for expression of IGFBP isoforms.

A visual semi quantitative grading scale was applied to assess the intensity of the immunoreactivity as follows: Zero (0) if the staining was absent, 1+, if it was weak and 2+, if it was strong. Only 2+ staining intensity was considered for analysis as previously described [15]. The immunopositivity of IGFBP-2, -3, -5 and -7 was assessed in more than 1000 cells from each tumor specimen. The IGFBP labeling index (LI) was expressed as percentage of cells that showed 2+ positive staining among the total number of cells that was counted.

Statistical Analysis

The demographic and clinical data of the patients were analysed using SPSS 15.0 for Windows software. Descriptive statistical analysis was carried out and the results for continuous variables are presented as mean±SD and categorical variables as number (%).

All continuous variables were tested for normal distribution and non-parametric tests were used wherever required. The correlation between continuous variables was performed using Spearman rank correlation analysis. Kaplan Meier survival analysis was performed to estimate median survival for overall and progression free survival duration. Comparison of mean isoform expression was performed by pairwise comparison analysis. The post-hoc pairwise comparison of survival pattern between groups was done using Log rank test. When the number of outcome event is less than 50 % for any given group, mean survival was reported. The significance of a continuous variable on survival duration was tested using Cox proportional hazard analysis to arrive at the predictors of survival and results

Table 1 Clinical profile of patients (n=53) with anaplastic astrocytoma in the retrospective cohort

Symptom	Frequency of occurrence	Percentage (%)
Raised ICP	31	58.5
Seizures	45	85
Motor/sensory deficits	22	41.5
Cognitive deficits	4	7.5
Acute deterioration	4	7.5

were expressed as P value and hazard ratio. A P value of <0.05 was considered statistically significant.

Results

In this retrospective cohort, the mean age of patients at presentation was 34.2 years (Median: 35 years; Range: 18–59 years). Majority of the patients were males (66 %; n=35). The clinical data are summarized in Table 1. Near total resection was done in 45 patients (84.9 %) and subtotal resection in 8 patients (15.1 %). All patients were administered radiotherapy postoperatively. 12 out of 53 patients received adjuvant chemotherapy, either PCV (Procarbazine, Lomustine and Vincristine) or temozolomide. The median follow up was 42 months (Range: 5–74 months). Out of 53 patients, 17 (32.1 %) had expired due to tumor progression. Twenty seven patients (50.9 %) had recurrence during the follow up. Symptomatic patients who had radiological recurrence were advised re-surgery, considering their general condition, operability of the lesion and KPS. Out of 27 patients, 7 underwent re-surgery. In other patients, surgery was deferred due to inoperability of the lesion, poor functional status and denial of the consent.

IHC analysis of IGFBP-2, -3, -5 and -7

The protein expression pattern of IGFBP-2, -3, -5 and -7 was analysed by IHC. The expression of the IGFBP-2, -3, -5 and -7 was mostly confined to the cytoplasm of neoplastic astrocytic cells. The pattern of staining was intra cytoplasmic, homogenous and diffuse for IGFBP-2, -3 and -5 and granular for IGFBP-7 (Fig. 1). Blood vessel wall staining for IGFBP-7 was seen in only two cases. Heterogeneity of staining was noted within individual tumour samples, which showed variations in intensity. None of the IGFBP isoforms were expressed in the normal brain tissues (n=5).

The median (mean±SD) LI for IGFBP-2, IGFBP-3, IGFBP5, IGFBP-7 were 0.000 (3.87±5.771), 20.000 (15.66±10.239), 15 (14.91±9.379), 10 (13.08±13.903), respectively. It was noted that the mean IGFBP-2 expression was significantly low in AA as compared to all other isoforms (P<0.001; Table 2). IGFBP-3 expression in AA was higher when compared to other isoforms such as IGFBP-2, -5 and -7.

Protein Expression Pattern of IGFBP Isoforms in Recurrent Tumors

Out of 27 recurrent cases, 7 had undergone re-surgery. 4 were AA and 3 were GBM on recurrence. IHC was

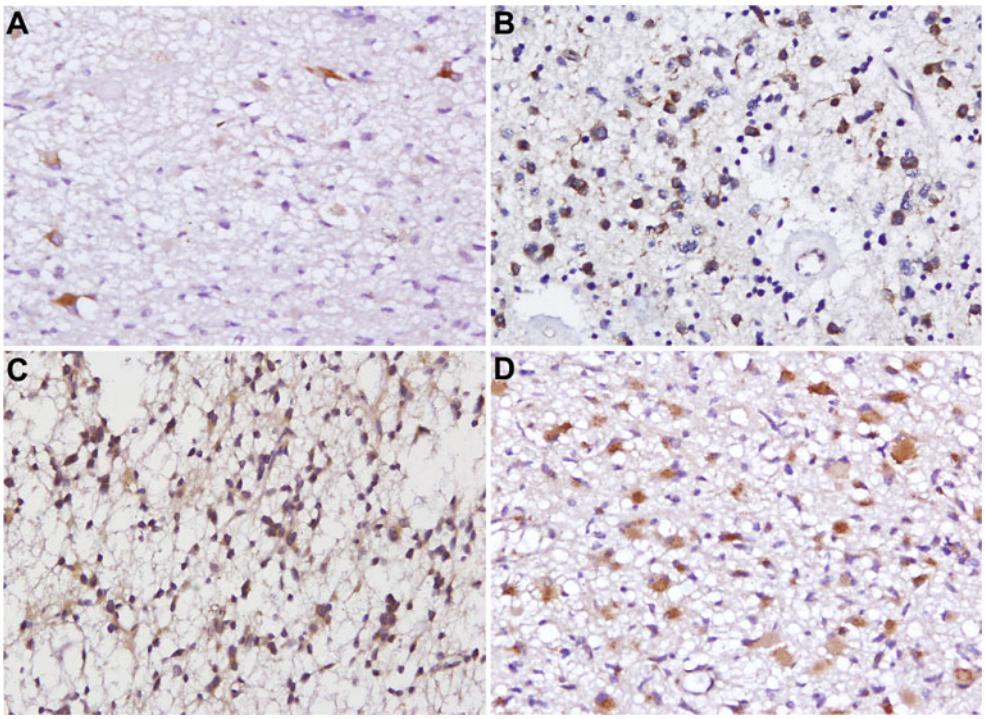

Fig. 1 Expression of various IGFBP isoforms in AA; IGFBP-2 **a**, IGFBP-3 **b**, IGFBP-4 **c** and IGFBP-7 **d**. All isoforms show cytoplasmic staining. Note that the expression of IGFBP-2 is significantly lower than the other isoforms**.** Original magnification x160

Table 2 The expression patterns of various isoforms

Isoform expression (n=53)	Minimum	Maximum	Mean±SD	P value—pair wise comparison of means
IGFBP-2	0	20	3.87±5.771	IGFBP-2 vs IGFBP-3 : P<0.0001
IGFBP-3	0	35	15.66±10.239	IGFBP-2 vs IGFBP-5 : P<0.0001
IGFBP-5	0	30	14.91±9.379	IGFBP-2 vs IGFBP-7 : P<0.0001
IGFBP-7	0	50	13.08±13.903	

performed on 5 out 7 recurrent tumor samples. An increased protein expression of the IGFBP isoforms was observed when compared to its corresponding primary tumor sample, regardless of whether the recurrent tumor was AA or GBM (Table 3). Representative micrographs of IHC results in recurrent tumors are shown in Figs. 2.

Effect of Clinical Variables and IGFBP-2, -3, -5 and -7 Protein Expression on Patient Survival

Univariate analysis revealed that none of the clinical variables (patient age, patient gender, KPS,) was associated with survival in this cohort of subjects with anaplastic astrocytoma. The extent of tumor resection was found to be a significant factor influencing both overall survival (P value=0.032) and progression free survival (P=0.031).

Interestingly, univariate Cox regression model demonstrated that among the IGFBP isoforms, only IGFBP-3 LI predicted a favorable prognosis for overall survival (hazard's ratio: 0.955; P=0.055) in this retrospective cohort. None of the IGFBP isoforms were associated with progression free survival.

All variables which were significant in univariate analysis (with P<0.1) were considered for multivariate analysis. A Cox proportional hazard analysis was performed initially with enter method followed by Wald forward stepwise analysis and hazard ratios were obtained. Multivariate analysis revealed that only extent of resection was associated with shorter survival (hazard's ratio: 0.296; P=0.028) However, the independent prognostic value of IGFBP-3 LI could not be elicited in this retrospective cohort.

Discussion

The IGFBP superfamily comprises six members (IGFBP-1 to IGFBP-6) which regulate tumor growth and invasion by binding and modulating the bioavailability of the Insulin growth factor ligands (IGF1&2) [11, 12]. The expression pattern of some IGFBP isoforms in gliomas has been documented in earlier studies with an emphasis on glioblastoma [11, 12]. Over expression of IGFBP-2 has been reported in glioblastoma [13, 14, 16]. IGFBP-2 has been shown to increase the invasive potential of glioblastoma cells [17]. There are many studies which have shown IGFBP-2 as an important prognostic marker in glioblastoma [18, 19]. An increased plasma level of IGFBP-2 has also been shown to be associated with adverse prognosis in high grade glioma patients [20]. In one report on the expression profiling of genes in glioblastoma, IGFBP-3 was classified as an up regulated gene [15]. In our previous cDNA microarray study, we had shown a differential expression of IGFBP-2 and -5 in astrocytoma tissues [19].

There are very few studies describing the pattern of expression of various IGFBP isoforms in different histologic subgroups of glioma [15, 21]. An earlier study by Wang et al has shown that expression of IGFBP-5, but not IGFBP-3, increases with glioma progression from lower grade to higher. In view of the strong correlation between overexpression of IGFBP-5 and histologic grade, the authors suggested that IGFBP-5 may play a role in glioma progression in addition to IGFBP-2. Our group has previously shown grade specific expression of various IGFBP isoforms, both at the mRNA and protein level in diffusely infiltrating astrocytomas. An increased expression pattern of IGFBP-2, -3 and -5 with increasing grades of malignancy and

Table 3 IGFBP protein expression (LI) in primary and recurrent samples of different cases (n=5)

CASE	CASE 1		CASE 2		CASE 3		CASE 4		CASE 5	
	Primary AA	Recurrent AA	Primary AA	Recurrent AA	Primary AA	Recurrent GBM	Primary AA	Recurrent AA	Primary AA	Recurrent AA
IGFBP-2	15	15	10	15	15	35	NEG	35	NEG	NEG
IGFBP-3	5	5	10	30	20	30	10	25	30	30
IGFBP-5	NEG	NEG	NEG	25	15	15	10	10	10	10
IGFBP-7	35	35	10	20	20	35	NEG	40	NEG	NEG

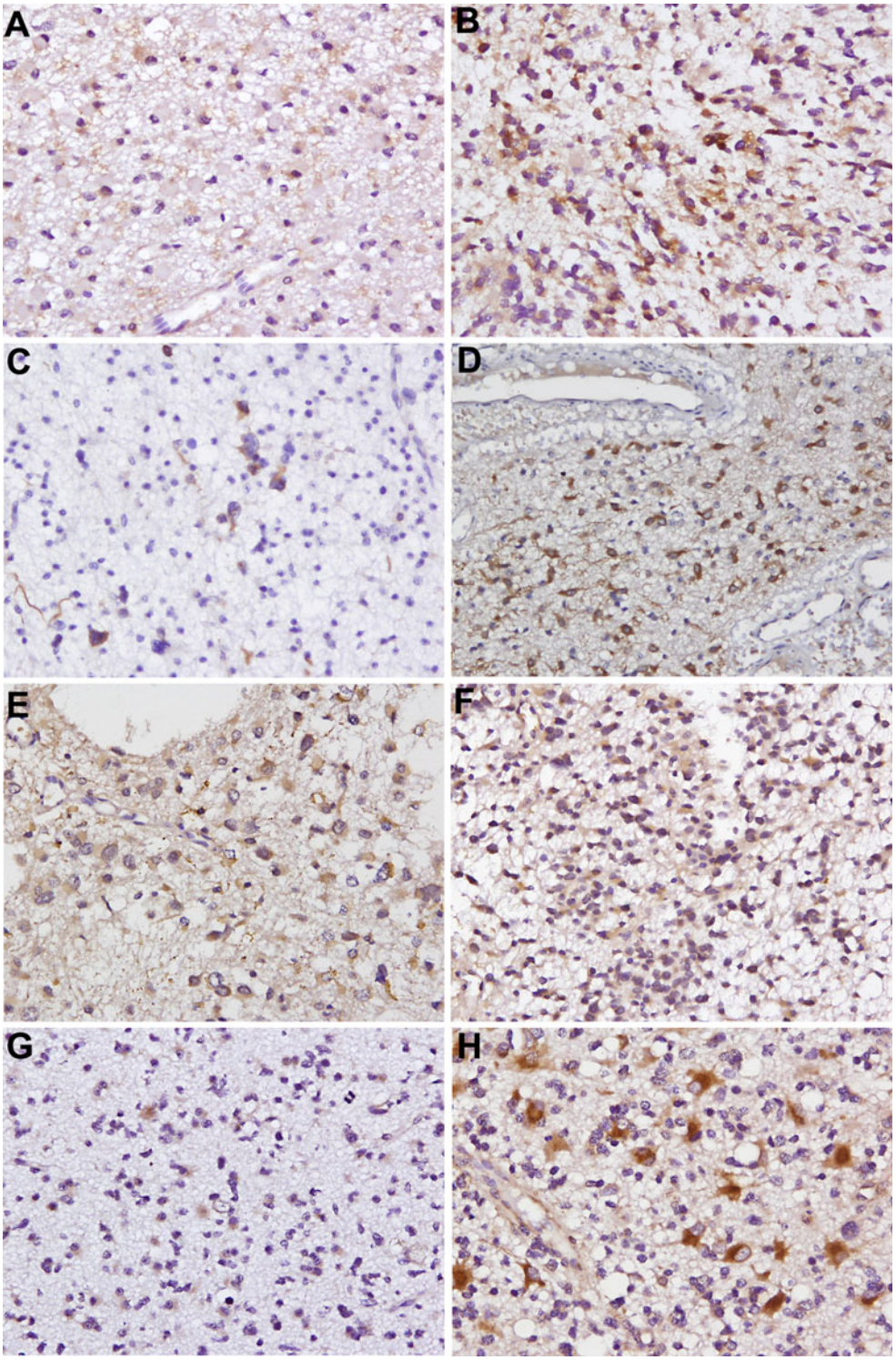

Fig. 2 Microphotographs showing increased expression of IGFBP isoforms with tumor recurrence; IGFBP-2- Primary **a**, recurrent **b**. IGFBP-3- Primary **c**, recurrent **d** IGFBP-5- Primary **e**, recurrent **f**, IGFBP-7- Primary **g**, recurrent **h**. Original magnification x160

maximum expression was noted in glioblastoma. In this study IGFBP-3 emerged as a strong independent adverse prognostic biomarker of glioblastoma [15].

The present study is the first of its kind to describe comprehensively the pattern of expression of IGFBP isoforms (IGFBP-2, -3, -5 and -7) exclusively in one homogeneous group of AA and correlate their expression with clinical outcome. Among AA tumors, we found the expression of IGFBP-2 was significantly low. Similar observations were reported earlier in our previous study comprising of a small number of AA tumors [15]. This is in stark contrast to the overexpression of IGFBP-2 and its poor prognostic impact in GBM reported in literature [13, 14, 16, 18, 19]. This indicates that IGFBP-2 is a specific molecular marker differentiating AA from GBM.

The present study demonstrated that IGFBP-3 expression correlated with overall survival with a trend towards significance (P=0.055, hazard's ratio=0.955) on univariate analysis. Increased expression of IGFBP-3 was associated with a better overall survival, but not with progression free survival. In our previous study, we have observed an opposite finding in glioblastoma patients, where we have shown IGFBP-2, -3 and -5 to be associated with shorter survival in the univariate model and in the multivariate model, IGFBP-3 was observed to be an independent prognostic marker for shorter survival [15]. These contradictory observations in the two different sets of high grade astrocytomas seem interesting and probably indicate that AAs differ completely in their biological profile when compared to glioblastomas.

Literature search shows the role of IGFBP-3 to differ completely in diverse human neoplasms. Both tumorigenic (growth promotion) and antitumerigenic (proapoptotic and anti proliferative) roles have been proposed. The role of IGFBP-3 has been well studied in other malignancies like breast cancer and over expression of IGFBP-3 has been shown to be associated with poor prognosis in breast cancer [22, 23]. Also, in vitro studies have shown that the potential mitogenicity of IGFBP-3 is mediated through its interactions with EGFR and RAS–p44/42 MAPK signaling in breast epithelial cells [24]. On the other hand, IGFBP-3 has been shown to potently inhibit proliferation of various cell types in an insulin-like growth factor (IGF)-independent manner. Kim HS et al has shown that IGFBP-3 induces apoptosis in an IGF-independent manner through the activation of caspases in human breast cancer cells [24]. This group also showed that, IGFBP-3 leads to G1 cell cycle arrest with inhibition of CDK2 and CDK4. Hence IGFBP-3 exerts its growth inhibitory action not only through induction of apoptosis but also the G1 cell cycle arrest in human breast cancer cells [25]. Recently IGFBP-3 has been shown to have tumor suppressive effects in lung cancer and gastrointestinal stromal tumors [26, 27]. In the present study, IGFBP-3 expression correlated with better survival among AA patients. It could be possible that expression of IGFBP-3 in AA tumors and association with better prognosis is an epiphenomenon, secondary to some other molecular alterations that determine its expression.

IGFBP-2 and IGFBP-5 have been shown to be associated with adverse clinical outcome in other systemic high grade tumors [28–30]. We have also previously seen this effect in glioblastoma patients [15]. However, in the present study, IGFBP-2 expression was the least and did not correlate with overall or progression free survival in AA patients, again confirming their differing roles in specific subsets of high grade gliomas.

On analyzing the pattern of IGFBP isoform expression in paired samples of recurrent tumors, it was noted that all the isoforms showed an increased expression with recurrence (irrespective of the tumor recurring as AA or as GBM). This suggests a clonal expansion of tumor cells expressing these molecules with tumor progression. To the best of our knowledge, such an observation with respect to these molecules has not been made earlier. Despite this clonal expansion of IGFBP expressing cells with tumor progression, IGFBP-3 seems to be a favourable prognostic biomarker for AA patients. Since this is contradictory to its behavior in glioblastoma, further studies are required to elucidate its functional role in different subsets of high grade glioma.

Conclusions

The present study is the first of its kind to describe comprehensively the pattern of expression of IGFBP isoforms (IGFBP-2, -3, -5 and -7) in anaplastic astrocytomas. IGFBP-2 and IGFBP-3 expression patterns and correlation to prognosis were distinct in anaplastic astrocytoma patients, contradictory to that in glioblastoma. The uniqueness of the expression patterns of these isoforms in AA probably underscores them as a distinct molecular entity.

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