ORIGINAL RESEARCH

FBLN4 as candidate gene associated with long-term and short-term survival with primary glioblastoma

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Background: Glioblastoma multiforme (GBM) is the most common malignant and lethal type of primary central nervous system tumor in humans. In spite of its high lethality, a small percentage of patients have a relatively good prognosis, with median survival times of 36 months or longer. The identification of clinical subsets of GBM associated with distinct molecular genetic profiles has made it possible to design therapies tailored to treat individual patients.

Methods: We compared microarray data sets from long-term survivors (LTSs) and short-term survivors (STSs) to screen for prognostic biomarkers in GBM patients using the WebArrayDB platform. We focused on *FBLN4*, *IGFBP-2*, and *CHI3L1*, all members of a group of 10 of the most promising, differentially regulated gene candidates. Using formalin-fixed paraffin-embedded GBM samples, we corroborated the relationship between these genes and patient outcomes using methylation-specific polymerase chain reaction (PCR) for *MGMT* methylation status and quantitative reverse transcription PCR for expression of these genes.

Results: Expression levels of the mRNAs of these 3 genes were higher in the GBM samples than in normal brain samples and these 3 genes were significantly upregulated in STSs compared to the levels in LTS samples ($P<0.01$). Furthermore, Kaplan–Meier analysis showed that the expression patterns of *FBLN4* and *IGFBP-2* serve as independent prognostic indicators for overall survival ($P<0.01$ and $P<0.05$, respectively).

Conclusion: To our knowledge, this is the first report describing *FBLN4* as a prognostic factor for GBM patient survival, demonstrating that increased GBM survival time correlates with decreased *FBLN4* expression. Understanding *FBLN4* expression patterns could aid in the creation of powerful tools to predict clinical prognoses of GBM patients.

Keywords: glioblastoma, *FBLN4*, *IGFBP-2*, tumor marker, prognosis

Background

GBM is the most common and lethal primary intracranial tumor originating from glial cells. Despite recent advances in therapy over the past 30 years, which include resection, local radiotherapy, and systematic chemotherapy, the median survival time is only 12–15 months.[1]

In this study, long-term survivors (LTSs) are defined as individuals with survival times greater than 36 months, while those with survival times less than 12 months are designated short-term survivors (STSs).[2] LTS patients are clinically very rare and comprise only 2%–12% of all GBM patients.[3–7] Understanding this subset of GBM patients might reveal useful biological aspects of this malignancy. Clinical characteristics of LTS patients include younger age, higher Karnofsky performance scores (KPS) at diagnosis, and extent of resection.[8,9] In addition, previous studies have

pinpointed several biomarkers that are closely correlated with LTS patients. *MGMT* promoter methylation, *p53* mutation, *EGFR* amplification, 1p/19q codeletion, *IDH1/2* mutation, and PTEN deficiency were observed preferentially in LTS GBM samples.[10–16] Although LTS GBM research at the molecular level has progressed greatly, biomarkers characterized previously have not sufficiently predicted LTS status in clinical trials. Microarray techniques could aid in the analysis of the genomic landscape to identify prognostic biomarkers for survival in GBM patients. However, analyses of microarray data sets require large numbers of samples to avoid bias. Unfortunately, the LTS sample size has been very limited in past studies. Therefore, it is important to generate data from larger cohorts to better detect robust differences between extreme LTS and general GBM patients.[17] In our study, we analyzed the microarray data integrated from multiple array sources using the WebarrayDB platform[18] to compensate for inadequate sample size. Among the genes upregulated in STS groups, we focused on a subset of significantly upregulated genes identified in several previous microarray studies,[15,19,20] including *FBLN4*, *IGFBP-2*, and *CHI3L1*.

FBLN4, also known as endothelial growth factor (EGF)-containing fibulin-like extracellular matrix protein 2 (EFEMP2), is an extracellular glycoprotein[21,22] that contains 6 EGF-like calcium-binding domains and belongs to the fibulin family.[23] IGFBP-2 belongs to the IGF family and is involved in molecular events in the regulation of diverse activities in GBM tumors.[24,25] CHI3L1, also known as YKL-40 or human cartilage glycoprotein 39, is a secreted glycoprotein that belongs to the 18-glycosyl-hydrolase family of proteins. CHI3L1, considered to be an angiogenic factor for promoting tumor vessel formation through VEGF production, contributes to invasion and radio/chemoresistance of in vivo tumors.[26–28]

In this study, we examined the expression of the genes *FBLN4*, *IGFBP-2*, and *CHI3L1* at the mRNA level based on quantitative polymerase chain reaction (qPCR) and microarray data analyses. *FBLN4* and *IGFBP-2* may represent potential biomarkers for predicting GBM prognosis.

Methods
Patients and tumor samples
Tumor tissue specimens from 77 GBMs were collected from the Department of Neurosurgery, First Affiliated Hospital of Jilin University, Changchun, China, from 2008 to 2013, as approved by the Institutional Review Board (IRB00008484). GBM tumors were obtained from 32 females and 45 males (mean age range at surgery: 17–81 years). All the patients lacked pretreatment history before surgery and all underwent subtotal or gross resection, as confirmed by postsurgical magnetic resonance imaging. All tumors were histologically classified into GBM (WHO grade IV) according to the 2007 WHO classification of tumors of the nervous system.[29] Nine control samples (from brains without GBM) were obtained from the same source as for GBM samples and these originated from patients undergoing surgery for brain trauma (n=7) and epilepsy (n=2). All control samples were reviewed to verify the absence of tumors.

Ethical approval
The research was performed in accordance with the Declaration of Helsinki and was approved by the ethics committee of the First Affiliated Hospital of Jilin University (IRB00008484). Written informed consent for participation in the study was obtained from all participants or their guardians.

Extraction, purification, and quality assessment of DNA and RNA
Hematoxylin and eosin (H/E) sections from FFPE specimens were reviewed by a pathologist to select the most informative blocks. Four 10 mm-thick sections per FFPE block were cut, followed by a H/E control slide. The tumor area selected for analysis was marked on the control slide to ensure, whenever possible, greater than 80% content of neoplastic cells within that block. Extraction of genomic DNA was performed using the QuickExtract™ FFPE DNA Extraction Kit (Epicentre, Madison, WI, USA) and quantified with a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Inc, Wilmington, DE, USA). RNA deparaffinization, extraction, and purification were performed using the QuickExtract FFPE RNA Extraction Kit protocol (Epicentre). The concentration of isolated RNA was measured using the Qubit® RNA BR assay (Invitrogen, Carlsbad, CA, USA) using the Qubit 2.0 fluorometer. Purity absorbance ratios ($A_{260}/_{280}$ and $A_{260/230}$) were measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

Real-time qPCR
Synthesis of complementary DNA was performed using the SuperScript™ II Reverse Transcriptase Kit (Invitrogen) using 300 ng total RNA using the manufacturer's protocol. All gene expression assays were run on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using recommended standard settings. All assays were prepared with the 1× SYBR® Green PCR Super mix (BioPioneer, San Diego, CA, USA). mRNA expression was normalized to the housekeeping

gene β-actin. The relative fold changes were calculated using the Pfaffl method[30] for each gene after correction using β-actin. The actual sequences of specific primers for *FBLN4*, *IGFBP-2*, *CHI3L1,* and *ACTB* (β-actin) were as follows: *FBLN4* sense: 5′-CCCAGAGATTTGGACTTG-3′; *FBLN4* antisense: 5′-GTTTGAGGCAGAGTTAGG-3′; *IGFBP-2* sense: 5′-AGGTTGCAGACAATGGCGAT-3′; *IGFBP-2* antisense: 5′-GTAGAAGAGATGACACTCGG-3′; *CHI3L1* sense: 5′-GAG GATGGAACTTTGGGTCTC-3′; *CHI3L1* antisense: 5′-TCAT TTCCTTGATTAGGGTGGT-3′; and β-actin sense: 5′-CCACGAAACTACCTTCAACTCCA-3′; β-actin antisense: 5′-GTGATCTCCTTCTGCATCCTGTC-3′.

The absence of primer dimer formation was demonstrated using agarose gel electrophoresis. Each sample was run in triplicate.

Methylation-specific PCR (MSP)
The extracted DNA (500 ng) was modified with bisulfate using the EZ DNA Methylation™ Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The *MGMT* primer sequences were 5′-TTTCGACGTTCGTAGGTTTTCGC-3′ (sense) and 5′-GCACTCTTCCGAAAACGAAACG-3′ (antisense) for methylated template detection and 5′-TTTGTGTTTTGA TGTTTGTAGGTTTTTGT-3′ (sense) and 5′-AACTCC ACACTCTTCCAAAAACAAAACA-3′ (antisense) for unmethylated template detection, as previously described.[31] qPCR assays were performed using an ABI Prism 7900HT Sequence Detection System, as previously described.[32] PCR products were separated using a 2% agarose gel. For qualitative assessment, a visible M primer band indicated a positive methylation status, whereas absence of an M primer MSP product was evaluated as a negative methylation status for that respective tumor specimen. Images of the agarose gels were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij).

Analysis of microarray data sets
Three independent external microarray data sets[15,19,20] containing all 253 GBM samples from 58 LTS patients and 195 STS patients were analyzed using the WebArrayDB cross-platform analysis suite and public data repository for candidate biomarker gene expression (Table 1).[18] Data were analyzed with the WebArrayDB platform using an analysis of covariance (ANCOVA) model to correlate gene expression with survival time. Genes were sorted in ascending order according to the *P*-values calculated for the survival time factor. Thereafter, the first 100 genes were clustered using

Table 1 Independent glioma RNA expression microarray data sets

	Platform		Sample	LTS	STS
GSE16011	HU133 plus 2.0 arrays	GPL570	Frozen sample	6	14
GSE53733	HU133 plus 2.0 arrays	GPL570	Frozen sample	24	15
GSE13041	HG-U133A arrays	GPL96	Frozen sample	22	71
	HU133 plus 2.0 arrays	GPL570	Frozen sample	0	8
	Human Genome U95Av2 array	GPL8300	Frozen sample	6	27

Notes: LTS: n=58; STS: n=135.
Abbreviations: LTS, long-term survivor; STS, short-term survivor.

the Database for Annotation, Visualization and Integrated Discovery (DAVID)[33] and Kyoto Encyclopedia of Genes and Genomes (KEGG).[34] We applied hierarchical clustering of 3 microarray data sets on survival information using the Kendall tau rank distance to rank dissimilarity for classification of GBM samples into the 4 subtypes identified using The Cancer Genome Atlas (TCGA) Data Portal.[35]

Statistical analysis
Variables are presented as mean values ± SD. For comparison of different groups, the ANCOVA test was used. Cox proportional hazards regression analyses were performed with the use of the design and survival package. Survival curves were calculated using the Kaplan-Meier method and analyzed using the log-rank test. Statistical analysis was performed using SPSS software version 17.0. A *P*-value less than 0.05 indicated statistical significance.

Results
Clinical characteristics
A total of 77 GBM patients treated within the defined study period were investigated. The mean age at diagnosis was 54.6 years (range: 17–81 years; LTS group: 49 years; STS group: 56 years). Most patients could perform activities of daily life independently (KPS scores ≥70) in the LTS group (13/15, 86.3%) and STS group (55/62, 88.7%). Total resection surgeries were performed in most patients within the LTS group (12/15, 80%) and STS group (47/62, 75.8%). Most of the patients also underwent active adjuvant treatment. Gender, KPS score, extent of resection, and method of adjuvant treatment were all similar between the LTS and STS groups (*P*>0.05) (Table 2).

Gene selection from microarray data sets
In order to select genes of potential interest, 3 independent microarray data sets were used (Table 1). These data sets included 8 normal controls, 58 LTS patients, and 135 STS patients. The 2 experimental groups' data were analyzed

Table 2 Clinical characteristics of GBM patients

Characteristics	LTS (n=15)		STS (n=62)		P-value
	Number of samples	%	Number of samples	%	
Age at diagnosis (years)					<0.01
Median (years)	49		56		
Range (years)	21–76		17–81		
>50	7	46.7	45	72.6	
≤50	8	53.3	17	27.4	
Gender					>0.05
Male	9	60.0	36	58.1	
Female	6	40.0	26	41.9	
KPS value					>0.05
90–100	9	60.0	40	64.5	
70–89	4	26.7	15	24.2	
<70	2	13.3	7	11.3	
Extent of resection					>0.05
Gross	12	80.0	47	75.8	
Subtotal	3	20.0	15	24.2	
Radiation therapy					>0.05
No	2	13.3	10	16.1	
Yes	13	86.7	52	83.9	
Chemotherapy					>0.05
No	1	6.7	3	4.8	
Yes	14	93.3	59	95.2	
MGMT methylation					<0.01
Methylated	11	73.3	24	38.7	
Unmethylated	4	26.7	38	61.3	

Notes: Multivariate analysis on prognosis of the patients; n=59.
Abbreviations: GBM, glioblastoma multiforme; KPS, Karnofsky performance score; LTS, long-term survivor; STS, short-term survivor.

Table 3 Classification of the 3 microarray data sets into the 4 subtypes of GBM

GBM subtype	GSE16011		GSE53733		GSE13041		Total	
	LTS	STS	LTS	STS	LTS	STS	LTS	STS
Classical	1	5	12	12	21	82	34	99
Mesenchymal	0	5	0	1	3	10	3	16
Neural	1	0	1	0	1	6	3	6
Proneural	4	4	11	2	3	8	18	14
Total	6	14	24	15	28	106	58	135

Abbreviations: GBM, glioblastoma multiforme; LTS, long-term survivor; STS, short-term survivor.

the mRNA levels using quantitative reverse transcription (qRT)-PCR. As shown in Table 4, when analyzing the mRNA levels of these genes in normal controls and GBM patients, we found an average increase of expression in tumor tissues compared to normal controls. Moreover, mRNA levels of the 3 genes were significantly different between GBM samples and normal controls ($P<0.05$). At the same time, we also found that the expression of all 3 genes was significantly upregulated in STS vs LTS patients ($P<0.05$).

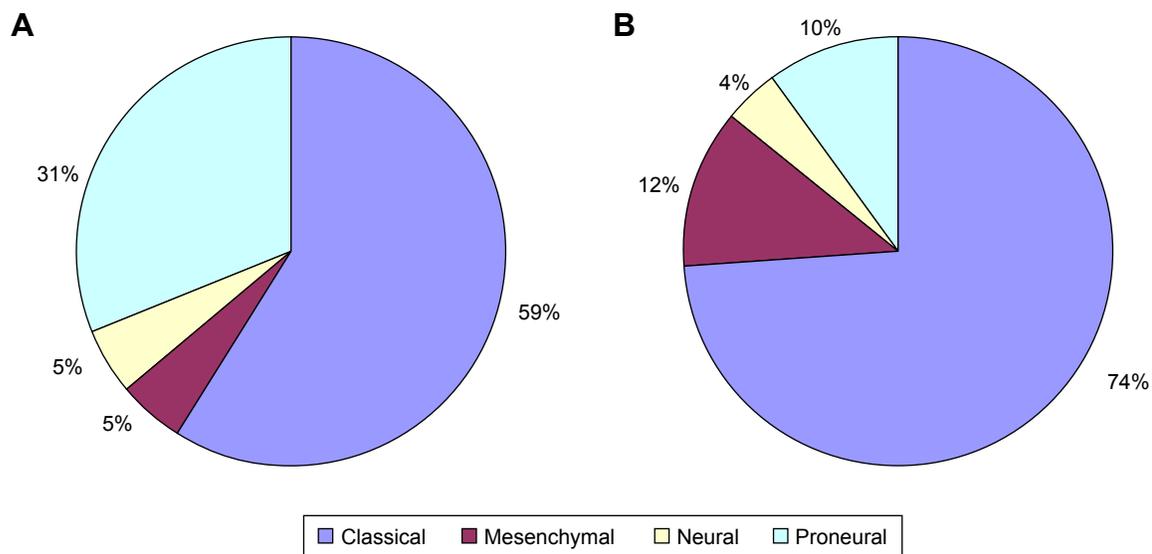## Analysis of methylation status of MGMT promoter

In 77 GBM samples, the methylation status of the *MGMT* promoter was tested using qMSP. *MGMT* promoter methylation was detected in 11 (73.3%) of the LTS samples and 24 (38.7%) of the STS samples. There was significant difference between the 2 groups ($P<0.05$) (Table 1).

Multivariate Cox regression analysis

We performed Cox regression analysis to evaluate the expression of genes and the *MGMT* methylation status in GBM patients. Multivariate survival analyses identified high expression of *FBLN4* and *IGFBP-2* ($P<0.05$), with *MGMT* promoter methylation status of the GBM patients acting as an independent prognostic factor for survival time (Table 5). However, *CHI3L1* expression did not function as an independent prognostic factor for survival time, although this result was not statistically significant due to the small sample size ($P=0.052$).

FBLN4 and *IGFBP-2* overexpression correlates with survival time in GBM patients

The search for a model describing the relationship between survival and the expression of *FBLN4* and *IGFBP-2* using maximally selected log-rank statistics identified a cutpoint model to be the most suitable; it was used to delineate 2 subgroups of GBM patients. Applying a 2-gene expression

using the WebArrayDB cross-platform analysis suite[18] using an ANCOVA model. Genes were sorted in ascending order according to the *P*-values for survival time. The top 100 genes that correlated most significantly with prognosis were compared with genes previously characterized by Gerber et al.[36] *FBLN4*, *IGFBP-2*, and *CHI3L1* were among the top 10 gene candidates in 2 studies. *IGFBP-2* and *CHI3L1* have also been reported to be closely correlated with glioma invasion and progression.[15,19] *FBLN4* had not been previously investigated as a biomarker of progression in GBM. Therefore, we focused our attention on these 3 genes in this study. Using the method proposed by Verhaak et al,[35] we used unsupervised hierarchical clustering to classify the 3 microarray data sets of LTS samples into 4 subtypes (Table 3) (Figure 1). In the 3 microarray data sets, 34 GBM samples belonged to the classical subtype, 3 to the mesenchymal subtype, 3 to the neural subtype, and 18 to the proneural subtype.

FBLN4, *IGFBP-2*, and *CHI3L1* mRNA levels in LTS and STS patients

To compare *FBLN4*, *IGFBP-2*, and *CHI3L1* expression levels in 15 LTS patients and 62 STS patients, we examined

A

B

Figure 1 Distribution of (**A**) LTS and (**B**) STS status in the data from the 3 microarray data sets among the 4 subtypes of GBM.
Notes: LTS: n=58; STS: n=135.
Abbreviations: GBM, glioblastoma multiforme; LTS, long-term survivor; STS, short-term survivor.

cutoff to a Kaplan–Meier survival curve estimation revealed decreased survival probability for patients with tumors expressing high levels of these gene products ($P < 0.05$), (Figure 2). The 2 subgroups exhibited significantly different outcomes relative to WHO grade IV patients.

Discussion

Although during the past 2 decades many attempts have been made to identify biomarkers with prognostic significance, predicting GBM patients' prognosis still remains a serious challenge. Nevertheless, the LTS phenomenon in GBM provides a unique opportunity to gain insights into the molecular characteristics underlying such a favorable prognosis.

In this study, we analyzed the data from 3 microarrays[15,19,20] to screen LTS and STS samples. Our study showed that LTS can be achieved in GBM patients with nonproneural tumors (40/58, 69.0%). Moreover, LTS patients can be observed within all GBM subtypes, as corroborated by previous

investigations.[36] This study also showed higher incidence of *MGMT* promoter methylation in the LTS group vs STS group (73.3% vs 38.7%). As a next step, we compared LTS and STS sample microarray data sets using the WebarrayDB platform and combined our results with those of another recent study[36] to evaluate the prognostic biomarkers in GBM patients. *FBLN4*, *IGFBP-2*, and *CHI3L1* ranked within the top 10 prognostic gene candidates in multiple microarray data sets. We further validated these 3 biomarkers in clinical samples using qPCR. We found that the mRNA expression levels of these genes were higher in GBM samples than in normal brain samples and all were significantly upregulated in STS samples vs LTS samples. Furthermore, both *FBLN4* and *IGFBP-2* expression patterns were independent prognostic factors for overall survival, as determined using the Cox proportional hazards regression model. Patients with high expression of *FBLN4* or *IGFBP-2* had poorer survival times than those with low expression of these genes. Furthermore,

Table 4 Comparison of mRNA expression of *FBLN4*, *IGFBP-2*, and *CHI3L1* in tumor tissue by qRT-PCR and microarray analysis

Gene	Unique ID	Gene title	Gene origin	P-value		
				LTS→normal	**STS→normal**	**STS→LTS**
FBLN4	206580_s_at	EGF-containing fibulin-like extracellular matrix protein 2	qRT-PCR	**2.95E-02**	**1.62E-03**	**3.53E-03**
			Microarray	4.68E-01	**6.83E-04**	**1.01E-04**
IGFBP-2	202718_at	Insulin-like growth factor binding protein 2	qRT-PCR	**3.05E-02**	**2.59E-03**	**5.12E-03**
			Microarray	**1.08E-02**	**8.28E-07**	**2.40E-04**
CHI3L1 (YKL-40)	209395_at	Chitinase 3-like 1	qRT-PCR	**1.86E-02**	**1.26E-02**	**1.04E-02**
			Microarray	4.14E-01	3.87E-03	3.18E-03

Notes: Significant values are shown in bold; *P*<0.01; *P*<0.05.
Abbreviations: GBM, glioblastoma multiforme; LTS, long-term survivor; qRT-PCR, quantitative reverse transcription polymerase chain reaction; STS, short-term survivor.

Table 5 Multivariate analysis on prognosis of the patients

Parameter	HR	95% CI	P-value
Gender	1.283	0.637–2.216	0.473
Age (>50), years	3.562	2.581–4.268	0.024
FBLN4	3.119	1.647–3.983	0.018
IGFBP-2	2.847	1.490–3.621	0.039
CHI3L1 (YKL-40)	2.051	2.409–5.277	0.052
MGMT methylation	3.781	2.195–5.263	0.009

Notes: LTS: n=15; STS: n=62. *P*<0.05.
Abbreviations: CI, confidence interval; HR, hazard ratio; LTS, long-term survivor; STS, short-term survivor.

CHI3L1 was not determined to be an independent prognostic factor, possibly due to the small sample size (*P*=0.052). Moreover, Kaplan–Meier analysis showed that both *FBLN4* and *IGFBP-2* can be independent prognostic indicators for overall survival (*P*<0.01 and *P*<0.05, respectively). To our knowledge, this is the first report showing that *FBLN4* is a prognostic factor in glioma patients.

FBLN4, also known as EGF-containing fibulin-like extracellular matrix protein 2 (EFEMP2), is localized to the *FBLN4* gene on chromosome 11q13a. It is an extracellular glycoprotein[21,22] that contains 6 EGF-like calcium-binding domains and belongs to the fibulin family.[23] FBLN4 is essential for connective tissue development and elastic fiber formation, and it plays an important role in vascular patterning and collagen biosynthesis.[37] Moreover, it is involved in the onset and progression of many diseases, including aortic aneurysms,[38,39]

cutis laxa,[40,41] and osteoarthritis.[42] FBLN4 has been observed to be a specific protein partner for mutant p53, and it displays both mutant p53-dependent and -independent oncogenic properties, with implications for both cancer biology and therapeutic intervention. To date, the role of FBLN4 in different types of tumor tissues has been controversial. *FBLN4* promoter methylation has frequently been found in lung cancers[43] and melanoma cells.[44] Other studies have reported that FBLN4 is overexpressed in tumors of the digestive system[45–47] and in gynecological tumors;[48–50] *FBLN4* overexpression correlates with tumor progression and poor prognosis in these studies. In contrast, *FBLN4* is downregulated in prostate cancer.[51] A recent study has reported that *FBLN4* is upregulated in glioma tissue and promotes glioma cell proliferation and invasion.[52] In our study, we also found that *FBLN4* expression was significantly increased in GBM samples vs normal samples (*P*<0.01) and was significantly higher in STS samples than in LTS samples (*P*<0.01). Therefore, *FBLN4* expression can be an independent prognostic indicator for overall survival (*P*<0.01).

IGFBP-2 belongs to the IGF family. IGFBP-2 exhibits higher expression in GBM samples than in other pathological types of glioma.[53,54] Moreover, IGFBP-2 is significantly associated with the Ki-67 labeling index in gastric carcinoma.[55] In addition, over the past 15 years, results of several studies reporting increased expression of IGFBP-2 have correlated it with poor clinical GBM patient outcomes.[56–59] Moreover, recent studies have reported that plasma IGFBP-2 levels could
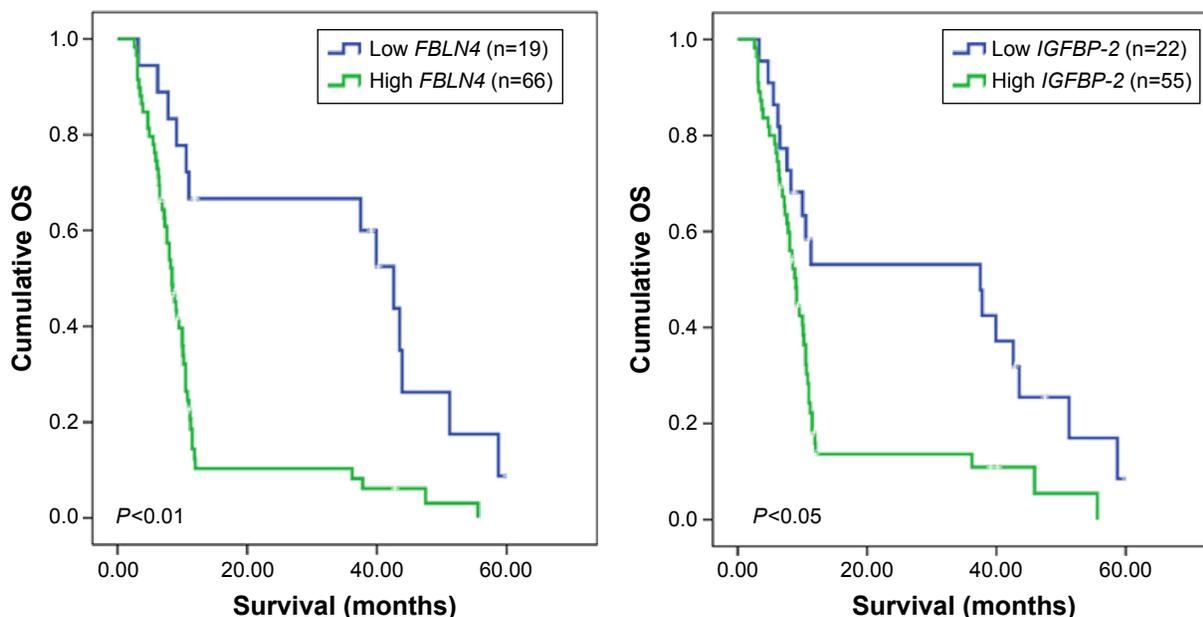
Figure 2 Kaplan–Meier estimates of OS in relation to *FBLN4* and *IGFBP-2* expression as determined by qPCR.
Notes: Cutoff value for dichotomization of *FBLN4* and *IGFBP-2* expression and *P*-values were determined by maximally selected log-rank statistics. (**A**) High *FBLN4* expression appeared to be strongly associated with poor OS in GBM patients; (**B**) *IGFBP-2* expression appeared to be a strongly positive prognostic factor in GBM patients (*P*<0.01).
Abbreviations: GBM, glioblastoma multiforme; OS, overall survival; qPCR, quantitative polymerase chain reaction.

predict the clinical outcomes of GBM patients.[25,60,61] IGFBP-2 expression has been associated with oncogenic effects via several mechanisms. IGFBP-2 modulates the status of PTEN through activation of the PI3K/AKT pathway.[62–64] IGFBP-2 drives glioma development and progression by activating the signaling pathway of integrins.[65–67] IGFBP-2 contributes to GBM progression by enhancing transcription of *VEGF* and *MMP-2* to promote tumor cell invasion.[68–70] IGFBP-2 plays a key role in the activation of the EGFR/STAT3 pathway to influence the invasive and migratory capacities of GBM cells. At the same time, IGFBP-2 modulates multiple key genes to enhance invasiveness of GBM cells.[71–74] In our study, we also found that IGFBP-2 exhibits higher expression in STS samples than in LTS samples ($P<0.01$). Furthermore, Kaplan–Meier analysis showed that IGFBP-2 level can be an independent prognostic indicator for overall survival ($P<0.05$).

Conclusion

We have found upregulated expression of the genes *FBLN4*, *IGFBP-2*, and *CHI3L1* in the STS group vs the LTS and normal groups. *FBLN4* and *IGFBP-2* show promise as useful clinical prognostic markers and could potentially improve patient health management. These provisional findings, however, require confirmation using a larger study.

Abbreviations

GBM, glioblastoma multiforme; LTS, long-term survivor; STS, short-term survivor; FFPE, formalin-fixed paraffin-embedded; H/E, hematoxylin and eosin; MSP, methylation-specific polymerase chain reaction.

Acknowledgments

Author contributions

FL, YPL, HY, and KZ carried out the molecular genetic studies and participated in the preparation of the manuscript. YL, PH, YJL, SC, and ZL collected samples. LB conceived the study and participated in its design and coordination, in addition to drafting the manuscript. All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work. All authors read and approved the final manuscript.

Disclosure

The authors report no conflicts of interest in this work.

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