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Research Article

Cancer Epidemiology, Biomarkers & Prevention

Grade-Specific Expression of Insulin-like Growth Factor–Binding Proteins-2, -3, and -5 in Astrocytomas: IGFBP-3 Emerges as a Strong Predictor of Survival in Patients with Newly Diagnosed Glioblastoma

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Abstract

Background: Insulin-like growth factor (IGF)–binding protein (IGFBP) isoforms have been implicated in the pathogenesis of human neoplasms including glioma. In view of this, we evaluated the expression of IGFBP isoforms (IGFBP-2, -3, and -5) during malignant progression of astrocytoma and their prognostic significance in glioblastoma.

Methods: The expression of IGFBP isoforms was analyzed in diffusely infiltrating astrocytomas by real-time quantitative PCR ($n = 203$) and immunohistochemistry ($n = 256$). Statistical methods were used to assess their grade-specific expression pattern and mRNA-protein intercorrelation. Survival analyses were done on a uniformly treated, prospective cohort of adult patients with newly diagnosed glioblastoma ($n = 136$) by using Cox regression models.

Results: The mean transcript levels of IGFBP-2 and -3 were significantly higher in glioblastomas (GBM) relative to anaplastic astrocytoma (AA), diffuse astrocytoma (DA), and controls whereas IGFBP-5 mRNA was higher in GBM relative to AA and controls ($P < 0.05$). By immunohistochemistry, the mean labeling index of all isoforms was significantly higher in GBM compared with AA, DA, and control ($P < 0.05$). A strong positive correlation was observed between their respective mRNA and protein expressions ($P < 0.01$). Multivariate analysis revealed IGFBP-3 expression (hazard ratio, 1.021; $P = 0.030$) and patient age (hazard ratio, 1.027; $P = 0.007$) to be associated with shorter survival in glioblastoma.

Conclusions: This study shows the associations of IGFBP-2, -3, and -5 expression with increasing grades of malignancy in astrocytomas. IGFBP-3 is identified as a novel prognostic glioblastoma biomarker. The strong correlation between their mRNA and protein expression patterns suggests their role in the pathogenesis of these tumors.

Impact: IGFBP isoforms have emerged as biomarkers with diagnostic and prognostic utility in astrocytomas.

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Introduction

Diffusely infiltrating astrocytomas are the most common intrinsic brain tumors in adults accounting for more than 60% of primary central nervous system neoplasms (1). They are further divided into three grades according to the current WHO classification scheme which includes diffuse astrocytoma (DA/grade II), anaplastic astrocytoma (AA/grade III), and glioblastoma (GBM/grade IV; ref. 2). Among them, GBM is the most malignant and the majority of affected patients exhibit rapid disease progression despite aggressive surgery, radiation, and chemotherapy with median survival times remaining in the 12- to 15-month range (3). However,

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the prognosis of individual patients is variable and ~10% are known to survive for more than 2 years (4). At present, the established survival predictors in patients with glioblastoma are: O^6-methylguanine-DNA methyltransferase promoter methylation status, age, Karnofsky performance score, extent of resection, and mini mental state examination (5, 6). However, the recent molecular and genetic profiling studies have provided a better understanding of prognostic and predictive factors of GBM along with underlying mechanisms of resistance to standard therapies (3).

Recently, the insulin-like growth factor (IGF) signaling pathway has been implicated in the progression of glioma (7, 8). The IGF signaling axis involves the complex coordinated actions of two ligands (IGF-I and IGF-II), three cell surface receptors (IGF-IR, IGF-IIR, and IR), IGF-binding proteins IGFBPs, and the proteases that affect the binding proteins. These interactions are thought to influence IGF bioavailability (7, 9). The mitogenic effects of IGFs are mainly mediated through interactions with IGF-IR followed by the activation of its downstream effector molecules.

Earlier studies have shown that human gliomas overexpress IGFs and IGFRs when compared with the normal brain (10, 11). Among the IGFBPs, the expression of isoforms-2, -3, and -5 have been studied in gliomas. IGFBP-2 overexpression has been reported in GBM (12-15), was found to increase the invasive potential of GBM cells (16), and has been associated with poor patient prognosis in GBM cases (15-17). A recent study reports increased plasma levels of IGFBP-2 to be associated with adverse prognosis in patients with high-grade glioma (18). Among other IGFBPs, increased expression of IGFBP-5 compared with IGFBP-3 has been reported in ascending grades of astrocytoma (19). In our previous cDNA microarray study, we have shown a differential expression of IGFBP-2 and -5 in astrocytoma tissues (20). In view of this and with emerging data on the role of some IGFBP isoforms in gliomas, the aim of this study was to evaluate the association of three IGFBP isoforms (IGFBP-2, -3, and -5) with the malignant progression of astrocytoma and assess their value as predictors of survival in patients with glioblastoma. To accomplish this, we have analyzed the expression of IGFBP-2, -3, and -5 both at the mRNA and protein level on tumor tissues of diffusely infiltrating astrocytoma, including DA, AA, and GBM. A subset of GBM tissues were from a large cohort of patients with newly diagnosed glioblastoma subjected to uniform standard therapeutic protocol and followed up prospectively. In this subset, the protein expressions of IGFBP-2, -3, and -5 in the tumor tissue were correlated with patient survival.

Materials and Methods

Patient and tissue samples

Samples of diffusely infiltrating astrocytoma of different grades (DA, AA, and GBM) were obtained from patients who underwent surgery at the National Institute of Mental Health and Neurosciences and Sri Sathya Sai Institute of Higher Medical Sciences, Bangalore, India. Control samples comprised a portion of anterior temporal cortex resected from patients who underwent surgery for intractable epilepsy. Tissues were freshly received from the neurosurgical operating rooms, bisected, and one half was placed in RNAlater (Ambion, Inc.), stored at −70°C and used for RNA isolation. The other half was fixed in 10% buffered neutral formalin, processed for paraffin sections, and was used for histopathology and immunohistochemistry. The mRNA expression of IGFBP-2, -3, and -5 was analyzed by real-time quantitative PCR (qPCR) on 223 samples (16 DA, 50 AA, 137 GBM, and 20 control brain tissues). The expression of these molecules at the protein level was analyzed on tumor tissue sections by immunohistochemistry on 268 samples (15 DA, 16 AA, 225 GBM, and 12 control brain tissues). Among these, 142 samples were analyzed in common by both qPCR and immunohistochemistry (3 DA, 10 AA, 125 GBM, and 4 control brain tissues). Among the GBM samples, 136 were from a set of patients with newly diagnosed glioblastoma, subjected to uniform treatment and followed up prospectively. In this cohort, both qPCR and immunohistochemistry was done on 102 samples and the rest were analyzed by immunohistochemistry only.

Prospective study of patients with newly diagnosed glioblastoma

The study has been scrutinized and approved by the ethics committee of two clinical centers (National Institute of Mental Health and Neurosciences and the Sri Sathya Sai Institute of Higher Medical Sciences) and patient consent was obtained prior to initiation of the study. In this prospective study, patients with newly diagnosed glioblastoma (n = 136) who underwent surgery in these two clinical centers between July 2006 to February 2009 were studied. Inclusion criteria for the study were (*a*) adult patients between the ages of 18 and 65 years with supratentorial lobar tumor, (*b*) patients who underwent macroscopic total/near total resection of the tumor as assessed by postoperative magnetic resonance imaging scan, and (*c*) patients with a postoperative Karnofsky's performance score of ≥70. The demographic data and clinical features of these patients were collected. All patients were treated uniformly with radiotherapy and chemotherapy. Radiotherapy was administered at a total dose of 59.4 Gy, given in 33 fractions along with concomitant chemotherapy with temozolomide, administered at a dose of 100 mg/d, which was continued daily for 45 days. Subsequently, five cycles of cyclical chemotherapy with temozolomide at a dose of 150 mg/m^2 body surface area for 5 days every 28 days was administered.

The patients were followed up clinically including magnetic resonance imaging. The clinical status in the form of neurologic deficits and Karnofsky's performance score were documented regularly. Overall survival was

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defined as the duration between surgery and death of the patient due to disease. Outcome analysis was done in September 2009. All patients had completed the adjuvant therapy protocol and were either on follow-up or had expired at the time of evaluation. The maximum follow-up period was 34 months. The median survival of patients of the whole group was 17 months. The 2-year survival rate of this cohort was 32%.

Real-time qPCR assay

Total RNA was isolated from tissues using TRI reagent (Sigma-Aldrich) as per the manufacturer's protocol. The concentration of RNA was estimated by measuring the absorbance at 260 nm and integrity was verified on a denaturing 1% MOPS-formaldehyde agarose gel followed by ethidium bromide staining. The relative quantification of the mRNA transcripts and expression levels of IGFBP-2, -3, and -5 genes was carried out using a two-step strategy. In the first step, cDNA was generated from total RNA derived from different tissue samples using the cDNA synthesis kit (Applied Biosystems). Subsequently, real-time qPCR was carried out in the ABI PRISM 7900 sequence detection system (Applied Biosystems) with the cDNA as template (equivalent to 10 ng of RNA) and gene-specific primer sets for each of the IGFBP genes using the DyNAmo HS SYBR Green qPCR kit (Finnzymes, Finland). Data were analyzed according to the relative quantification model proposed by Pfaffl (21). All measurements were made in triplicate, and for each real-time qPCR primer set, the reaction efficiency estimates were derived from standard curves that were generated using serial dilutions of the pooled cDNA set used for the study. For normalization, the mean expression levels of four genes, ribosomal protein L35a (*RP-L35a*), 1-acylglycerol-3-phosphate *O*-acyltransferase 1 (*AGPAT1*), ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C1 (subunit 9; *ATP5G1*), and glycyl-tRNA synthetase (*GARS*) were used as internal controls because their expression levels were found to be unaltered in our previous microarray data (20, 22). Control brain tissue samples (n = 20) from patients with epilepsy were individually used as reference. To assess grade-specific upregulation of IGFBP-2, -3, and -5 in diffusely infiltrating astrocytomas, qPCR was carried out on 223 samples that included 16 DA, 50 AA, and 137 GBM along with 20 control samples. The primer sequences for all the genes are shown in Table 1.

Histopathology and immunohistochemistry

Histologic sections of normal brain and tumor tissues were examined by light microscopy using H&E stain. Tumor sections of diffusely infiltrating astrocytoma were graded using the WHO grading scheme (1, 2). Paraffin sections (4 μm) from the tumor tissue and control samples were collected on silane-coated slides and immunohistochemistry for the protein expression of IGFBP-2, -3, and -5 were carried out on 268 samples that included 12 control brain tissues, 15 DA, 16 AA, and 225 GBM tumors. The polyclonal IGFBP antibodies were from Santa Cruz Biotechnology. The details of the primary antibodies are IGFBP-2 (C-18: sc-6001 diluted to 1:100), IGFBP-3 (H-98: sc-9028, diluted to 1:50), and IGFBP-5 (H-100: sc-13093 diluted to 1:25). Antigen retrieval was done by heat treatment of the deparaffinized sections in a microwave oven for 25 to 35 minutes at 700 W in citrate

Table 1. Sequences of the primers used for qPCR

Sequence no.	Gene	Sequence (5′ → 3′)	Primer length	GenBank accession no.*	Amplicon length
1	IGFBP2_F	GACAATGGCGATGACCACTCA	21	NM_000597.2 (571-691)	
	IGFBP2_R	GCTCCTTCATACCCGACTTGA	21		120†
2	IGFBP3_F	AGAGCACAGATACCCAGAACT	21	BC064987 (694-798)	
	IGFBP3_R	TGAGGAACTTCAGGTGATTCAGT	23		104‡
3	IGFBP5_F	CGGGGTTTGCCTCAACGAA	19	NM_000599 (1064-1180)	
	IGFBP5_R	TCTTGGGGGAGTAGGTCTCCT	21		116‡
4	RPL35a_F	ACGCCCGAGATGAAACAG	18	BC017093 (209-428)	
	RPL35a_R	GGGTACAGCATCACTCGGA	19		219‡
5	AGPAT1_F	TGTCCCCATAGTCATGTCCTC	21	NM_006411.2 (933-1105)	
	AGPAT1_R	GGAAAACAGTGAGCATGGAGT	21		172‡
6	ATP5G1_F	CCAGACGGGAGTTCCAGAC	19	NM_005175.2 (283-440)	
	ATP5G1_R	GACGGGTTCCTGGCATAGC	19		157‡
7	GARS_F	TCCTCTGTTTGAAGGGCAAG	20	NM_002047.1 (2525-2710)	
	GARS_R	AAATCAGGCAGCCACTGAAG	20		185†

*Numbers in parentheses denote the position of the amplicon in the GenBank sequence.
†Intraexonic.
‡Interexonic.

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buffer (10 mmol/L; pH 6.0). After the initial processing steps, sections were incubated overnight with primary antibody at 4°C. This was followed by incubation with the linked streptavidin- biotinylated secondary antibody (Universal LSAB, DAKO, Denmark) for IGFBP-2 and with supersensitive non-biotin horseradish peroxidase detection system (QD440-XAK, Biogenex) for the other antibodies. 3, 3′-Diaminobenzidine (Sigma-Aldrich) was used as the chromogenic substrate. GBM tumors that showed markedly increased mRNA levels of IGFBP-2, -3, and -5, respectively, by qPCR experiments served as positive controls. A negative control slide in which the primary antibody was excluded was incorporated with each batch of staining. A visual semiquantitative 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. The immunopositivity of IGFBP-2, -3, and -5 was assessed in more than 1,000 cells from each tumor specimen. The IGFBP labeling index (LI) was expressed as a percentage of cells that showed 2+ positive staining among the total number of cells that were counted.

Statistical analysis

Grade-specific expression of IGFBP-2, -3, and -5 (mRNA and protein) in astrocytoma tissues. All continuous variables were tested for normal distribution and they were found to be non-normal. To determine the grade-specific upregulation, a nonparametric test, Kruskal-Wallis one-way ANOVA on ranks, followed by Dunn's post hoc test was done to adjust the α level to minimize the type I error rate. The results were expressed in the form of median and mean ± SD. $P < 0.05$ was considered to be statistically significant. Spearman rank-correlation coefficient was used to assess the pairwise correlation between mRNA and protein expression of IGFBP-2, -3, and -5 in all grades of astrocytoma and control tissues (n = 142; DA = 3, AA = 10, GBM = 125, control = 4).

Correlation of IGFBP-2, -3, and -5 protein expressions with survival in patients with newly diagnosed glioblastoma. Because the extent of surgical resection was uniform for all patients in this cohort (macroscopic total/near total resection), and postoperative Karnofsky's performance scores were uniformly ≥70, the only clinical variable included for analyses was patient age. The significance of continuous variables (patient age, IGFBP-2, -3, and -5 LIs) were assessed using univariate Cox regression models. Variables that were associated with survival in the univariate analyses ($P < 0.10$) were considered for potential inclusion into the multivariate model. Forward stepwise selection was used to determine the final Cox regression model. Results were reported using the P values and the estimated hazard ratio (HR) with their 95% confidence intervals. $P < 0.05$ was considered significant and all exact two-sided P values were reported.

Results

Grade-specific expression pattern of IGFBP-2, -3, and -5: a distinct discrimination between GBM and other grades of astrocytoma

qPCR analysis of IGFBP-2, -3, and -5. The expression patterns of IGFBP-2, -3, and -5 across 223 samples that

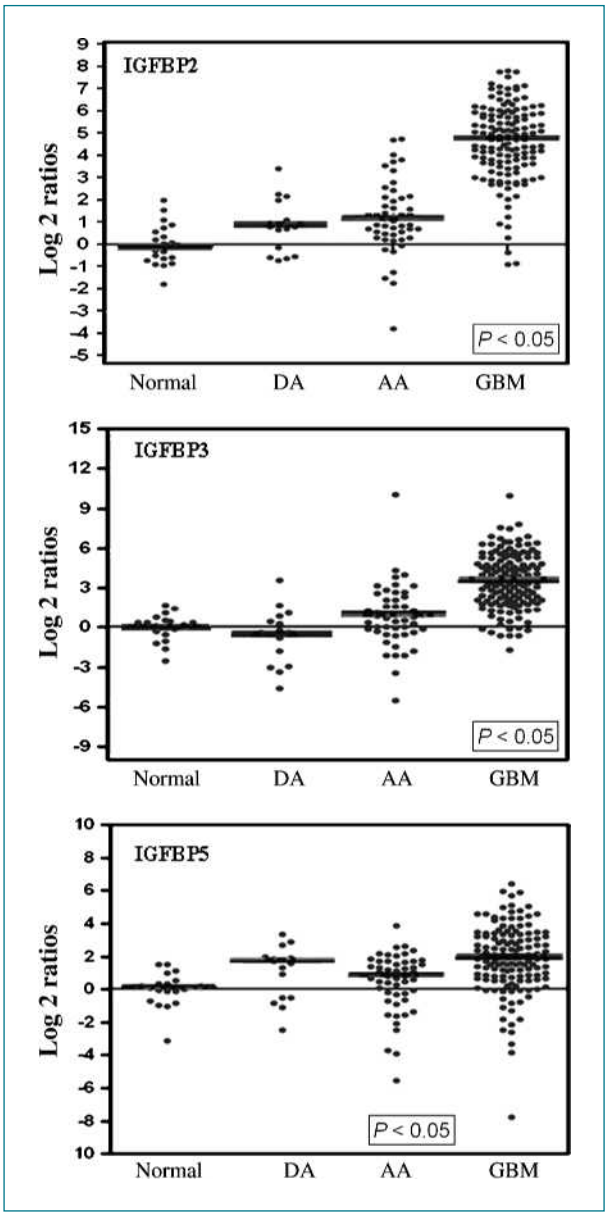

Figure 1. mRNA expression pattern of IGFBP-2, -3, and -5 in various grades of astrocytoma. Scatter plots showing the differentially regulated IGFBP isoform genes in diffusely infiltrating astrocytoma. Log 2-transformed gene expression ratios obtained from qPCR analyses are plotted for IGFBP-2, IGFBP-3, and IGFBP-5 on *Y*-axis against control, DA, AA, and GBM samples on the *X*-axis. A significant difference in the median log 2 ratios of IGFBP isoforms was observed between GBM, AA, DA, and control by Kruskal-Wallis one-way ANOVA followed by Dunn's post hoc test ($P < 0.05$). Bars, group medians.

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Table 2. Individual group differences in the LI (immunohistochemistry) and log 2 ratios (qPCR) of IGFBP-2, -3, and -5

Variables	Normal	DA	AA	GBM	Kruskal-Wallis test	
					χ^2; *P*	Post hoc *P**
IGFBP-2 LI	0.0000 ± 0.0000 (0.0000)	0.0000 ± 0.0000 (0.0000)	2.500 ± 8.756 (0.0000)	30.070 ± 17.150 (30.000)	81.92; <0.0001	<0.05[†] <0.05[‡] <0.05[§]
IGFBP-2 log 2 ratio	0.0000 ± 0.935 (–0.116)	0.816 ± 1.195 (0.789)	1.210 ± 1.606 (1.100)	4.602 ± 1.724 (4.815)	125.30; <0.0001	<0.05[†] <0.05[‡] <0.05[§]
IGFBP-3 LI	0.0000 ± 0.0000 (0.0000)	6.333 ± 10.93 (0.0000)	12.500 ± 13.780 (10.000)	34.240 ± 17.020 (35.000)	73.53; <0.0001	<0.05[†] <0.05[‡] <0.05[§]
IGFBP-3 log 2 ratio	0.0000 ± 1.017 (0.123)	–0.624 ± 2.105 (–0.386)	0.866 ± 2.343 (0.819)	3.542 ± 2.123 (3.462)	89.00; <0.0001	<0.05[†] <0.05[‡] <0.05[§]
IGFBP-5 LI	0.0000 ± 0.0000 (0.0000)	6.000 ± 10.390 (0.0000)	7.500 ± 10.000 (0.0000)	24.820 ± 13.680 (25.000)	64.09; <0.0001	<0.05[†] <0.05[‡] <0.05[§]
IGFBP-5 log 2 ratio	0.0000 ± 1.038 (0.128)	1.039 ± 1.634 (1.621)	0.425 ± 1.818 (0.814)	1.844 ± 2.077 (1.909)	36.05; <0.0001	<0.05[†] >0.05[‡] <0.05[§]

NOTE: Values in parentheses are the median.
*Dunn's post hoc *P* value.
[†]Normal vs. GBM.
[‡]DA vs. GBM.
[§]AA vs. GBM.

included 16 DA, 50 AA, 137 GBM tumors, and 20 control brain tissues using the SYBR Green–based qPCR assay was analyzed as described in Materials and Methods. We have chosen to use mean expression levels of four genes, i.e., *RPL35a, AGPAT1, ATP5G1*, and *GARS* for normalization of the gene expression data in our studies. These are not the standard normalization genes reported in the literature for glioma studies. We tested the expression levels of the standard genes such as glyceraldehyde-3-phosphate dehydrogenase and β-actin. These genes were found to have a regulated pattern between normals and tumors. Hence, we analyzed the microarray data and identified several unregulated genes that were consistent across all the tumors. These four genes were the most consistently unregulated genes between tumors compared with normal tissues. The microarray data were validated in large number of tumors by qPCR in comparison with control brain tissues (22). Because there are no standard reference normalization gene(s) reported for glioma, we propose that these genes could be used as routine normalization genes in glioma studies.

Significant differential mRNA expressions of IGFBP-2, -3, and -5 were observed in diffusely infiltrating astrocytomas (Fig. 1). Individual group differences in log 2 ratios were statistically significant as shown in the Table 2. The median (mean ± SD) log 2 ratio of IGFBP-2 in GBM was 4.815 (4.602 ± 1.724), whereas in AA, DA, and controls, it was 1.100 (1.210 ± 1.606), 0.789 (0.816 ± 1.195), and −0.116 (0.0000 ± 0.0.935), respectively. The comparison of median log 2 ratios between groups by Kruskal-Wallis one-way ANOVA followed by Dunn's post hoc test showed that the difference between GBM and control, GBM and DA, and GBM and AA were significant ($P < 0.05$).

For IGFBP-3, the median (mean ± SD) log 2 ratio in GBM was 3.462 (3.542 ± 2.123), whereas in AA, DA, and controls, it was 0.819 (0.866 ± 2.343), −0.386 (−0.624 ± 2.105), and 0.123 (0.0000 ± 0.1.017), respectively. The comparison of median log 2 ratios between groups by Kruskal-Wallis one-way ANOVA followed by Dunn's post hoc test showed that the differences between GBM and control, GBM and DA, and GBM and AA were significant ($P < 0.05$).

For IGFBP-5, the median (mean ± SD) log 2 ratio in GBM was 1.909 (1.844 ± 2.077) compared with AA (0.814; 0.425 ± 1.818), DA (1.621; 1.039 ± 1.634), and control (0.128; 0.0000 ± 1.038). The comparison of median log 2 ratios between groups by Kruskal-Wallis one-way ANOVA followed by Dunn's post hoc test showed that the difference between GBM and control and GBM and

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AA were significant ($P < 0.05$). However, the difference between GBM and DA was not statistically significant ($P > 0.05$).

Immunohistochemical analysis of IGFBP-2, -3, and -5. The protein expression patterns of IGFBP-2, -3, and -5 were analyzed by immunohistochemistry. Representative micrographs of immunohistochemical results in different grades of astrocytoma (DA, AA, and GBM) are shown in Fig. 2. The control brain sections stained negatively for these IGFBPs (data not shown). Among astrocytomas, the expressions of IGFBP-2, -3, and -5 were mostly confined to the cytoplasm of neoplastic astrocytic cells. The pattern of staining was intracytoplasmic, homogenous, and diffuse for IGFBP-2, -3, and -5 (Fig. 2: A-C1, D-F, and G-I, respectively). Interestingly, in GBM tumors, the cells lining pseudopalisading necrosis strongly expressed IGFBP-2 (Fig. 2-C2). Heterogeneity of staining was noted within individual tumor samples, which showed variations in intensity.

A significant differential protein expression of IGFBP-2, -3, and -5 was observed in diffusely infiltrating astrocytomas (Fig. 3). Individual group differences in LIs were statistically significant as shown in Table 2. The median (mean ± SD) LI for IGFBP-2 in GBM was 30.000 (30.070 ± 17.150), whereas in AA, DA, and controls, it was 0.0000 (2.500 ± 8.756), 0.0000 (0.0000 ± 0.0000), and 0.0000 (0.0000 ± 0.0000), respectively. The comparison between groups by Kruskal-Wallis one-way ANOVA followed by Dunn's post hoc test showed that the differences between GBM and control, GBM and DA, and GBM and AA were significant ($P < 0.05$).

For IGFBP-3, the median (mean ± SD) LI in GBM was 35.000 (34.240 ± 17.020), in comparison with AA (10.000; 12.500 ± 13.780), DA (0.0000; 6.330 ± 10.930), and control (0.0000; 0.0000 ± 0.0000). The differences between GBM and control, GBM and DA, and GBM and AA were statistically significant ($P < 0.05$).

For IGFBP-5, the median (mean ± SD) LI in GBM was 25.000 (24.820 ± 13.680) in comparison with AA (0.0000; 7.5008.000 ± 10.000), DA (0.0000; 6.000 ± 10.390), and normal (0.0000; 0.0000 ± 0.0000). Dunn's post hoc test for multiple comparisons showed that the differences

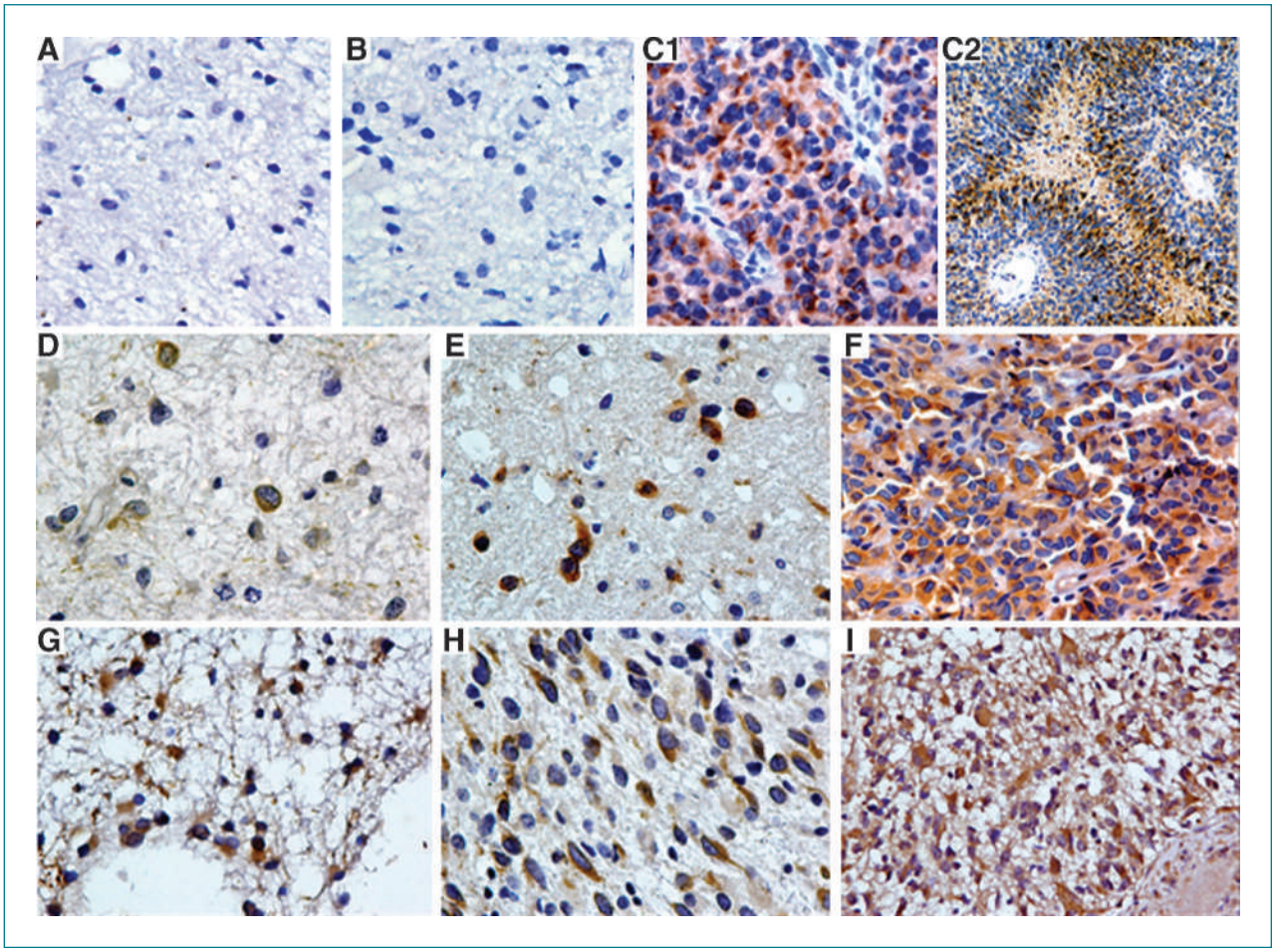

Figure 2. Immunohistochemical staining pattern of IGFBP-2, -3, and -5. Representative micrographs showing staining of IGFBP-2 (A, B, C1, and C2), IGFBP-3 (D, E, and F), and IGFBP-5 (G, H, and I). DA staining (A, D, and G), AA staining (B, E, and H), and GBM staining (C1, C2, F, and I). C2 represents pseudopalisading necrosis region in a GBM section. All original magnifications are ×400 except in C2, which is ×100.

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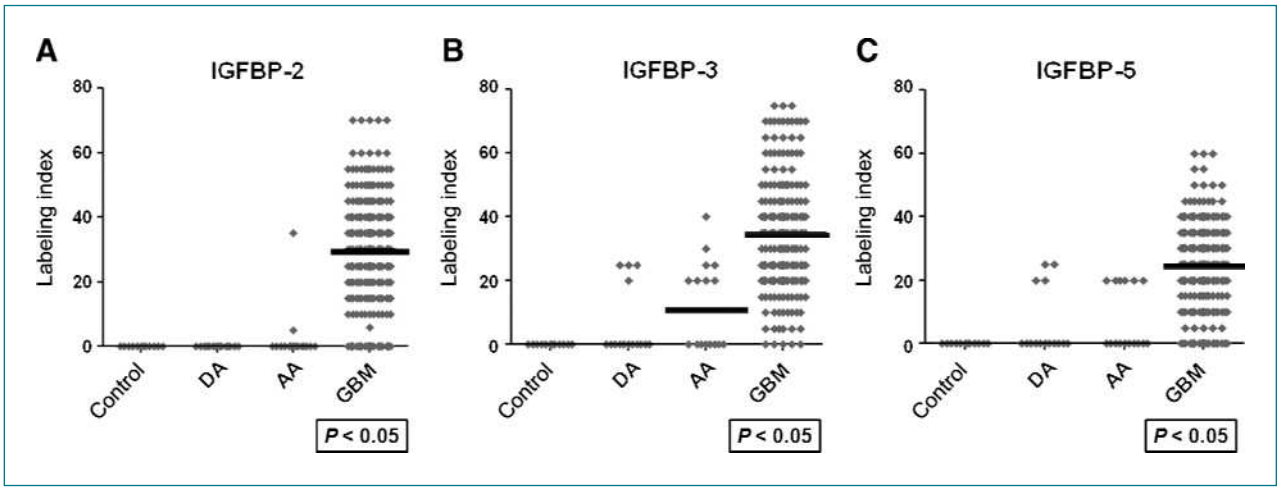

Figure 3. Protein expression pattern of IGFBP-2, -3, and -5 in various grades of astrocytoma. A, B, and C, scatter plots showing differential expression of IGFBP isoforms in diffusely infiltrating astrocytoma. The LIs of different samples obtained from immunohistochemistry analyses are plotted for IGFBP-2, IGFBP-3, and IGFBP-5 (*Y*-axis) against control, DA, AA, and GBM samples (*X*-axis). A significant difference in the median LIs of IGFBP isoforms was observed between GBM, AA, DA, and control by Kruskal-Wallis one-way ANOVA followed by Dunn's post hoc test ($P < 0.05$). Bars, group medians.

between GBM and control, GBM and DA, and GBM and AA were significant ($P < 0.05$).

Overexpression of IGFBP-2, -3, and -5 mRNA as well as the protein was seen in a grade-dependent manner in diffusely infiltrating astrocytomas. A strong positive correlation was observed between the mRNA and protein expressions of IGFBP-2 ($\rho = 0.498$; $P < 0.001$), IGFBP-3 ($\rho = 0.215$; $P < 0.010$), and IGFBP-5 ($\rho = 0.500$; $P < 0.001$; Fig. 4).

Effect of age, IGFBP-2, -3, and -5 protein expression on survival in patients with newly diagnosed glioblastoma

Univariate Cox regression models showed statistical significance for the continuous variables age, IGFBP-2, -3, and -5 LIs (Table 3A). Patient age (HR, 1.026; $P = 0.008$), IGFBP-2 LI (HR, 1.016; $P = 0.028$), IGFBP-3 LI (HR, 1.023; $P = 0.016$), and IGFBP-5 LI (HR, 1.018; $P = 0.052$) were all associated with worse clinical outcome.

The variables age, IGFBP-2, -3, and -5 LIs were associated with survival in the univariate analysis at the predetermined 0.1 significance level, and were therefore considered for inclusion in the multivariate Cox model. By enter method, it was observed that patient age was the most significant independent predictor of poor outcome (HR, 1.026; $P = 0.010$). Subsequently, using forward stepwise (Wald) selection of variables that determines the final multivariate Cox model, it was noted that patient age (HR, 1.027; $P = 0.007$) and IGFBP-3 LI (HR, 1.021; $P = 0.030$) predicted worse clinical outcome (Table 3B).

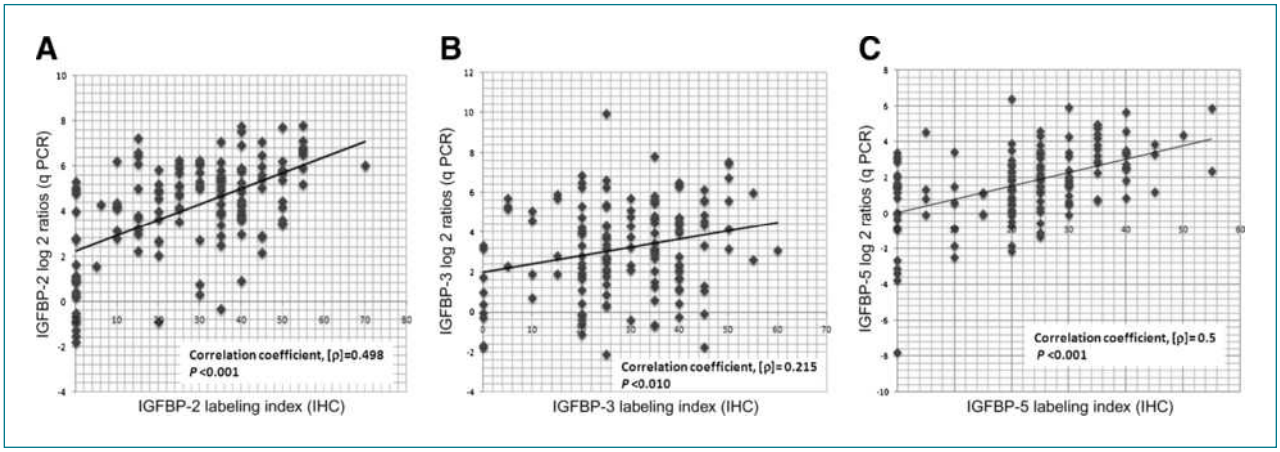

Figure 4. A scatter plot showing a positive correlation between the mRNA and protein expressions of IGFBP isoforms: Spearman's rank correlation coefficient for (A) IGFBP-2, $\rho = 0.498$; $P < 0.001$; (B) IGFBP-3, $\rho = 0.215$; $P < 0.010$; and (C) IGFBP-5, $\rho = 0.500$; $P < 0.001$. The linear regression line that best fits the data is shown.

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Table 3. Results of Cox regression analyses

Variable	HR (95% CI)	*P*
(A) Univariate Cox regression analysis		
Age (years)	1.026 (1.007-1.046)	0.008
IGFBP-2 LI	1.016 (1.002-1.030)	0.028
IGFBP-3 LI	1.023 (1.004-1.043)	0.016
IGFBP-5 LI	1.018 (1.000-1.036)	0.052
(B) Multivariate Cox regression analysis		
Age (years)	1.027 (1.007-1.047)	0.007
IGFBP-3 LI	1.021 (1.002-1.041)	0.030

Discussion

This family of IGFBPs comprises six members (IGFBP-1 to IGFBP-6) which bind and regulate the functions of IGFs. They modulate the bioavailability of IGFs, and in turn, regulate tumor growth and invasion (7, 23). Among the various isoforms, the expression and role of IGFBP-2 in gliomas has been extensively studied (12-18, 24), and there are few reports on the expression pattern of IGFBP-3 and -5 in gliomas (19, 25). The present study shows a grade-specific differential overexpression of IGFBP-2, -3, and -5 in diffusely infiltrating astrocytoma tissues along with a linear correlation between their mRNA and protein expressions. More importantly, their prognostic significance is established on a large cohort of patients with newly diagnosed glioblastoma who received standard therapy including, surgery, radiation and temozolamide as per the accepted international norms (26), and followed up.

Grade-specific expression pattern of IGFBP-2, -3, and -5

Analysis of the expression pattern of IGFBP-2, -3, and -5 in the present study clearly establishes a statistically significant grade-specific overexpression of all the three isoforms in diffusely infiltrating astrocytomas. It is noteworthy that the protein expression of these isoforms, as determined by immunohistochemistry, showed significant concordance with their corresponding mRNA expression as observed by qPCR results. This suggests a possible role for these isoforms in the pathogenesis and malignant progression of astrocytomas.

Gene expression profiling and immunohistochemistry experiments have shown IGFBP-2 to be highly expressed in GBM tumors when compared with low-grade glioma and normal brain, thus correlating the expression of this biomarker with the aggressive growth of this tumor (12-16, 24). In accordance with this, Marucci et al. have shown that gene and protein expression of IGFBP-2 correlates with the overexpression of CDC-20 and suggest the use of these two markers in combination for the diagnosis of GBM in small tissue biopsies (27). IGFBP-2 has also been shown to enhance matrix metalloproteinase-2 gene expression resulting in an increased invasion of GBM tumor cells (16). In another report, IGFBP-2 and platelet-derived growth factor-β together have been shown to be responsible for the progression of diffuse gliomas into a malignant phenotype (28). The present study shows a statistically significant overexpression of IGFBP-2 transcript as well as protein in GBM tumors when compared with DA, AA, and control brain. We have noted IGFBP-2 immunoreactivity within the cytoplasm of tumor cells, in agreement with the previous studies (12, 14, 27). In GBM tumors that had pseudopalisading necrosis, a strong IGFBP-2 staining was observed in the perinecrotic cells. In an earlier study, IGFBP-2 was shown to be colocalized with vascular endothelial growth factor receptor by *in situ* hybridization in perinecrotic cells surrounding pseudopalisading necrosis (29), indicating a possible involvement of IGFBP-2 in tumor angiogenesis. It is well established that hypoxic conditions result in necrosis within the core of the tumor. The observation that IGFBP-2 staining is seen in the perinecrotic area suggests the induction of this gene by hypoxia. Indeed, induction of IGFBP-2 has been shown in hypoxic conditions including hypoxic injury to the rat brain (30), giving strength to the argument that IGFBP-2 is expressed in GBMs under hypoxic conditions. Other experimental studies have shown that IGFBP-2 overexpression increases the invasion of glioma cells (16). Taken together, IGFBP-2 seems to be an important hypoxia-induced gene which is essential for tumor, angiogenesis, and invasion.

IGFBP-3 has been shown to have dual roles in tumor behavior. Both proapoptotic/antiproliferative and growth promotion actions have been proposed (31, 32). IGFBP-3 has been identified as a hypoxia-induced gene, in a study on a malignant glioma cell line (U-251) by cDNA microarray analysis (25). In this study, the mRNA and protein expression of IGFBP-3 showed a differential overexpression in diffusely infiltrating astrocytomas with ascending grades of malignancy, with the highest expression in GBM tissues. This suggests a plausible role for IGFBP-3 in tumor aggressiveness and malignant progression of astrocytomas. Our results differ from a previous observation in which the protein expression of IGFBP-5, but not IGFBP-3, was shown to increase with glioma anaplastic progression (19). However, in a recent report on the expression profiling of genes in GBM tumors, IGFBP-3 was classified as an upregulated gene (33). A strong cytoplasmic expression of IGFBP-3 was observed within the neoplastic astrocytes, which is similar to the reported cytoplasmic localization in human breast cancers (34). The present study is the first of its kind to show a strong cytoplasmic overexpression of IGFBP-3 in a grade-specific manner in astrocytomas.

We observed that the relative mRNA expression of IGFBP-5 was significantly higher in GBM tissues in comparison to control and AA tissues, albeit statistically not significant relative to DA tissues. However, its protein

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expression showed significant overexpression with ascending grades of malignancy in astrocytomas. This is in agreement with the previous report of IGFBP-5 expression in gliomas, which has shown its overexpression with increasing grades of malignancy (19). In an earlier study involving the application of a protein lysate array, IGFBP-5 was found to be overexpressed in GBM and was noted to be closely clustered with IGFBP-2, indicating that both proteins might contribute to glioma invasion and/or other common functions (35).

IGFBP3 is a strong independent predictor of survival in patients with newly diagnosed glioblastoma

In the present study, we have correlated the protein expression of IGFBP-2, -3, and -5 with survival on a large prospective and uniformly treated cohort of adult patients with newly diagnosed glioblastoma. The expression of these isoforms was found to be significantly associated with shorter survival by univariate Cox regression analysis. Subsequently, multivariate Cox proportional hazards model showed IGFBP-3 expression (HR, 1.021; $P = 0.030$) and patient age (HR, 1.027; $P = 0.007$) to be associated with worse clinical outcome in patients with glioblastomas.

The present study is the first of its kind to show that IGFBP-3 protein overexpression is significantly associated with shorter survival in human glioblastomas. The prognostic significance of IGFBP-3 in glioblastoma has not been previously explored. Interestingly, IGFBP-3 overexpression has been shown to be associated with poor prognosis in patients with breast cancer (36). Further strengthening this observation, there is mounting evidence through *in vitro* studies that the potential mitogenicity of IGFBP-3 is mediated through its interactions with epidermal growth factor receptor and RAS-p44/42 mitogen-activated protein kinase signaling in breast epithelial cells (37).

The prognostic value of IGFBP-2 has been proposed in earlier studies (15, 17, 18). It has been reported that IGFBP-2, along with IQGAP-1, is an important prognostic biomarker in high-grade astrocytic neoplasms (17). Importantly, plasma levels of IGFBP-2 have been correlated with recurrence and progression-free survival in high-grade gliomas (18). By using a larger prospective cohort of patients who received uniform therapy, this study establishes IGFBP-2 as an important biomarker in predicting poor prognosis in patients with newly diagnosed glioblastoma, although not an independent biomarker for predicting survival. Therefore, the value of IGFBP-2 as a predictor of survival could be more significant in conjunction with other molecular markers as shown in previous reports (17).

The expression level of IGFBP-5 is known to contribute to the development of breast cancer and has been proved to be a prognostic factor in breast cancers (38). Wang et al. have shown IGFBP-2 and IGFBP-5 to be useful markers to predict lymph node metastasis in patients with small (T1) invasive breast carcinomas (39). In the present study, IGFBP-5 showed a lower predictive value than the other two isoforms. However, its prognostic potential could improve when considered in combination with other molecular markers.

In conclusion, the results of the present study indicate that the differential overexpression of IGFBP-2, -3, and -5 with increasing grades of malignancy and the strong interdependence between their mRNA and protein expression patterns suggest their possible role in pathogenesis and malignant progression in diffusely infiltrating astrocytomas. Such a high correlation warrants the analysis of gene copy number for these IGFBP isoforms in astrocytomas. Most importantly, IGFBP-3 protein overexpression in tumor tissues emerges as a strong independent predictor of shorter survival in patients with newly diagnosed glioblastoma. Further studies are required to elucidate their functional role to exploit these isoforms as candidates for new therapeutic targets.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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