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Tissue microarray analysis for epithelial membrane protein-2 as a novel biomarker for gliomas

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Abstract

Epithelial membrane protein-2 (EMP2) expression is noted in many human cancers. We evaluated EMP2 as a biomarker in gliomas. A large tissue microarray of lower grade glioma (WHO grades II–III, $n = 19$ patients) and glioblastoma (GBM) (WHO grade IV, $n = 50$ patients) was stained for EMP2. EMP2 expression was dichotomized to low or high expression scores and correlated with clinical data. The mean EMP2 expression was 1.68 in lower grade gliomas versus 2.20 in GBMs ($P = 0.01$). The percentage of samples with high EMP2 expression was greater in GBMs than lower grade gliomas (90.0 vs. 52.6%, $P = 0.001$). No significant difference was found between median survival among patients with GBM tumors exhibiting high EMP2 expression and survival of those with low EMP2 expression (8.38 vs. 10.98 months, $P = 0.39$). However, EMP2 expression ≥2 correlated with decreased survival (r = −0.39, $P = 0.001$). The EMP2 expression level also correlated with Ki-67 positivity (r = 0.34, $P = 0.008$). The mortality hazard ratio for GBM patients with EMP2 score of 3 or higher was 1.92 (CI 0.69–5.30). Our findings suggest that elevated EMP2 expression is associated with GBM. With other biomarkers, EMP2 may have use as a molecular target for the diagnosis and treatment of gliomas.

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Conflict of interest MW. and L.K.G. are inventors on the University of California patents related to EMP2 as a target for antibody therapy. They are also the founders of Paganini Biopharma. The remaining authors have no personal or institutional financial interest in drugs, materials, or devices described in this study.

Keywords

Biomarker; Epithelial membrane protein-2; Glioblastoma; Prognosis; Glioma

Introduction

Epithelial membrane protein-2 (EMP2) is a member of the growth arrest-specific gene 3/peripheral myelin protein-22 (GAS3/PMP22) subfamily, which together with tetraspanins and connexins comprise three subfamilies of the large 4-transmembrane family [11]. Its expression is increased in breast, ovarian and endometrial cancers in which it has been shown to correlate with poor survival and/or advanced disease [9, 10, 17, 41]. In contrast, EMP2 is expressed at low or undetectable levels in normal brain tissue [33].

EMP2 has gained recent interest in the study of gliomas, where its expression is upregulated at the levels of both transcription and protein expression [8, 33]. EMP2 expression is particularly enriched on the outer cell membrane of glioblastoma (GBM) cells [8, 33]. EMP2 enhances tumor growth in vivo partly by up-regulating $\alpha v\beta 3$ integrin surface expression, activating focal adhesion kinase and Src kinase, and promoting cell migration and invasion [43]. EMP2 expression is significantly associated with increased Src kinase activation in human samples and results in increased tumor cell invasion in intracranial murine models [33]. In these models, EMP2 also promoted a more aggressive phenotype.

The purpose of the current study was to assess the potential of EMP2 as a biomarker and prognostic indicator for patients with gliomas. We compared EMP2 expression levels between lower grade [World Health Organization (WHO) grade II–III] gliomas and GBMs (WHO grade IV) using a microarray analysis, and related differences in EMP2 expression to patients' overall survival (OS). We hypothesized that EMP2 is more highly expressed in GBM compared to lower grade glioma, and that higher EMP2 expression is associated with shorter OS.

Materials and methods

Immunohistochemistry

A tissue microarray (TMA) containing glioma tumor samples from 69 patients (19 lower grade glioma and 50 GBM) who underwent craniotomy for tumor resection between July 2008 and February 2013 was stained for cell surface expression of EMP2 using human EMP2 antisera, as previously described [42]. Samples of normal pituitary tissue and meningioma (WHO grade I) tumors were included in the TMA as negative and positive controls, respectively. Staining was completed using standard immunohistochemistry (IHC) procedures. TMA sections were incubated at 95 °C for 20 min in 0.1 M citrate at pH 6.0 for antigen retrieval. EMP2 was detected using rabbit human EMP2 antisera at 1:1500 dilution followed by visualization using Dako EnVision+ System-HRP (DAB) (Agilent Technologies, Denmark) according to the manufacturer's instructions. Staining intensity was quantified on a 0– histological scoring scale by two independent pathologists who were blinded to the identity of the samples. Figure 1 shows representative images for each of the

expression scores in lower grade gliomas and GBMs. The pathologists' EMP2 expression scores were then dichotomized as either low (expression scores 0–1) or high (expression scores 2–3) based on the results of a prior study, which found that EMP2 expression scores of 2 or higher were predictive of decreased survival [33].

Secondary molecular and genetic markers of interest included antigen Ki-67 positivity, epithelial-like growth factor receptor III (EGFRvIII) expression, isocitrate dehydrogenase 1 (IDH1) mutation and 1p/19q chromosome codeletion. Ki-67 positivity was used as a cellular marker of proliferation, and has been previously associated with poorer prognosis and shorter survival [19, 28, 37]. *EGFR* is among the most commonly altered genes in high-grade glioma, with the *EGFRvIII* variant observed in around 25% of cases and strongly associated with tumor aggressiveness [1, 15, 20, 27]. IDH1 mutations are observed in around 80% of low-grade or secondary high-grade gliomas and associated with more favorable prognosis [3, 44]. Chromosome arm 1p/19q co-deletion is another pathognomonic biomarker characteristic of oligodendrogliomas [2]. It is closely associated with IDH1 mutation and predicts favorable prognosis [7, 34, 45]. Ki-67 positivity, EGFRvIII expression, and IDH1 mutation status were assessed via IHC. 1p/19q co-deletion was assessed using fluorescence in situ hybridization (FISH).

Clinical measures

Patient information and clinical outcomes were retrieved from the electronic medical record. Clinical measures of interest included patient age at surgery, tumor diagnosis based on the surgical pathology report, OS from time of diagnosis, and primary or secondary GBM. All research was reviewed, approved, and carried out in accordance with the recommendations of the University of California, Los Angeles Institutional Review Board (IRB#10-000655). Prior to surgery, all patients gave written informed consent to the use of their tumor samples for research purposes in accordance with the Declaration of Helsinki. All accessed clinical data were de-identified prior to analysis.

Statistical analysis

Statistical analyses were performed using SPSS v.22.0 (IBM Corporation, Armonk, NY, USA). Univariate analyses were conducted using Fisher's exact tests and Mann-Whitney U tests to compare pairs of categorical and continuous variables, respectively. Kaplan-Meier survival curves and the log-rank test were used to evaluate OS. Multivariate analysis was completed using Cox proportional hazard regression to compare OS in GBM patients [5]. Pearson product-moment correlation coefficients were used to compare the relationship between OS and EMP2 expression levels. Standard confidence intervals were used to quantify uncertainty in reported analyses. All tests were two tailed, with statistical significance evaluated using a critical alpha criterion of $P < 0.05$.

Results

Patient demographics

The mean age for patients with lower grade glioma was less than that of patients with GBM (41.95 vs. 53.68 years, $P = 0.006$). Tumor recurrence rates in the lower grade glioma and

GBM groups were 38.5% ($n = 5$) and 76.7% ($n = 33$), respectively ($P = 0.01$). Of the 50 patients diagnosed with GBM, 41 were diagnosed with primary GBM and 9 were diagnosed with secondary GBM. Patient demographics and characteristics are summarized in Table 1.

EMP2 expression in gliomas

The percentage of lower grade glioma samples with high EMP2 expression was 52.6%, compared to 90.0% of GBM samples with high EMP2 expression ($P = 0.001$) (Table 2). Figure 2 contrasts the mean EMP2 expression scores between lower grade glioma and GBM samples. Mean EMP2 histological expression scores were 1.68 (95% CI 1.32–2.05) in lower grade gliomas compared to 2.20 (95% CI 2.03–2.37) in GBM samples ($P = 0.01$).

Correlation of EMP2 with overall survival and other glioma biomarkers

Across all 69 glioma cases, EMP2 expression score of ≥2 ($r = -0.39$, $P = 0.001$) and high Ki-67 positivity ($r = -0.42$, $P = 0.001$) were negatively correlated with OS, while EGFRvIII positivity ($r = 0.52$, $P = 0.04$), 1p/19q co-deletion ($r = 0.50$, $P = 0.02$) and IDH1 mutation ($r = 0.42$, $P = 0.009$) were positively correlated with OS.

The specific relationships between these glioma biomarkers and OS in lower grade glioma and GBM patients are summarized in Table 3. EMP2 expression was correlated with high Ki-67 positivity ($r = 0.34$, $P = 0.008$). Table 4 summarizes the specific associations observed between EMP2 expression and other glioma biomarkers in lower grade glioma and GBM patients.

EMP2 and overall survival in glioblastoma

The median OS for GBM patients with low EMP2 expression was 10.98 months compared to 8.38 months for GBM patients with high EMP2 expression; however, this trend was not significant ($P = 0.39$) (Table 5). Figure 3 compares OS curves for GBM patients who had either low or high EMP2 expression. These subgroups of GBM patients did not differ in OS ($P = 0.74$). Next, we computed an adjusted mortality hazard ratio (HR) for GBM patients using Cox regression in which patient age, sex, diagnosis (primary, secondary or recurrent GBM), high EMP2 expression, and tumor recurrence were included as explanatory variables (Table 6). The HR for GBM patients with a high EMP2 expression level was 1.92 but did not reach statistical significance (95% CI 0.69–5.30). The HR for patients with recurrent GBM was 24.13 (95% CI 6.1–95.1).

Discussion

A recent trend in cancer biology is the development of personalized medicine and individualized treatment regimens through the identification of molecular markers [30]. The ability of biomarkers to provide information on disease course and treatment response in GBM has been previously evaluated. One well-known example in GBM biology has been the epigenetic silencing of O^6-methylguanine-DNA methyltransferase (MGMT) DNA-repair gene, which is associated with better treatment response to alkylating agents [18]. The identification of other GBM biomarkers could help physicians and patients make informed decisions regarding therapeutic interventions, provide personalized treatment regimens, and

stratify patient prognoses. In this study, we investigated EMP2 expression in lower grade gliomas and GBM to elucidate its diagnostic and prognostic value. We observed increased EMP2 expression in GBM tumor samples compared with lower grade gliomas, which suggests that EMP2 could have limited diagnostic or prognostic utility as a glioma biomarker. However, we did not find significant associations between EMP2 expression level and overall survival within patients diagnosed with GBM.

EMP2 as a diagnostic marker

The pathologic distinction between WHO grade III (anaplastic astrocytoma) and grade IV (GBM) gliomas may be difficult to discern, and variations in the grading of these tumors has been demonstrated [4, 24, 32]. Thus, identification of a biomarker that can distinguish lower grade glioma from GBM is imperative in making the appropriate diagnosis and providing patients with accurate prognosis and treatment options. We demonstrated a difference in EMP2 expression between lower grade (WHO grades II–III) gliomas and GBMs. A greater percentage of lower grade glioma samples had an EMP2 expression score of 1, while a greater proportion of GBM samples had an expression score of 2. This difference was significant when EMP2 expression levels were dichotomized into low and high EMP2 expression categories. Mean EMP2 expression score was also significantly different between lower grade gliomas and GBMs. A prior study reported that 53% of GBM tumors expressed an EMP2 score of ≥2 [8]. Here, we found that 90% of GBM tumors expressed a EMP2 expression score ≥2. Conversely, Rickman et al. reported finding increased *EMP2* mRNA expression in WHO grade I gliomas when compared to higher grade gliomas in their evaluation of 6,800 genes via microarray analysis [35]. The discrepancy between our findings and those of Rickman et al. may be explained in part by the fact that the latter quantified mRNA levels, whereas we reported protein expression measured via IHC [16]. Future research is needed to clarify this issue. While we did not stratify EMP2 expression levels individually by specific WHO glioma grade (I–IV), our results suggest that EMP2 protein levels may have utility in distinguishing lower grade gliomas from GBMs.

Complementary findings have also been reported by Qin et al., who demonstrated increased EMP2 expression via Western blot analysis in GBM tumors and cell lines when compared to normal brain parenchyma [33]. Our study differs from the aforementioned work in three important aspects: (1) our TMA included grade II—III glioma samples, (2) we performed a survival analysis to evaluate the prognostic utility of EMP2 expression, and (3) we investigated the relationship between EMP2 expression and a number of currently known GBM biomarkers. In the authors' opinion, lower grade glioma samples serve as a more useful benchmark against which to compare GBM tissue for the purpose of evaluating potential diagnostic utility of glioma biomarkers, since the clinical decision faced by medical providers is more often differentiating GBMs from other gliomas (e.g., anaplastic astrocytoma) and working with patients to develop an informed treatment course based on prognosis.

EMP2 as a prognostic marker

Heterogeneity in survival length amongst GBM patients suggests the presence of distinct molecular features that dictate tumor aggressiveness or response to treatment [26, 31].

Investigations into EMP2's biochemical roles support its potential to be exploited as a prognostic marker in GBM. EMP2 regulates cell trafficking as well as cellular display of extracellular membrane receptors and glycolipids [40]. Furthermore, EMP2 physically associates with and regulates the activity of integrin–FAK signaling complexes, specifically associating with αvβ3 integrin, which has been shown to correlate with GBM progression and invasion into brain parenchyma [6, 12, 39]. As such, EMP2 has been implicated in cell migration, invasion and neoangiogenesis in a number of human cancers [14, 22, 33, 38]. However, our results did not demonstrate significant associations between EMP2 expression and overall post-diagnosis survival within patients diagnosed with GBM. Our sparse sample of GBM tumors, especially those with low EMP2 expression scores, limited our statistical power and ability to assess prognostic utility of EMP2 expression among patients with GBM. In particular, sample size was a limiting factor for our Kaplan-Meier survival and Cox hazard regression analyses.

A number of established biomarkers for gliomas are described in the literature [8, 23, 25, 29]. MGMT, isocitrate dehydrogenase 1 and 2 (IDH1/2), epidermal growth factor receptor (EGFR), phosphatase and tensin homolog (PTEN), loss of heterozygosity of chromosome arms 1p or 19q, and 1p/19q co-deletion have been investigated extensively for their ability to aid clinical management of GBM. Direct comparison of the prognostic utilities of different biomarkers is confounded by study reproducibility and external validity. Nevertheless, MGMT promoter methylation, 1p/19q co-deletion and IDH1 mutations have been shown conclusively to confer favorable prognosis in gliomas [13, 18, 21, 36]. To date, the most notable prognostic molecular classifications of GBMs are related to mutations in TERT promoter and IDH, as well as 1p/19q deletions [7]. Thus far, the vast majority of other studied biomarkers have shown only marginal prognostic utility with no significant impact on disease management [25].

Our results lead us to believe that EMP2 may provide some value as a diagnostic biomarker for GBM if used in combination with other established markers. In reviewing associations between glioma biomarkers and OS, we found EMP2 expression scores of ≥2 to be as strongly correlated with OS as IDH1 mutation and 1p/19q co-deletion, and in the opposite direction. An interesting question is whether EMP2 has the potential to serve as a therapeutic target for GBM treatment [33]. To this end, much further research is needed to elucidate the molecular roles of EMP2 in cellular processes and tumorigenesis in gliomas. Advancing our molecular genetic understanding of GBM will ultimately help clarify clinical subgroups and design patient-specific treatment paradigms tailored to molecular genetic tumor profiles.

Limitations

First, correlation of TMA samples to patient data was performed retrospectively. Thus, patients who did not continue to receive care at our institution after their initial surgery may have deficiencies in their clinical data. This has the potential to both affect outcomes and interpretation of our results. Second, TERT promoter sequencing was not routinely available at our institution during the study period, and the lack of TERT promotor mutation status in our data limits comparison of our results to some GBM classification schemes. Third,

sample size in the present study made it infeasible to stratify cases between grade I—II and grade III gliomas. Sample size was also restrictive when comparing GBM samples with high EMP2 expression to the minority of samples with low EMP2 expression, and hindered our ability to identify EMP2-related differences in survival among GBM patients. A larger scale normalized prospective study would allow for both lower grade glioma and GBM samples to be collected with a more adequate sample size and long-term follow-up. A study design of this type would better facilitate assessment of the prognostic value of EMP2 expression on GBM survival. Finally, recurrent GBM tumor status was the only significant predictor of early mortality, with a 24-fold increase in mortality risk for recurring GBM. Recurrent GBM tumor status implies that the patient already failed primary treatment and underwent multiple craniotomies and resections. Our inclusion of such recurrent tumor cases may have affected our survival analyses due to older age and greater surgical and overall morbidity in this subset of patients.

Conclusions

EMP2 expression may help differentiate lower grade gliomas from GBMs, and may have limited prognostic potential as a predictor of survival in patients with gliomas. The correlation of EMP2 expression with survival is not particularly strong, nor is it superior to other known markers biomarkers, but at best, it can be expected to be as good as Ki67. When used in conjunction with currently known biomarkers, EMP2 may have potential to increase the accuracy of diagnosis and prognosis in patients with GBM, and in this regard may prove beneficial in helping to inform the optimal course of care. A useful biomarker is a quick, cheap, and reproducible measure that stratifies patients outcome, or more importantly, response to therapy. Thus, future investigations are needed to determine whether EMP2 meets these criteria. In addition, future prospective studies involving larger cohorts are needed to validate and expand upon these findings, particularly to evaluate differences in EMP2 expression among patients with lower grade gliomas.

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Compliance with ethical standards

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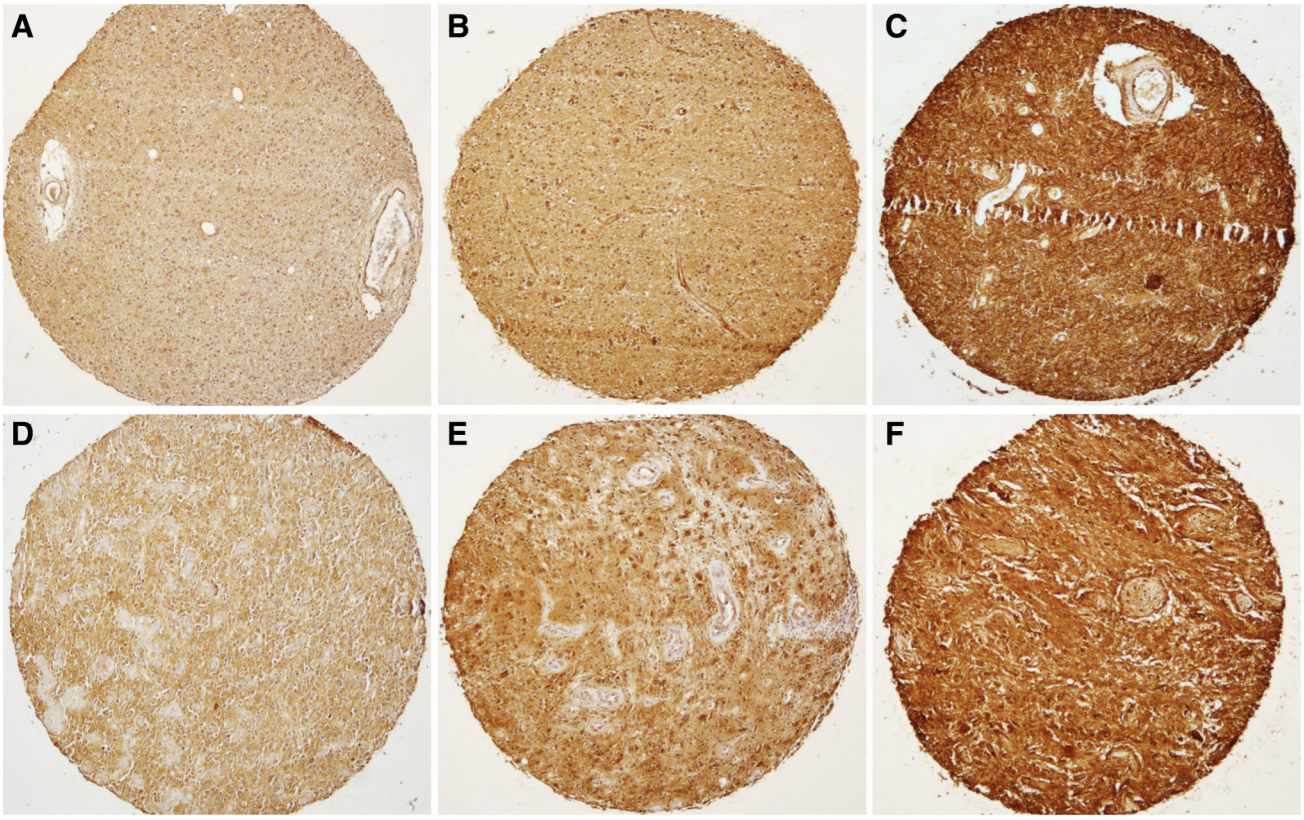

Fig. 1.

Representative photomicrographs (×10 objective) of EMP2 immunohistochemical staining showing expression scores of 1+ (**a**, **d**), 2+ (**b**, **e**), and 3+ (**c**, **f**) in lower grade gliomas and GBMs, respectively. All photographs were taken at the same exposure settings

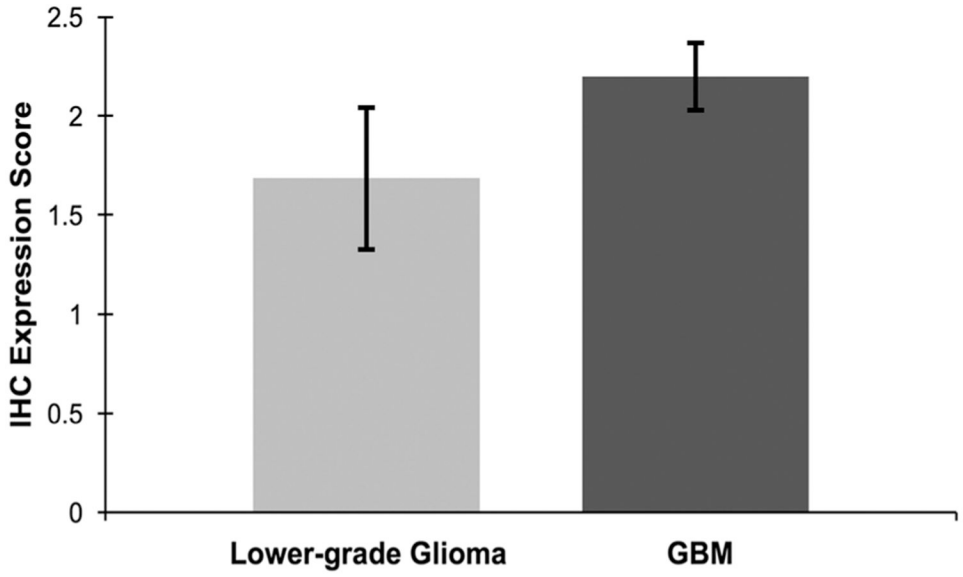

Fig. 2.

Average EMP2 expression score of lower grade glioma versus GBM with corresponding 95% confidence interval error bars. Mann-Whitney *U* test demonstrated a significant difference between the EMP2 expression scores in lower grade glioma and GBM ($P = 0.01$)

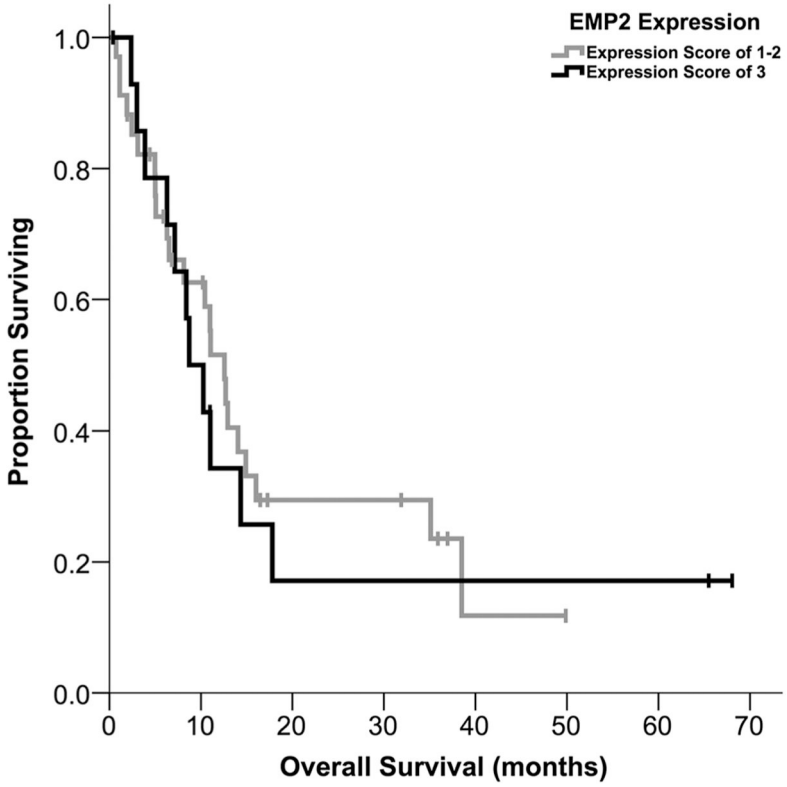

Fig. 3.

Overall survival in GBM patients expressing low versus high EMP2 expression. A Kaplan-Meier survival curve showing overall survival in patients with EMP2 expression of ≤2 (*gray*) and EMP2 expression of 3 (*black*). Log-rank analysis did not demonstrate a significant difference between GBM samples with low EMP2 expression against those with high EMP2 expression ($P = 0.74$)

Table 1

Patient demographics and characteristics

Variable	Overall	Lower grade glioma	GBM	*P* value
Patients, *n*	69	19	50	
Age (years), mean ± SD	50.03 ± 15.86	41.95 ± 10.78	53.68 ± 16.29	0.006
Sex, *n* (%)				0.16
Female	28 (39.4)	10 (52.6)	17 (34.0)	
Male	43 (60.6)	9 (47.4)	33 (66.0)	
Extent of resection, *n* (%)				0.28
Subtotal	18 (28.1)	7 (38.9)	11 (25.0)	
Gross total	46 (71.9)	11 (61.1)	33 (75.0)	
Tumor recurrence, *n* (%)				0.01
No	18 (31.0)	8 (61.5)	10 (23.3)	
Yes	40 (69.0)	5 (38.5)	33 (76.7)	

Table 2

EMP2 expression level in lower grade glioma vs. GBM

Expression score	Lower grade glioma No. (%)	GBM No. (%)	*P* value
1	9 (47.4)	5 (10.0)	
2	7 (36.8)	30 (60.0)	
3	3 (15.8)	15 (30.0)	
Low EMP2 expression	9 (47.4)	5 (10.0)	0.001
High EMP2 expression	10 (52.6)	45 (90.0)	

Table 3

Overall survival time in months for GBM patients stratified by EMP2 expression

Expression score	Mean ± SD	Median	Range
Overall	13.83 ± 15.58	9.45	0.33–68.05
1 (n = 5)	15.45 ± 13.04	10.98	0.28–38.50
2 (n = 30)	12.54 ± 12.89	7.47	0.33–49.87
3 (n = 15)	15.87 ± 21.17	8.71	0.39–68.05
Low EMP2 expression	15.45 ± 13.04	10.98	P = 0.39
High EMP2 expression	13.31 ± 15.73	8.38	

SD standard deviation

Table 4

Adjusted mortality hazard ratio for GBM patients

Covariates	Adjusted HR	95% CI	*P* value
Age	1.04	1.00–1.08	0.09
Female sex	1.45	0.66–3.17	0.36
Secondary GBM	1.76	0.38–8.08	0.47
Recurrent GBM	24.13	6.12–95.11	0.001
EMP2 expression score of 3	1.92	0.69–5.30	0.21
Tumor recurrence	2.87	0.89–9.23	0.08

HR hazard ratio, *CI* confidence interval

Table 5

Correlation between genetic studies and overall survival in lower grade glioma and GBM

Parameter	Correlation coefficient	*P* value	*n*
EMP2 expression score of ≥2	−0.39	0.001	69
Ki-67 positivity	−0.42	0.001	60
GFAP positivity	0.09	0.49	59
MGMT methylation	0.17	0.23	54
PTEN loss	−0.21	0.20	41
EGFR amplification	−0.14	0.40	37
IDH1 mutation	0.42	0.009	37
IDH2 mutation	0.20	0.37	22
Chromosome 1p LOH	0.34	0.13	21
Chromosome 19q LOH	0.30	0.18	21
Co-deletion of chromosome 1p/19q	0.50	0.02	21
EGFRvIII positivity	0.52	0.04	16

EMP2 epithelial membrane protein-2, *GFAP* glial fibrillary acidic protein, *MGMT* O^6-methylguanine-DNA methyltransferase, *PTEN* phosphatase and tensin homolog, *EGFR* epidermal growth factor receptor, *IDH* isocitrate dehydrogenase, *LOH* loss of heterozygosity

Table 6

Correlation between genetic studies and EMP2 expression in lower grade glioma and GBM

Parameter	Correlation coefficient	*P* value	*n*
Ki-67 positivity	0.34	.008	60
GFAP positivity	0.01	.92	59
MGMT methylation	0.02	.88	54
PTEN loss	−0.04	.81	41
EGFR amplification	−0.13	.45	37
IDH1 mutation	−0.12	.49	37
IDH2 mutation	−0.05	.81	22
Chromosome 1p LOH	−0.37	.10	21
Chromosome 19q LOH	−0.37	.10	21
Co-deletion of chromosome 1p/19q	−0.39	.08	21
EGFRvIII positivity	−0.12	.66	16

EMP2 epithelial membrane protein-2, *GFAP* glial fibrillary acidic protein, *MGMT* O^6-methylguanine-DNA methyltransferase, *PTEN* phosphatase and tensin homolog, *EGFR* epidermal growth factor receptor, *IDH* isocitrate dehydrogenase, *LOH* loss of heterozygosity