

Activation of the mTOR signaling pathway in peritumoral tissues can cause glioma-associated seizures

Yang Yuan¹ · Wang Xiang¹ · Liu Yanhui¹ · Liang Ruofei¹ · Luo Jiewen¹ · Jiang Shu¹ · Mao Qing¹

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Abstract Epileptic seizures, the most common symptom accompanying glioma, are closely associated with tumor growth and patient quality of life. However, the association between glioma and glioma-related epilepsy is poorly understood. In fact, findings related to the location of epileptogenicity have been inconsistent in previous studies. We investigated seizure foci in patients with glioma and the corresponding association between glioma-related epilepsy and the tumoral and peritumoral microenvironment. Clinical characteristics, extracellular electrophysiology, immunohistochemistry, and western blots were conducted on 12 patients with glioma; nine patients had histories of preoperative seizures while three did not. Samples from included patients were used to identify seizure foci and mTOR pathway status. Electrophysiological recordings were conducted on 36 samples (tumor, peritumoral, and normal brain tissues) from 12 patients. Interictal-like discharges (ILDs) were observed in seven of nine peritumoral tissues obtained from patients with glioma that had experienced perioperative seizures. No ILDs were observed in any other sample groups. Western blots and immunohistochemistry for mTOR pathway proteins (mTOR and S6k) suggested that the mTOR pathway was activated in peritumoral tissues of patients with seizure history, but inactivated in patients without seizure history. Our results suggest that mTOR pathway expression in peritumoral

tissues is associated with tumor-related seizures, thus providing a potential target for therapeutics aimed at simultaneously controlling gliomas and seizures.

Keywords Glioma · Glioma-associated seizure · Epilepsy · Patch clamp · Brain slice

Abbreviations

GAS Glioma-associated seizure
GBM Glioblastoma multiforme
HGG High grade glioma
OS Overall survival
PFS Progression-free survival

Introduction

Although gliomas are the most prevalent type of malignant brain tumor [1, 2], they currently have no effective treatment. It has been reported that nearly 80 % of patients with glioma have (or will) experience a seizure at least once during the course of the disease, making seizure activity the most common symptom accompanying gliomas [11, 16, 17]. Moreover, it has been suggested that postoperative epilepsy prognosis significantly affects the quality of life for patients with glioma [18].

Previous studies have disagreed on the location of epileptogenicity in patients experiencing glioma-associated seizures (GAS) [12, 15]. For example, the tumorocentric hypothesis suggests that seizures arise from, or are caused by, the tumor itself [10]. On the other hand, the epileptocentric hypothesis postulates that changes in the extracellular milieu cause cortical hyperexcitability [9].

The mechanism of epileptogenesis in patients with glioma also remains unclear [1]. Recent evidence suggests

✉ Jiang Shu
jiangshu2000@126.com

✉ Mao Qing
qingmao2000@163.com

¹ Department of Neurosurgery, West China Hospital, Sichuan University, 37 Guo Xue Xiang, Chengdu, Sichuan 610041, China

that tumor growth and seizure activity could stimulate each other; thus, it is possible that gliomas and epilepsy share one or more signaling pathways [14]. One possible candidate is the mammalian target of rapamycin (mTOR) pathway, as it has been reported to be involved in glioma genesis [6] as well as several neurological diseases including epileptogenesis [3]; however, whether disruption in mTOR signaling can induce GAS has not been investigated.

In this study, we hypothesized that seizure activity could be localized to peritumoral tissue in patients with glioma. Further, we postulated that activation of the mTOR pathway in peritumoral neurons would lead to neuronal hyperexcitability and epileptic activity. Thus, to identify the possible relationship between the mTOR pathway and seizure activity in patients with glioma, we used the patch clamp method to record activity from gliomas in intraoperative tissues.

Methods

Patient data and sample collection

All patients included in this study were pathologically diagnosed with gliomas and all surgeries were performed in the

West China Hospital at Si Chuan University. For extracellular electrophysiological examinations, tumor samples were immediately immersed in ice-cold artificial cerebral spinal fluid (ACSF). Tumor samples were also obtained for western blotting and immunohistochemistry, which were numbered and transferred into jars containing liquid nitrogen and stored for future use. Glioma patients that had a history of at least one typical seizure, or who had electroencephalogram (EEG)-confirmed epilepsy with a histologically confirmed glioma were defined as having a GAS.

Extracellular electrophysiology

Fresh tumors and peritumoral tissues obtained intraoperatively from patients with gliomas were immersed in ice-cold ACSF containing (in mM) 119 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.3 MgSO₄, 1 NaH₂PO₄, 26.2 NaHCO₃, and 11 glucose; pH 7.4 and bubbled with 95 % O₂ + 5 % CO₂. Coronal slices (400 μm) were then obtained and submerged in ACSF at 25 °C (ACSF contained (in mM): 125 NaCl, 3 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 2 CaCl₂, 1.3 MgSO₄, and 25 glucose) where they recovered for 30 min. Slices were then transferred to a recording chamber, and data were acquired using Clampex 10.04 software. Recording electrodes were fabricated from BF150-75-10 borosilicate glass (Sutter Inc) via a glass microelectrode puller (P97

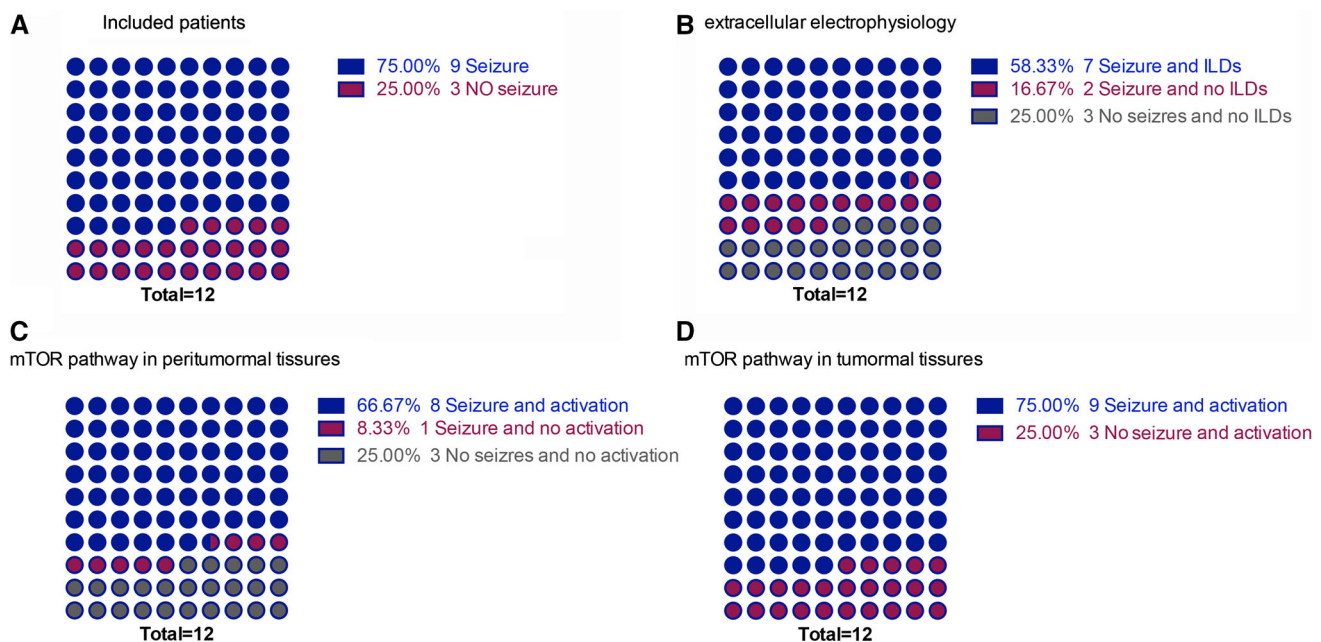


Fig. 1 Patient characteristics shown as a proportion of the entire patient population. **a** Nine patients (*blue*) included in this study had preoperative seizures, while three (*violet*) did not. **b** Epileptogenicity extracellular recordings of 12 peritumoral tissues. Of the nine patients with preoperative seizures, seven displayed spontaneous interictal-like discharges (ILDs) while two of them did not. No ILDs were detected in the peritumoral tissues collected from the three patients

without history of seizures. **c** mTOR pathway status in peritumoral tissue samples; eight of the nine patients with seizure exhibited mTOR pathway activation in peritumoral tissues, while the other patient with a history of seizure displayed no mTOR activity. Likewise, no mTOR activation was detected in the three patients without history or seizure. **d** mTOR pathway status in the 12 included tumor samples; all exhibited mTOR activation (color figure online)

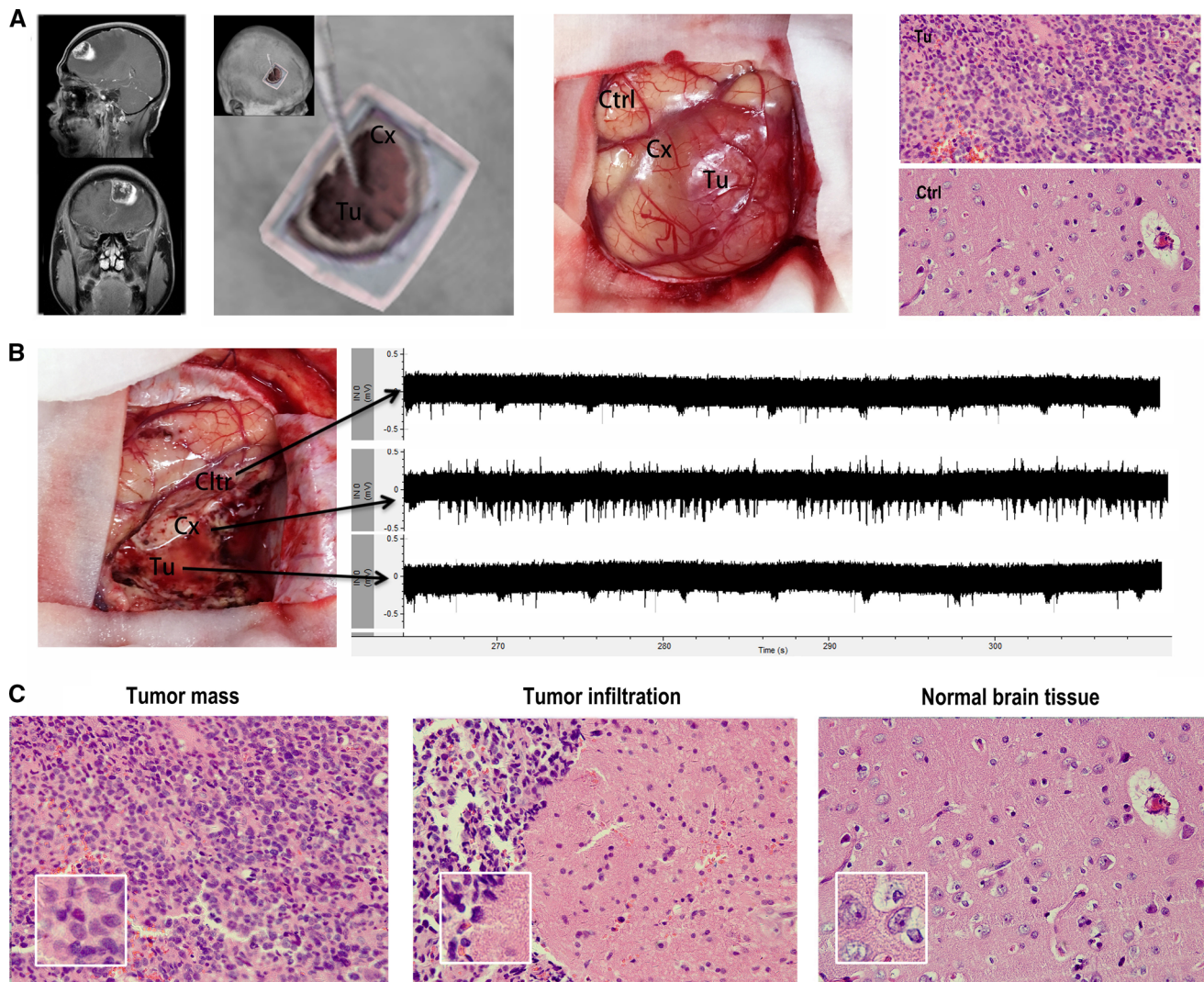


Fig. 2 a Example of a patient with a glioblastoma patient with history of preoperative seizures; contrast T1 magnetic resonance and digital images showing the tumor-infiltrated cortex. HE staining showing tumor abnormalities compared to normal tissues.

b Extracellular recordings of interictal-like discharges (ILDs) from a slice of peritumoral tissues. Electrode locations: *Ctrl* normal brain tissue, *Tu* tumor tissue, *Cx* superficial peritumoral tissue

(Sutter Inc)). All data were filtered at 5 kHz, digitized at 10–20 kHz, and analyzed using Clampfit 10.0 software (Molecular Devices).

Western blotting and immunohistochemistry

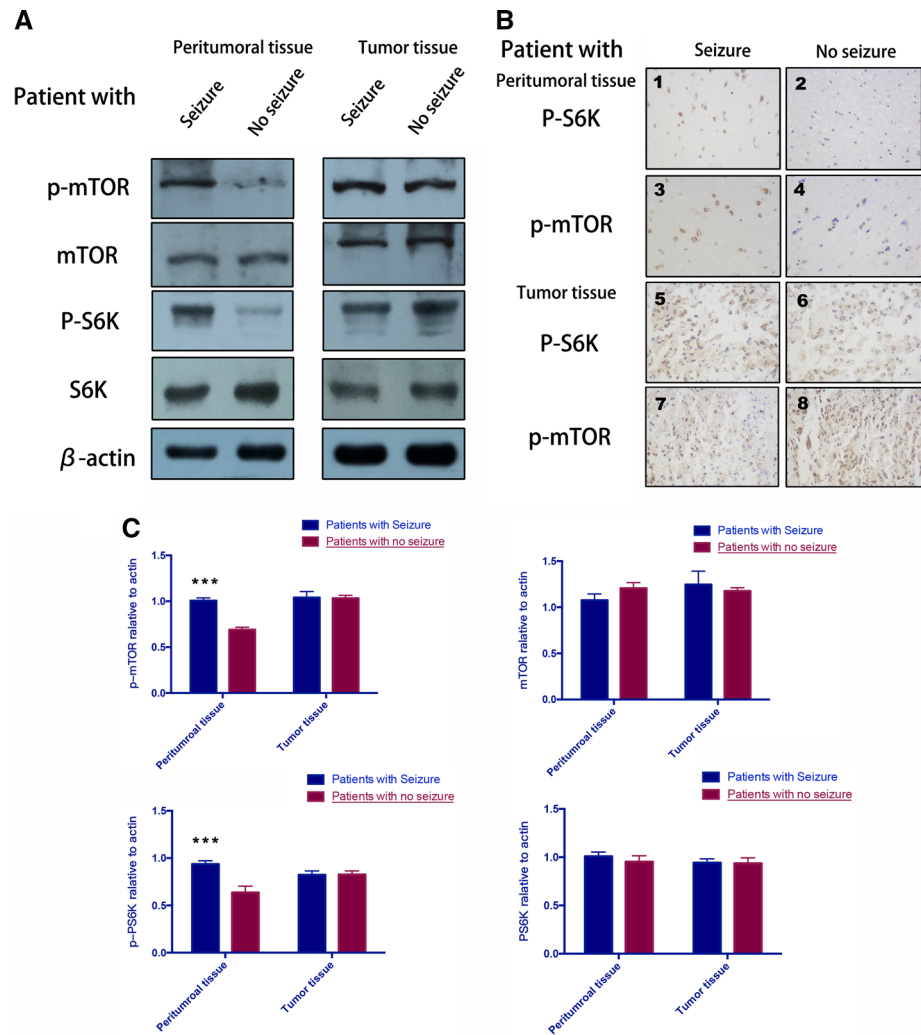
Primary antibodies used were anti-phospho-S6 ribosomal protein (5364, Cell Signaling Technology, USA), anti-phospho-mTOR (2448, Cell Signaling Technology, Boston, USA), and anti-NeuN (24307, Cell Signaling Technology, USA). Western blots and immunohistochemistry were conducted according to the manufacturers' protocols. Briefly, for immunohistochemistry, staining was performed using an automated immunostainer (Benchmark Ultra, Ventana, Tucson, AZ, USA) following the standard protocol

recommended by the manufacturer. For western blots, proteins were resolved using polyacrylamide gel electrophoresis (BioRad) under denaturing conditions, followed by their transfer to polyvinylidene fluoride membranes (Millipore, MA, USA). Membranes were blocked overnight in 5 % milk (Santa Cruz Biotechnology, TX, USA) in Tris-buffered saline with 0.1 % Tween 20 (TBST; pH 7.5) at 4 °C. Membranes were then washed three times for 5 min each in TBST and incubated with primary antibodies.

Statistical analyses

All statistical data were analyzed by SPSS version 20.0. A type I error of $\alpha = 5\%$ was defined. Univariate analyses were performed using the Chi-square test for dichotomous

Fig. 3 Western blot and immunohistochemistry staining identifying mTOR pathway status. **a** Western blot of both tumor and peritumoral tissue samples collected from patients with and without history of seizure. **b** immunohistochemistry staining for mTOR, p-MOT, S6K, and p-S6k in peritumoral tissues. **c** All results were normalized using beta actin protein. Histogram showing phosphorylated mTOR pathway proteins significantly increased in peritumoral tissues of patients with glioma-associated seizures ($p < 0.05$)



variables and the Mann–Whitney U test was performed on continuous non-parametric data.

Results

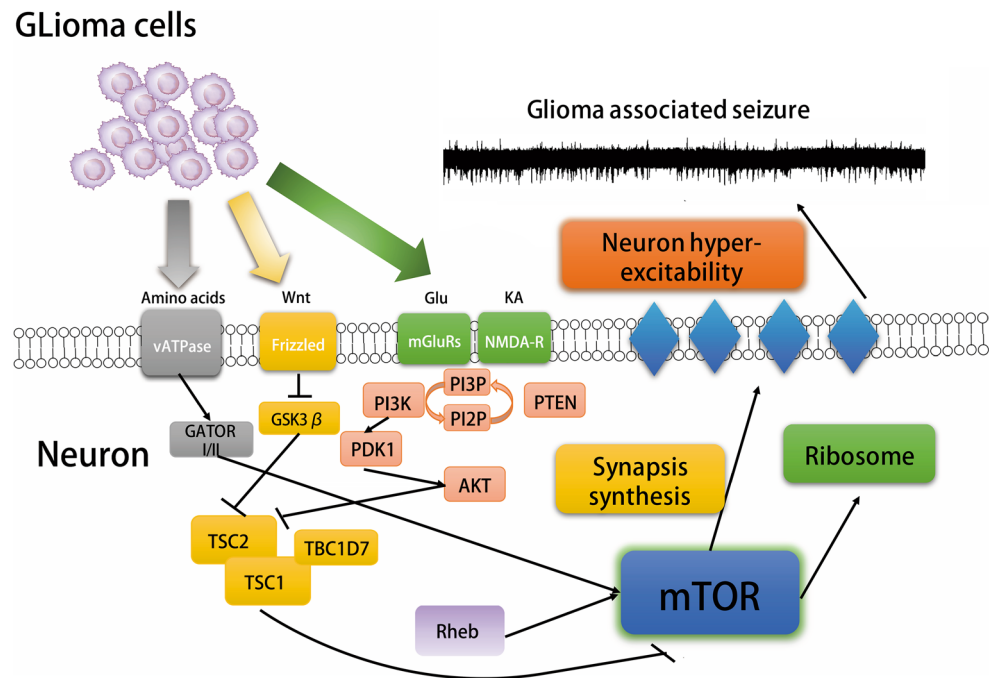
A total of 12 patients with pathologically confirmed gliomas were included in this study; nine patients had preoperative seizures and three patients reported no preoperative seizures, five of them were low-grade gliomas and seven of them were high-grade gliomas. Patient characteristics are summarized in Fig. 1. Of the 12 patients, seven were female and five were males. All patients, regardless of seizure status, underwent tumor resection surgeries. Tumor tissues, peritumoral tissues, and relative, normal brain tissues were collected for future use.

To determine the localization of epileptogenicity, extracellular electrophysiology was conducted on samples obtained from the included patients. Interictal-like discharges (ILDs)

were observed in seven of the nine peritumoral tissues obtained from patients with glioma and preoperative seizures. Their peak amplitude was 0.30027 mV and antipeak amplitude was -0.42867 mV, slope was 1.50238 mV/ms. Hematoxylin and eosin (HE) staining showed difference between tumors and normal brain tissue (Fig. 2a). Such Interictal-like discharges (ILDs) were not observed in slices obtained from the three glioma patients without preoperative seizures or from tumor tissues (Fig. 2b).

To identify the possible cause of neuronal hyperexcitability, we next performed western bolt and IHC analyses on the freshly collected frozen samples (tumor and peritumoral tissues) with anti-phospho-S6 ribosomal protein and anti-phospho-mTOR protein. The western blot analysis for the patients with GAS identified activation of the ps6-mTOR pathway in peritumoral tissues (Fig. 3a). For patients without GAS, the ps6-mTOR pathway remained inactivated in peritumoral brain tissues. Moreover, weak staining for p-S6 and p-mTOR was observed in

Fig. 4 Illustrations of the relationship between glioma-associated seizure and mTOR pathway status. Glioma cells likely affected adjacent interneurons or pyramidal cells, inducing pathological mTOR pathway activation. mTOR activation in interneurons or pyramidal cells may accelerate protein synthesis, cell growth and proliferation, and synaptic plasticity, which may induce neuronal excitability and cause epileptogenesis



patients without GAS in peritumoral tissue areas, but p-S6 and p-mTOR were intensely positive in eight of the nine patients with GAS (Figs. 1, 3b). Finally, all tumor samples were positive for p-S6 and p-mTOR antibodies.

Discussion

Epileptic activity is common in patients with glioma [5, 18, 19], and recent studies have identified the two conditions as sharing common pathogenic mechanisms and pathways [4]. Increasing evidence suggests that glioma growth stimulates seizures, that and seizure activity could contribute to tumor growth. Here, we performed electrophysiological investigations on tissues obtained from 12 patients with glioma and demonstrated that glioma cells that had infiltrated the peritumoral neocortex generated spontaneous interictal discharges. This finding supports the key physiological theory that GAS stems from peritumoral tissue instead of from the tumor core itself. An important mechanism mediating this key microenvironmental interaction is the mTOR pathway, and our results have identified that different mTOR pathway statuses in peritumoral tissue are associated with the likelihood of GAS occurrence (Fig. 4).

Theoretically, the depolarization of large numbers of pyramidal cells, which are located in the third layer of the cortex, may contribute to the initiation of spontaneous ILDs and the ultimate development of seizure activity [13]. Here, we found that peritumoral interneuron firing preceded ILDs, supporting the theory that the seizure focus in

patients with glioma is localized in peritumoral tissue. It should be noted that one hypothesis suggested that GAS could arise from a tumor core if the glioma contained neuronal components. However, no ILDs were found in any of our 12 glioma tissue samples.

In terms of mTOR signaling, this pathway has previously been identified as being involved in the pathology of many neurological diseases [8]. For instance, Jae Seok Lim demonstrated that somatic brain mutations in mTOR cause focal cortical dysplasia type II, leading to intractable epilepsy [7]. In the current study, in addition to our localization of the seizure focus in patients with glioma, we found a change in the activation of the mTOR pathway in peritumoral tissue in patients with GAS, but not in those without GAS.

Two mechanisms of GAS remain unclear: (1) how glioma cells disrupt interneurons and cause mTOR pathway activation and (2) how the mTOR pathway is involved in epilepsy. Previous studies have implicated that mTOR signaling may involve protein synthesis, cell growth and proliferation, and synaptic plasticity, which may induce neuronal excitability and be responsible for epileptogenesis [8]; however, the role of the mTOR pathway in GAS requires further study.

Finally, our study has some limitations that should be addressed. First, the number of patients in the clinic with glioma was limited; thus, our electrophysiological characterization was conducted on a small number of tissue samples. Moreover, because of the small sample size, and in consideration of ethical issues, western blot and IHC analyses were not conducted on samples from normal brain

tissues. Finally, although mTOR signal pathway activation in peritumoral tissue was demonstrated in patients with GAS, the genetic mechanisms that mediate this effect remain unclear and were beyond the scope of the current study; thus, this will be need to be addressed in future investigations.

Conclusions

Epilepsy is a feature of at least 60 % of all cases of gliomas. In our study, seizure activity in patients with GAS was localized to peritumoral tissues. Further, we demonstrated that activation of the mTOR pathway in peritumoral tissues correlated with GAS. Our results provide a target for potential therapeutics aimed at simultaneously controlling gliomas and seizures in this patient population.

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Compliance with ethical standards

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

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