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CLINICAL STUDY

Peritumoural glutamate correlates with post-operative seizures in supratentorial gliomas

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Abstract To examine the impact of glutamate on post-operative seizures and survival in a cohort of patients with grade II to IV supratentorial glioma. A retrospective analysis was performed on 216 patients who underwent surgery for supratentorial gliomas. Primary explanatory variables were peritumoural and/or tumoural glutamate concentrations, glutamate transporter expression (EAAT2 and SXC). Univariate and multivariate survival analysis was performed with primary outcomes of time to first post-operative seizure and overall survival. Subgroup analysis was performed in patients with de novo glioblastomas who received adjuvant chemoradiotherapy. 47 (21.8%), 34 (15.8%) and 135 (62.5%) WHO grade II, III and IV gliomas respectively were followed for a median of 15.8 months. Following multivariate analysis, there was a non-significant association between higher peritumoural glutamate concentrations and time to first post-operative seizure (HR 2.07, CI 0.98–4.37, p = 0.06). In subgroup analysis of 81 glioblastoma patients who received adjunct chemoradiotherapy, peritumoural glutamate concentration was significantly associated with time to first post-operative seizure (HR 3.10, CI 1.20–7.97, p = 0.02). In both the overall cohort and subgroup analysis no glutamate cycle biomarkers were predictive of overall survival. Increased concentrations of peritumoural glutamate were significantly associated with shorter periods of post-operative seizure freedom in patients with de novo glioblastomas treated with adjuvant chemoradiotherapy. No glutamate cycle biomarkers were predictive of overall survival. These results suggest that therapies targeting glutamate may be beneficial in tumour associated epilepsy.

Keywords Glioma · Epilepsy · Seizure · Glutamate · Tumour

Introduction

Seizures commonly occur in patients with supratentorial gliomas. Such tumour associated epilepsy (TAE) is not controlled by antiepileptic drug therapy in approximately a third of patients [1–4]. Poor seizure control is associated with a worsening of quality of life and cognitive function within this population [5]. The precise neurobiological mechanisms underpinning the development of epilepsy and pharmacoresistance in patients with gliomas are uncertain.

Alterations within glutamate homeostasis however, appear to play a pivotal role in epileptogenesis in patients with gliomas, particularly in the peritumoural region [6–9]. Under physiological conditions, to prevent aberrant signalling, glutamate is cleared rapidly from the extracellular space by excitatory amino acid transporters (EAAT),

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predominantly EAAT2, in astrocytes [10]. Glutamate is also shuttled back into the extracellular space in exchange for cystine by system xC (SXC) transporter in order to participate in metabolism of the potent anti-oxidant glutathione [11].

Glioma cells have been found to upregulate SXC expression [9, 12] leading to generation of greater amounts of protective glutathione, which may confer a survival advantage to tumour cells in hypoxic microenvironments. The resultant increase in extra-cellular glutamate excessively activates glutamate receptors causing excitotoxicity to peritumoural structures and promoting tumour growth and invasion [9, 13–16]. Tumours also mediate the down regulation of EAAT2, which impairs the region's ability to re-uptake glutamate, creating an environment favouring tumour growth [17]. These changes to the glutamate cycle enhance the survival, growth and invasive abilities of gliomas in cell based and murine models.

Increased peritumoural glutamate concentrations were shown to induce epileptiform activity in glioma implanted mice [6] under the influence of glutamate release from the SXC transporter [7]. Our research group recently examined glutamate homeostasis in 288 patients with supratentorial gliomas [8], finding an association between higher tumoural and peri-tumoural glutamate concentrations and pre-operative seizures. It is unknown whether glutamate may also play a role in post-operative seizures, or overall survival. However, such a role would provide a therapeutic target in glioma and TAE treatment.

In the present analysis, we examined the association between glutamate cycle changes, post-operative seizures and survival in a cohort of patients with grade II to IV supratentorial glioma followed up post-operatively. We hypothesized that these changes in the glutamate cycle might also be associated with post-operative seizures and shorter overall survival in patients with gliomas.

Materials and methods

Patient selection

Subjects were selected from a database of 221 patients who underwent craniotomy at the Royal Melbourne Hospital (RMH) or Melbourne Private Hospital (MPH) between 2003 and 2011 for supratentorial glioma, and had peritumoural and/or tumoural tissue sampled for glutamate cycle biomarker analysis (defined below). 175 patients from this database had already been included in an early analysis by our research group examining glutamate and pre-operative seizures [8]. Demographic, glutamate molecular analysis and pre-operative seizure information were the only data points utilized from this previous study.

Inclusion criteria were (a) histopathological diagnosis of WHO grade II, III or IV glioma (as determined by an anatomical neuropathologist), and (b) available follow-up to determine at least one month of post-operative seizure history.

Clinico-pathological data collection

Clinical data were collected from medical records and the Australian Cancer Grid database as part of the BioGrid Australia™ clinical informatics system [18]. The author collecting data was blinded to molecular results. The clinicopathological variables collected were gender, age and histopathological diagnosis at the time of glutamate cycle biomarker analysis, date of first histological diagnosis, side and lobe of lesion. Collected oncological treatment data included the extent of surgery, post-operative chemotherapy and radiotherapy use. Surgery was classified as gross macroscopic resection, subtotal resection (50–95 %), partial resection (less than 50 %) or biopsy. Extent of resection was most commonly determined by post-operative MRI, and was obtained from the multi-disciplinary meeting record or neurosurgical correspondence.

The peritumoural biopsy was within 1 cm of the tumour margin, judged intra-operatively by the surgeon, determined by macroscopic appearance and MRI-guided surgical navigation. Radiologically it was defined as the region outside the FLAIR signal for low grade gliomas and the region outside contrast enhancement for high grade gliomas. The location of peritumoural tissue was determined on an individual basis. The majority of peritumoural samples were taken from white matter.

Seizures and epilepsy were defined in accordance with international classification [19]. TAE was defined as one or more seizures occurring in the presence of a supratentorial glioma. Pre-operative seizures were defined as seizures, attributable to the glioma, occurring before the patient's first surgery. Time to first post-operative seizure in the first 12 months following glutamate biomarker analysis was collected. When an exact time was not documented in the medical record, it was estimated by the mid time point over the interval that the seizure occurred. Estimation of time to first seizure occurred in 16.7 %.

Patients were followed up until July 2014 or until death or referral to a palliative care service and discharge from specialist follow-up. Overall survival was defined as time from initial histological diagnosis until last follow-up.

Epilepsy treatment

An anti-epileptic drug was commenced following first seizure attributable to a glioma. Pre-operatively, most patients without a seizure were also prescribed a prophylactic AED

by the treating neurosurgeon. Post-operatively, all patients were followed by either a neurosurgeon and/or medical oncologist who undertook initial seizure management. Patients with seizures difficult to control were referred to an epileptologist. In general, patients were initially treated with single anti-epileptic drug. Dose adjustment and drug changes were made based on clinical response in terms of seizure control and adverse effects. Serum AED levels were monitored as clinically indicated. Prophylactic AED was generally ceased after a period of 3–6 months of seizure freedom.

Molecular analysis

The primary independent variables were tumoural and peritumoural glutamate cycle biomarkers. Biopsy of brain adjacent to tumour provided the peri-tumoural tissue sample. Biopsy location was judged by the neurosurgeon with the aid of stereotactic MRI-guided navigation. Tumour and peritumoural tissue samples were analysed for glutamate cycle biomarkers including protein concentration of glutamate, EAAT2 transporter and SXC transporter, as previously described [8, 20]. High-performance chromatography was utilized to determine tissue concentration of glutamate. Western blotting was used to quantify the expression of EAAT2 and SXC transporters using integrated optical density (IOD) for protein bands normalized against IOD values for actin [8].

Outcomes

The primary endpoints were time to first post-operative seizure up to 12 months following resection, and overall survival. The 12 month seizure outcome was chosen to minimize the confounding factor of survival on seizure incidence, given the high proportion of glioblastoma patients in the cohort and small number alive after 12 months.

Subgroup analysis

Patients with de novo glioblastomas who received subsequent chemo-radiotherapy were selected for a subgroup analysis. This group represented a relatively more homogenous population which is commonly enrolled in clinical trials.

Statistical analysis

Univariate survival analysis was performed with cox regression. A log transformation was used to convert glutamate cycle biomarkers (glutamate, EAAT2 and SXC) to a normal distribution. A p value <0.2 was chosen to select variables to be included in multivariate analysis using cox regression. A backwards stepwise regression was performed to remove the most non-significant variables from the model. A p value of <0.05 was considered to indicate statistical significance on multivariate analysis. Hazard ratios are presented with 95% confidence intervals (CIs). Kaplan–Meier curves were utilized to display overall survival with a log-rank test employed to compare survival curves. Given there is no normal range for glutamate concentrations, a level greater or equal to the median was deemed 'high' for Kaplan–Meier analysis. Given the high drop out of patients (30% by 6 months and 31% by 12 months) due to death, the 'time to event' approach was deemed more statistically appropriate than an actuarial method for analysis of seizure freedom. All analyses were performed with SPSS-version 22 (SPSS Inc., Chicago, IL).

Ethics

This study protocol was approved by the Melbourne Health Human Research and Ethics Committee (HREC 2006.199). All patients in this study (or their families) gave written informed consent to participate. The authors confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Results

Patient cohort

Of the 221 subjects in the database, 216 were included for analysis (Table 1). Five patients were excluded; one had cerebellar glioma, one had ependymoma, and three had less than one month of follow-up. One hundred and seventy patients from this cohort were included in a previous published analysis [8].

Patients were followed up for a median of 15.8 months. Grade II gliomas had a follow-up of 63.7 months; Grade III gliomas 32.1 months; Grade IV gliomas 10.1 respectively. 81 patients with de novo glioblastomas treated with chemoradiotherapy were examined in a subgroup analysis (Supplementary Table 1). 68 (83.9%) within this subgroup received Stupp protocol adjuvant therapy, and those who didn't were treated before this regimen became standard of care in 2005 [21].

Anti-epileptic drug use

Post-operative AED data was available in 205 (94.9%) of patients. 28 (12.9%) patients had their AED withdrawn for at least 6 months due to seizure control. 93 patients from the entire cohort had post-operative seizures and 94.6% patients

Table 1 Baseline characteristics of the study population

	N	%
Number of patients	216	
Gender (female)	95	43.5
Age at diagnosis (years, SD)	53.91 (16.97)	
Tumour histology		
A	22	10.2
OA/OD	25	11.6
AA	26	12
AOA	8	3.7
GBM	135	62.5
WHO tumour grade		
II	46	21.3
III	33	15.3
IV	137	63.4
Tumour side		
Right	111	46.8
Left	101	51.4
Bilateral	4	1.9
Tumour location		
Frontal	99	45.8
Occipital	9	4.2
Parietal	33	15.3
Temporal	75	34.7
Pre-operative seizures	96	44.4
Post-operative AED prescribed	203	94.0
Post-operative AED not prescribed	2	0.9
Unknown	11	5.1
Type of surgery		
Biopsy	17	7.9
Partial resection	21	9.7
Subtotal resection	83	38.4
Gross total resection	86	39.8
Unknown	9	4.2
Chemotherapy	128	59.3
Unknown	37	17.1
Temozolamide	116	53.7
Unknown	37	17.1
Radiotherapy	180	83.3
Unknown	0	0.0

SD standard deviation, *A* astrocytoma, *OA* oligoastrocytoma *OD* oligodendroglioma, *AA* anaplastic astrocytoma, *AOA* anaplastic oligoastrocytoma, *GBM* glioblastoma, *AED* anti-epileptic drug

were receiving AED at the time of post-operative seizures. In the 205 subjects with AED data, phenytoin was utilized in 77.6%, levetiracetam in 31.2%, valproate in 19.0% and carbamazepine in 19.0%. Clonazepam, lamotrigine, topiramate, oxcarbazepine, clobazam, phenobarbitone, primidone, pregabalin, zonisamide and perampanel were all each used in less than 5% of subjects. 60.5% of subjects (124/205) were only prescribed a single AED.

Post-operative seizures

At the 12 month post-operative point, 81 patients (37.5%) had had at least one post-operative seizure. On univariate analysis, temporal lobe location (HR 1.68), pre-operative seizures (HR 2.14), extent of surgical resection, higher peritumoural EAAT2 (HR 1.82) and higher peritumoural glutamate concentration (HR 1.36) were all associated with shorter time to first seizure (Table 2) and included in multivariate analysis. No other glutamate cycle biomarkers in tumoural or peritumoural tissue were predictive of time to first seizure, including tumoural glutamate concentration (HR 1.0). Following multivariate analysis, only extent of surgical resection was associated with time to first post-operative seizure ($p=0.04$). Although it approached significance, there was no association between peritumoural glutamate concentrations and time to first post-operative seizure (HR 2.07, CI 0.98–4.37, $p=0.06$). No other variables remained in the final backward regression model.

In the de novo glioblastoma with chemoradiotherapy subgroup analysis, pre-operative seizure (HR 1.83, CI 0.96–3.50, $p=0.08$) and higher peritumoural glutamate concentration (HR 3.10, CI 1.20–7.97, $p=0.02$, $n=56$) were associated with shorter time to first post-operative seizure (Table 3). In a multivariate model ($n=56$), higher peritumoural glutamate concentration remained (HR 3.10, CI 1.20–7.97, $p=0.02$) the only variable significantly associated with the occurrence of post-operative seizures (Fig. 1).

Survival

Overall median survival was 62.5 months (interquartile range, IQR 39.2–83.5), 32.1 months (IQR 15.7–104.3) and 10.1 months (IQR 4.6–18.0) for WHO grade II, III and IV gliomas respectively. When examining the entire glioma cohort, no tumour or peritumoural glutamate biomarkers predicted overall survival (Supplementary Table 2) (Fig. 2).

Predictors of survival were analysed in the more homogenous de novo glioblastoma with adjuvant therapy population. Overall median survival for this cohort was 14.5 months (IQR 9.7–21.7). Age at diagnosis (HR 1.0, CI 1.00–1.04, $p=0.06$), lobe of lesion ($p=0.004$), extent of resection ($p=0.05$), tumour SXC transporter expression (HR 0.632, CI 0.32–1.26, $p=0.19$, $n=44$) and peritumoural glutamate concentration (HR 0.745, CI 0.52–1.08, $p=0.12$, p $n=56$) were associated with overall survival on univariate analysis. No glutamate cycle biomarkers were significantly prognostic in a multivariate model (Supplementary Table 3).

Table 2 Clinicopathological factors associated with time to first post-operative seizure

	n	HR	CI 95 %	p value
Gender (female)	216	1.194	0.766–1.862	0.43
Age at diagnosis	216	1.003	0.990–1.016	0.67
Tumour histology	216			0.28
A		0.829	0.404–1.699	0.61
OA/OD		0.546	0.407–1.609	0.55
AA		0.904	0.496–1.858	0.90
AOA		2.523	1.001–6.356	0.05
GBM		–	–	–
Temporal lobe	216	1.657	1.064–2.580	0.03
Preoperative seizure	216	2.140	1.367–3.350	0.001
Surgery resection	207			0.19
Biopsy		1.243	0.514–3.003	0.63
Partial		2.137	1.063–4.298	0.03
Subtotal		1.355	0.822–2.236	0.23
Gross macroscopic			–	–
Tumour				
Glutamate (log)	199	1.025	0.742–1.417	0.88
EAAT2 (log)	131	1.383	0.704–2.716	0.35
SXC (log)	124	1.014	0.469–2.194	0.97
Peri-tumoural				
Glutamate (log)	131	1.356	0.932–1.975	0.11
EAAT2 (log)	73	1.816	0.747–4.416	0.19
SXC (log)	73	1.066	0.385–2.951	0.90

A astrocytoma, *OA* oligoastrocytoma, *OD* oligodendroglioma, *AA* anaplastic astrocytoma, *AOA* anaplastic oligoastrocytoma, *GBM* glioblastoma, *EAAT* excitatory amino acid transporter, *SXC* system Xc

Discussion

This study is novel in investigating the association between tumoural and peritumoural glutamate cycle biomarkers and post-operative seizures and survival in patients with supratentorial gliomas. The major findings were that while glutamate cycle biomarkers were not associated with post-operative seizures or survival in the overall patient population, a higher peritumoural glutamate level was associated with seizure recurrence in those with de novo glioblastomas treated with chemoradiotherapy.

In gliomas, glutamate homeostasis is altered both within tumour and in the peri-tumoural region. Higher extra-cellular concentrations of glutamate have been reported in tumour and peritumoural tissue using microdialysis [22–24] and high performance liquid chromatography [6, 8]. In our previous study, the peritumoural region had significantly higher glutamate concentrations than the tumour itself, suggesting a degree of peritumoural independence [8].

Peritumoural tissue is increasingly regarded as the key structure responsible for epileptogenesis with studies suggesting ictal onset zone is in the region 1–2 mm from the tumour edge [25]. This tissue surrounding brain tumours harbours an altered microenvironment compared with normal brain [24]. An imbalance in glutamate neurotransmission has long been proposed as a key mechanism in network hyperexcitability [26, 27]. However, only recently, has upregulated glutamatergic neurotransmission been linked to seizures in rodents and pre-operative humans with gliomas [6–9]. One possible mechanism of epileptogenesis is the over excitation of peritumoural extra-synaptic NMDA receptors by excess glutamate [28].

We have shown in subgroup analysis that peritumoural glutamate concentration at time of resection predicts seizures over the subsequent 12 months. This subgroup was chosen as they represented a homogenous group with similar tumour type and adequate performance status to all receive adjuvant chemoradiotherapy. Although the subgroup size was small, the significant signal in this well controlled cohort supports the epileptogenic nature of peritumoural glutamate. One potential mechanism explaining this association is that peritumoural glutamate levels stay elevated over the course of post-operative period and directly maintain a hyperexcitable local network. Alternatively, initial peaks in peritumoural glutamate concentration may help prime a local network for seizures, potentially via priming of glutamate receptors [28].

The failure of the analysis of the whole tumour cohort to reach statistical significance may be related to the heterogeneous nature of the population. The role of glutamate

Table 3 Clinicopathological factors associated with time to first post-operative seizure—subgroup analysis

	n	HR	CI 95 %	p value
Gender (female)	81	0.990	0.512–1.915	0.98
Age at diagnosis	81	1.003	0.977–1.030	0.83
Temporal lobe location	81	0.886	0.455–1.724	0.72
Pre-operative seizure	81	1.830	0.960–3.491	0.07
Surgery resection	81			0.99
Biopsy		0.000	0.000–0.000	0.98
Partial resection		0.994	0.330–2.998	0.99
Subtotal resection		0.939	0.468–1.880	0.86
Gross total resection		–	–	–
Tumour				
Glutamate (log)	78	1.009	0.615–1.655	0.97
EAAT2 (log)	48	1.008	0.402–2.527	0.99
SXC (log)	44	1.398	0.395–4.951	0.60
Peri-tumoural				
Glutamate (log)	56	3.096	1.202–7.970	0.02
EAAT2 (log)	31	1.235	0.327–4.669	0.76
SXC (log)	31	3.814	0.493–29.493	0.20

EAAT excitatory amino acid transporter, *SXC* system Xc

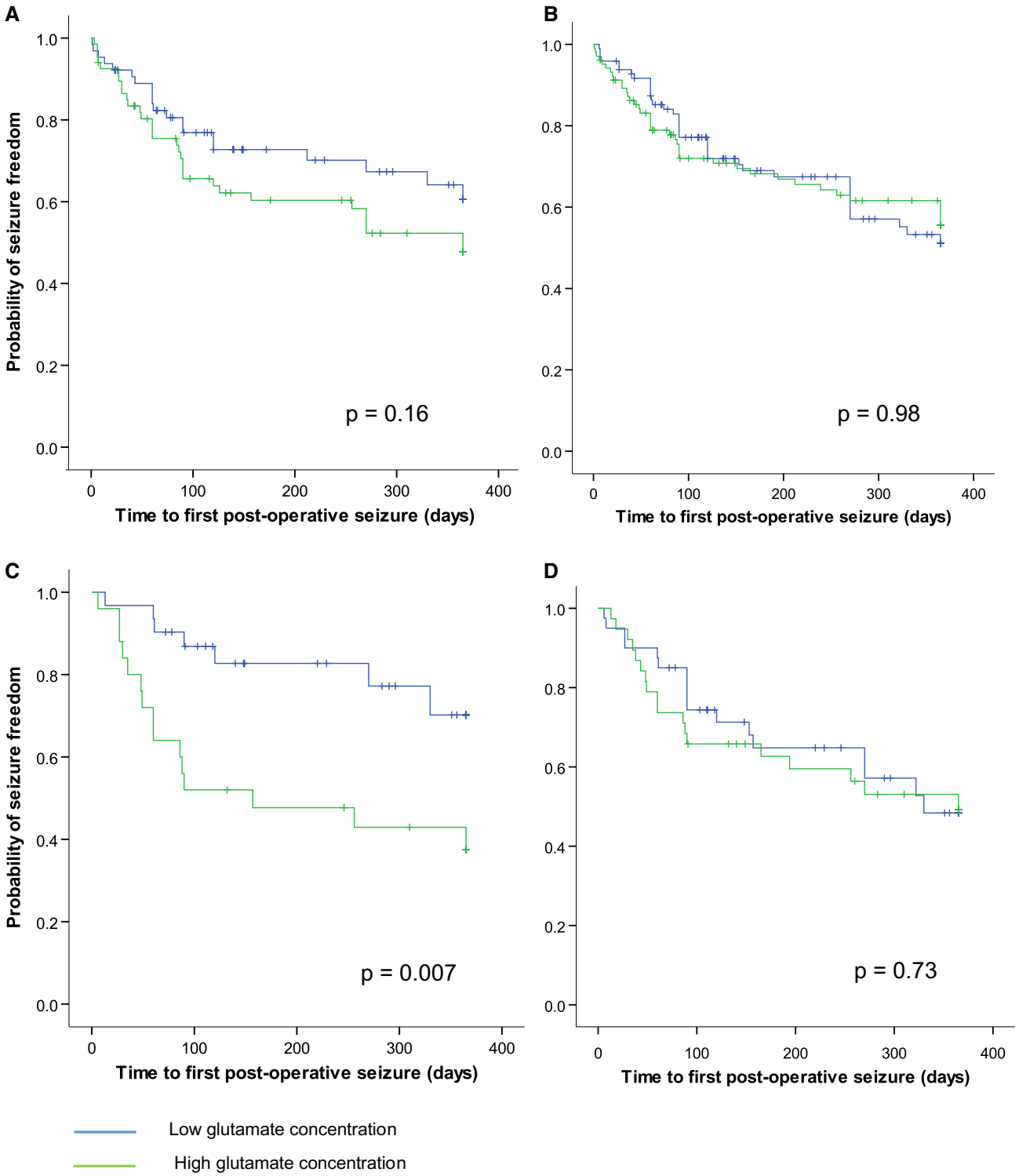

Fig. 1 Probability of seizure freedom according to glutamate concentrations Time to first post-operative seizure Kaplan–Meier survival curves. **a** According to peritumoural glutamate concentration n = 131, p = 0.16. **b** According to tumoural glutamate concentration n = 200, p = 0.98. **c** According to peritumoural glutamate concentration in de novo glioblastoma with adjuvant therapy subgroup, n = 56, p = 0.007. **d** According to tumoural glutamate concentration in de novo glioblastoma with adjuvant therapy subgroup, n = 78, p = 0.73

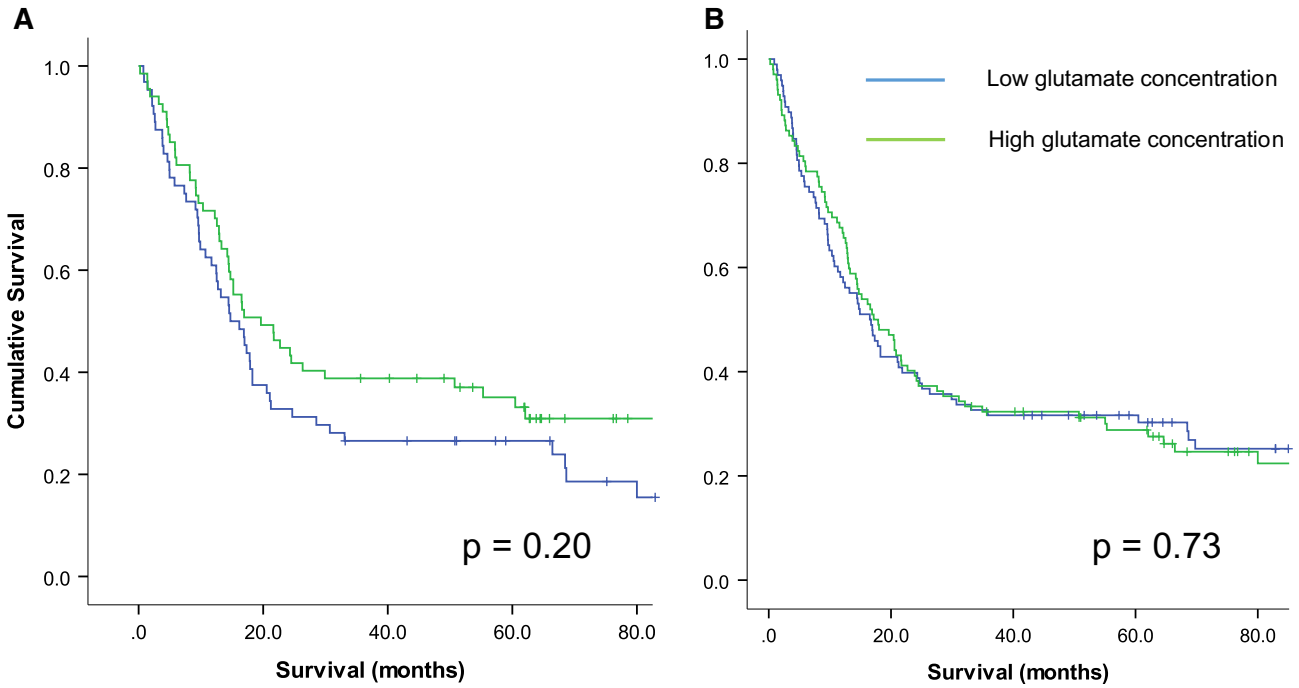

Fig. 2 Overall survival according to **a** peritumorual glutamate concentration, n = 131, p = 0.19, **b** tumoural glutamate concentration, n = 213, p = 0.73

cycle adaptations in lower grade gliomas have not been well studied and likely only contribute in part to the pathogenesis of TAE, with other molecular factors such as isocitrate dehydrogenase mutations [29] and impaired GABAergic inhibition [30, 31] also likely playing an important role. In addition, chemotherapy and radiotherapy have been described to have a positive impact on seizure control [32–34] and these therapies differed widely across the cohort and may have clouded the influence of glutamate.

In contrast to our work on pre-operative tumour related seizures [8], EAAT2 and SXC expression did not correlate with seizures post-operatively. Within gliomas, SXC is responsible for the majority of extra-cellular glutamate [11] and the small sample of peritumoural cases analysed for transporter expression may have been insufficient to detect a correlation.

In our analysis, 'time to first post-operative seizure' was selected as a primary outcome measure. This was in favour of the examining seizure frequency or freedom, as is commonly assessed in epilepsy drug trials. This 'time to event survival analysis' was chosen as the most appropriate method given the variability in follow-up, owing largely to the short survival of patients with grade IV gliomas.

There is a large amount of cell line and animal data suggesting that glutamate homeostasis promotes survival and tissue invasion for the glioma cells. Upregulation of SXC leads to the highly protective glutathione, an important antioxidant. Increased glutathione confers resistance against chemoradiotherapy [35], AMPA receptor stimulation induces tumour cell migration [13] while glutamate secretion triggers neurotoxic cell death and gives a growth advantage to gliomas [15]. Importantly, blockade of SXC, AMPA and NMDA and upregulation of EAAT2 suppress glioma growth and improves tumour survival [12–17]. Reduced expression of SXC has also been associated with prolonged overall survival in a small human cohort with grade IV gliomas [9].

Despite these preclinical findings, we did not find any of our measured glutamate cycle biomarkers to be prognostic, both when examining our cohort as a whole and within the de novo glioblastoma with adjuvant therapy subgroup. Our results are in line with clinical studies that reported lack of benefit from anti-glutamate therapy for gliomas. These included two early phase clinical trials that examined the role of sulfasalazine, a SXC antagonist as adjuvant therapy for patients with recurrent [36] and newly diagnosed high grade glioma [37]. A lack of survival benefit and significant adverse effects in both studies have limited future studies of this well studied SXC antagonist in high grade glioma patients. In addition, two studies analysed the impact of talampanel, an AMPA receptor antagonist, in glioblastoma patients and found no survival benefit [38, 39]. Our findings add doubt to the role of glutamate in determining glioblastoma survival in humans.

The main limitation of this study is its retrospective nature and small sample size. Tumour progression and subsequent therapy, both important influences on seizures, were not documented owing to the inherent inaccuracy in collecting

this variable retrospectively. During the follow-up period of our study, molecular markers such as IDH1, 1p19q and ATRX were not routinely assessed. Given their now recognized prognostic implications, the absence of these markers weakens our survival analysis. Anti-epileptic treatments and the approach to drug adjustments varied across the cohort; yet importantly, only a minority of post-operative seizures occurred off AED (5.4 %). Characterization of seizure semiology (e.g. focal, generalized, status epilepticus) were not consistently available, but would have be a clinically useful addition to our analysis. Finally, the study spanned a long time period, during which neuro-oncological practices had inevitably changed, creating heterogeneity within the cohort. The examination of a larger sample would allow confirmation of our subgroup findings.

Our analysis adds to a growing body of evidence supporting glutamate in the pathogenesis of tumour associated seizures. If glutamate is indeed a useful biomarker to direct therapy against, choose patients for treatment and measure response, a non-invasive alternative to brain sampling is required [40]. Prospective imaging studies that examine post-operative seizures, tumour progression and glutamate over the course of disease will be important in further characterizing the link between glutamate adaptations and seizures. Such prospective studies will be necessary to define populations that may benefit from targeted epilepsy treatments. One exciting target is the AMPA receptor given the antagonist, perampanel, has recently been approved for use in Australia after showing efficacy as adjunctive therapy in refractory focal epilepsy.

Conclusion

Glutamate cycle biomarkers in tumoural and peritumoural tissue did not predict overall survival and adds further doubt to the role of glutamate in determining glioma survival in humans. In contrast, increased concentrations of peritumoural glutamate were associated with shorter periods of post-operative seizure freedom in patients de novo glioblastomas treated with adjuvant chemoradiotherapy. These findings suggest a hyperexcitable peritumoural network due to elevated glutamate and support this region as a target for future therapies against TAE.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards

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