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Glutamate Promotes Cell Growth by EGFR Signaling on U-87MG Human Glioblastoma Cell Line

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Abstract Accumulating evidences suggest that glutamate plays a key role in the proliferation and invasion of malignant glioblastoma (GBM) tumors. It has been shown that GBM cells release and exploit glutamate for proliferation and invasion through AMPA glutamate receptors. Additionally, amplification of the epidermal growth factor receptor (EGFR) gene occurs in 40–50% of GBM. Since, PI3K/Akt is considered one of the main intracellular pathways involved in EGFR activation, AKT functions could trigger EGFR signaling. Thus, we investigated whether EGFR-phospho-Akt pathway is involved on the glutamate inducing U-87MG human GBM cell line proliferation. For these purpose, we treated the U-87MG cell line with 5 to 200 mM of glutamate and assessed the number of viable cells by trypan blue dye exclusion test. An increase in cell number (50%) was found at 5 mM glutamate, while the addition of DNQX (500 μM), an antagonist of AMPA receptor, inhibited the effect of glutamate on the U87-MG cells proliferation. Also, at 5 mM glutamate we observed an increase on the EGFR and phospho-Akt contents evaluated by immunohistochemistry. Moreover, U-87MG cells treated with glutamate exhibited an increase about 2 times in the EGFR mRNA expression. While, in the presence of the anti-EGFR gefitinib (50 μM) or the PI3K inhibitor wortmannin (5 μM), the U-87MG proliferation was restored to control levels. Together, our data suggest that glutamate signaling mediated by AMPA receptor induces U-87MG human GBM cell line proliferation via EGFR-phospho-Akt pathway.

Keywords Glioblastoma multiforme · Glutamate · EGFR · Phospho-Akt · Cell proliferation

Abbreviations

CNS	central nervous system
DMEM	Dulbecco's modified essential medium
DNQX	6,7-Dinitroquinoxaline-2,3-dione
EGFR	epidermal growth factor receptor
FCS	fetal calf serum
GBM	malignant glioblastoma
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-biphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate

Introduction

Malignant Glioblastoma (GBM) accounts for 25% of all primary central nervous system (CNS) tumors in adults and is associated with poor prognosis. The prognosis for intermediate grade glioma patients has been improved by

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the combination of therapies such as surgery, radiation, and chemotherapy, but the overall survival has not increased in patients with GBM [1–3]. The poor prognosis of GBM patient's seems to be related to its highly proliferative and invasive behavioral through the healthy nervous tissue [4, 5].

Amplification of the epidermal growth factor receptor (EGFR) gene occurs in 40–50% of GBM [6–15], and the tumor cells usually overexpress EGFR [16, 17]. EGFR stimulation by the growth factor leads to activation of phosphatidylinositol 3-kinase (PI3K), which catalyzes the conversion of phosphatidylinositol 4,5-biphosphate (PIP2) into phosphatidylinositol 3,4,5-triphosphate (PIP3). Finally, membrane-associated PIP3 attracts and activates the protein serine-threonine kinase Akt [18, 19].

Regarding the glial origin of GBMs, these tumors exhibit receptors for a large variety of neurotransmitters [20–22]. Particularly, glutamate receptors, including ionic (NMDA, AMPA and Kainate) and metabotropic receptors (G-protein-coupled-receptors) have been implicated on glioma behavior [20, 21, 23–28]. Accordingly, GBMs with enhanced release of glutamate could take a growth advantage killing neurons and expanding freely through the brain [29]. Glutamate may also stimulate tumor growth and migration by an autocrine or paracrine pathway [25, 28, 30]. Indeed, the presence of ionotropic receptors (NMDA and AMPA) seems to be essential to glioma survival and migration in vitro [24, 25]. Currently, it has been proposed that the activation of ionic glutamate receptors, more specifically AMPA receptors, may be involved in the stimulation of the anti-apoptotic signaling cascade of the serine/threonine kinase Akt [31]. In glial culture systems it was demonstrated that the activation of glutamate receptors were capable to induce an increase on the expression of the erbB1 receptor genes, which include, among others, EGFR [32]. Therefore, in the present study, we investigate whether glutamate trigger cell growth in U-87MG human GBM cell line through EGFR-phospho-Akt signaling.

Materials and Methods

Cell Line and Cell Line Maintenance

The U-87MG human GBM cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in complete medium consisting of Dulbecco's modified essential medium (DMEM; Gibco BRL, NY, USA) containing 2% (*w/v*) *L*-glutamine and 10% (*v/v*) fetal calf serum (FCS; Cultilab, SP, Brazil), at a temperature of 37°C, a minimum relative humidity of 95%, and an atmosphere of 5% CO_2 in air. For experiments, exponentially growing cells were detached from the culture flasks either using ethylenediamminetetraacetic acid (EDTA)-trypsin (Sigma-Aldrich, MO, USA), or by scraping with a rubber policeman. For experiments, the FCS concentration was reduced to 0.5% in accordance to Yoshida et al. [33]. Cell viability greater than 95% was confirmed by trypan blue exclusion.

Glutamate and Drug Treatment

The U-87MG cell line was treated with glutamate, DNQX (6,7-Dinitroquinoxaline-2,3-dione; Sigma-Aldrich, MO, USA), gefitinib (Astra Zeneca, Macclesfield, Cheshire, UK) and wortmannin (Calbiochem, San Diego, USA) during 48 h. Glutamate (Sigma-Aldrich, MO, USA) was dissolved in the culture medium (FCS 0.5%) to obtain concentrations of 5 to 200 mM. DNQX was dissolved in the culture medium alone (FCS 0.5%) and in 5 mM glutamate solution to obtain a concentration of 500 μM. gefitinib and wortmannin were dissolved in DMSO at a stock solution and diluted in culture medium (FCS 0.5%) to obtain appropriate concentrations determined by preliminary studies: 50 μM for gefitinib and 5 μM for wortmannin. Control cells were cultured, during 48 h, in culture medium (FCS 0.5%) without added glutamate. Then, cell viability assay, immunohistochemistry and RNA extraction procedures were performed.

Cell Viability

After the 48 h glutamate treatment (5, 25, 50, 75, 100, 150 and 200 mM), DNQX (500 μM), gefitinib (50 μM) or wortmannin (5 μM) cell viability was assessed by cell counting as described previously by Sigalas [34]. Cultures were incubated with 0.4% trypan blue solution (Sigma-Aldrich, MO, USA) for 10 min. Only dead cells, with a damaged cellular membrane, are permeable to trypan blue. The number of trypan blue permeable cells and non-stained cells were counted in four randomly chosen fields. By counting the cells in the field and calculating the ratio blue/white, we can express the viability of the culture as the number of viable cells.

RT-PCR Assay

Total RNA from 10^5 cells per treatment was extracted by the method of Guanidine isothiocyanate [35]. For cDNA generation two micrograms of RNA were incubated with 20 units of avian myoblastosis virus reverse transcriptase (Boehringer, Mannheim, Germany) for 1 h at 37°C. Then PCR amplification was carried out with EGFR forward primer 5'-AGCCATGCCCGCATTAGCTC-3' and EGFR reverse primer 5'-AAAGGAATGCAACTTCCCAA-3' and with the GAPDH constitutive primer forward 5'-CTGCTT CACCACCTTCTTGA-3' and GAPDH constitutive primer Reverse: 5'-ATCACTGCCACCCAGAAGACT-3' in a

GenAmp PCR System 9600 (Perkin Elmer, Foster City, CA, USA) for 35 cycles. Cycle program was 30 s at 94°C, 1 min at 54°C and 1 min at 72°C. PCR product amplification was run in 2% agarose electrophoresis gel. Semi quantitative densitometry gel banding analysis was processed in Scion software 4.032 (National Institute of Healthy) using the gel plot2 macro. Briefly, the optical density of the bands was determined and the relative amounts of mRNA for EGFR were calculated as EGFR/GAPDH ratios.

Immunohistochemical Technique

Slides were seeded with 2×10^4 cells per well and treated with glutamate accordingly described above. After treatment cells were fixed with cold acetone and dried at room temperature. Immunohistochemical procedure was carried on accordingly to manufactures instructions (Vectastain ABC System; Dako, Vector, CA, USA). Briefly, endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide-methanol solution. Thereafter, slides were incubated for 20 min in protein block serum-free (Dako, Carpinteria, CA, USA). The EGFR primary antibodies (Dako, Carpinteria, CA, USA) and Akt (R&D Systems, Minneapolis, MN, USA) were applied, and the slides incubated for 30 min at 37°C and overnight at 4°C in a humidity chamber. Subsequently, slides were incubated with biotinylated secondary antibody (Vector, CA, USA) for 30 min. After incubation with VECTASTAIN® ABC Reagent for 30 min, peroxidase activity was developed with DAB Substrate–Chromogen System (Merck, WS, NJ, USA) identifying bound antibody. After a final wash in distilled water, the slides were lightly counterstained with hematoxylin, dehydrated in graded alcohol, cleared with xylene, and mounted with xylene-based permanent mounting medium.

For all specimens, control slides were processed identically and at the same time, except that primary antibody was not applied. Therefore, all differences between the experimental tissue and the control tissue are ultimately due to DAB identification of the relevant protein.

Digital Image Capture

Images from three fields were captured from each section at × 400 magnification through a microscope-mounted digital camera (Sony Corp, Tokyo, Japan) built on a Leica/CME microscopic (Leica, Wetzlar, Germany). The images were saved TIFF format and transferred onto an image analysis computer workstation for further analysis.

Immunohistochemistry Analysis

The immunohistochemistry analyses were realized by direct visualization. Briefly, score were carried on accordingly to Schmidt et al. [36]. The score were made for both extent (percentage of positive tumor cells: 0%, score=0; <5%, score=1; 5–20%, score=2; 21–50%, score=3; 51–75%, score=4, >75%, score 5) and intensity (absent, score=0; weak, score=1; moderate, score=2; strong, score=3). Both scores were multiplied to give a composite score (0–15) for each tumor cell culture.

Statistical Analysis

Data presented are mean ± SD of at least three experiments performed in triplicate. One-way ANOVA followed by Dunnett's test, when appropriate, were used to indicate statistical significance. The differences were considered significant when $p<0.05$ or $p<0.001$ and was indicated in the graphics.

Results

Effect of Glutamate on the U-87MG Cell Proliferation

To examine how glutamate influences GBM growth, we treated the U-87MG cell line during 48 h with 5 to 200 mM of glutamate and then the number of viable cells was assessed by trypan blue dye exclusion test. A substantial increase in cell number (approximately 50%) was found in the medium containing 5 mM glutamate. Also, 2 mM glutamate induced increase in U-87MG cell number (Data not shown). Conversely, 25 mM and higher doses of glutamate (50, 75, 100, 150 and 200 mM) caused significant decrease on cell viability ($p<0.01$) (Fig. 1). The increase in cell viability observed with 2 or 5 mM of glutamate was not reproduced in normal serum concentration (FCS 10%) (Data not shown).

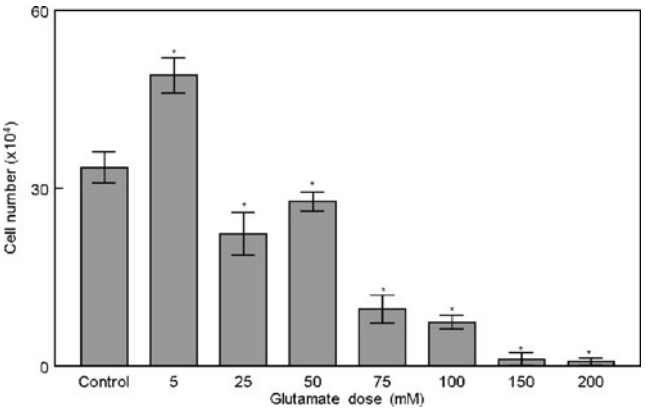

Fig. 1 Effect of glutamate on U-87MG human GBM cell proliferation. Number of non-treated (control) or glutamate treated (5, 25, 50, 75, 100, 150 or 200 mM) cells were assessed by cell count after 48 h using trypan blue exclusion. Data was plotted as the mean ± SD of three different experiments. *Significantly different from control ($p<0.01$)

The further addition of DNQX (500 μM), an antagonist of AMPA receptors, markedly inhibited the effect of glutamate on U-87MG cell proliferation. Actually, we observed a decrease of 67% in cell viability when the cells were treated with glutamate and DNQX (Fig. 2). In the presence of DNQX alone the cell viability decreased approximately 95%, suggesting the involvement of AMPA receptors on the U-87MG cell growth. These results indicate that glutamate regulated cell proliferation by activating AMPA receptors.

Effect of Glutamate Treatment on the EGFR- phospho-Akt Signaling

In order to investigate if glutamate treatment mediates the increase in cell proliferation by EGFR signaling, we decided to verify EGFR content after glutamate treatment. Thus, the U-87MG cell line was treated with 5 mM glutamate alone, 5 mM glutamate plus 500 μM DNQX, or 500 μM DNQX alone for 48 h, and afterwards the EGFR was evaluated by immunohistochemistry. The immunohistochemistry analysis revealed a significant increase in the content of EGFR with 5 mM of glutamate. Indeed, the glutamate treatment induced an increase of 1.4 times in EGFR on the U-87MG cell line. In the presence of DNQX, EGFR content decreased to control level (Figs. 3 and 4).

Since glutamate increased the EGFR contents in U-87MG cell line, we investigated if this effect was mediated by induction of EGFR expression. Forty-eight hours after 5 mM glutamate treatment we performed semi-quantitative RT-PCR experiments. When U-87MG cells were treated with glutamate it was observed a significant increase in the EGFR mRNA expression ($p<0.05$). Accordingly, glutamate treatment has increased 2 times the expression of EGFR mRNA (Fig. 5).

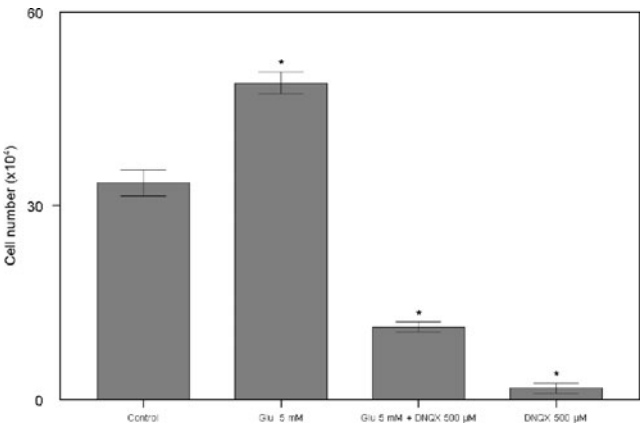

Fig. 2 Effect of glutamate and DNQX on U-87MG human GBM cell proliferation. Number of non-treated (control), glutamate (5 mM), glutamate (5 mM) plus DNQX (500 μM); and DNQX (500 μM) treated cells were assessed by cell count after 48 h using trypan blue exclusion. Data was plotted as the mean ± SD of three different experiments. *Significantly different from control ($p<0.01$)

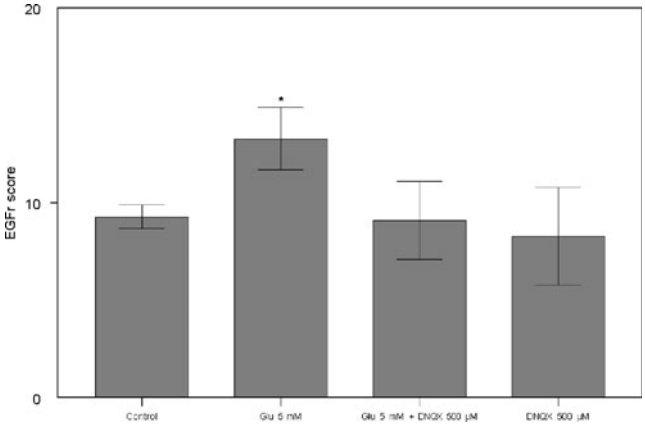

Fig. 3 Effect of glutamate and DNQX on EGFR content in U-87MG human GBM cell line. Immunostaining score was calculated for non-treated (control), glutamate (5 mM), glutamate (5 mM) plus DNQX (500 μM); and DNQX (500 μM) treated cells. Data was plotted as the mean ± SD of three different experiments. *Significantly different from control ($p<0.05$)

We next examined the effect of glutamate on the EGFR signaling using the anti-EGFR gefitinib. The further addition of gefitinib (50 μM) inhibited the effect of glutamate on U87-MG cell proliferation, restoring the control levels. In the presence of gefitinib alone the cell viability decreased approximately 48% (Fig. 6). These results suggest that glutamate regulated cell proliferation by activating EGFR.

Given that, our results suggest that EGFR is involved as a downstream effector for glutamate signaling mediated by AMPA receptors in GBM cells and, PI3K/Akt is considered one of the main intracellular pathways involved in EGFR activation [7, 37], the AKT functions could trigger glutamate signaling. To test this hypothesis, we examined whether phospho-Akt contents can be affected by glutamate acute treatment. Immunohistochemistry analysis revealed an increase about 1.8 times after 48 h of glutamate treatment in phospho-Akt content on U-87MG cells. While, in the presence of DNQX, phospho-Akt contents were restored to control levels (Figs. 7 and 8). Moreover, the addition of the PI3K inhibitor—wortmannin (5 μM), which suppress the phosphorylation of Akt [31], significantly reduced the number of viable cells ($p<0.01$). In the presence of glutamate the further addition of wortmannin restored cell proliferation to control levels (Fig. 9). Together, our data suggest that glutamate signaling mediated by AMPA receptor induces U-87MG human GBM cell line proliferation via EGFR-phospho-Akt pathway. In addition, we performed similar experiments on a primary GBM cell culture and in the U-138MG cell line. These data demonstrated, in a clear way, that, despite differences in

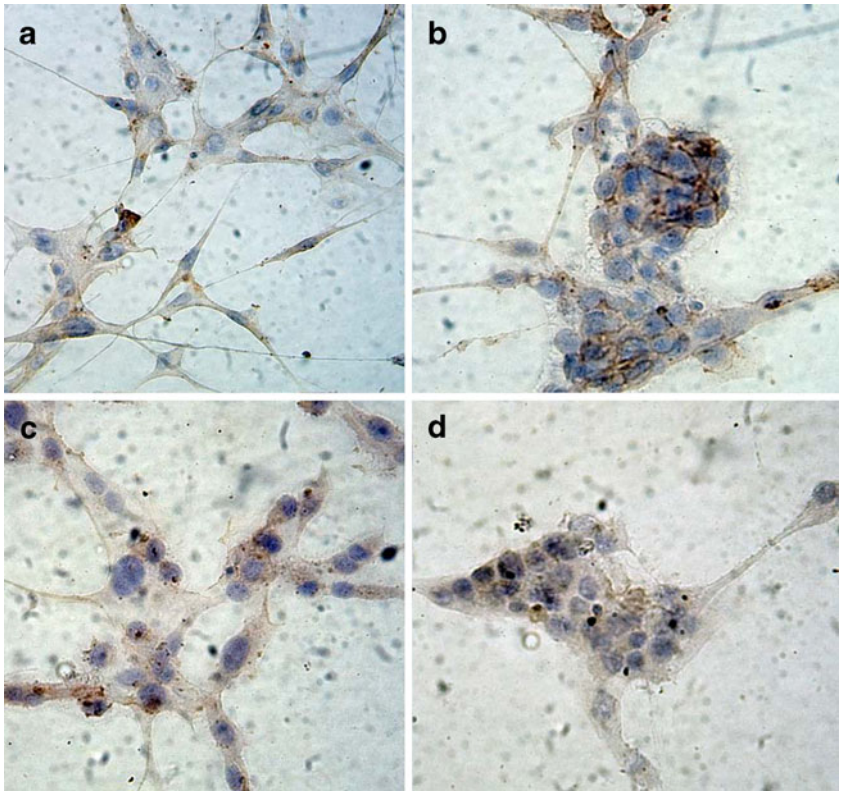

Fig. 4 Representative photomicrographs of the EGFR content evaluated by immunohistochemistry in U-87MG human GBM cell line. (A) Non-treated, (B) glutamate (5 mM), (C) glutamate (5 mM) plus DNQX (500 μM); (D) and DNQX (500 μM) treated cells. EGFR antibody clone H11, recognizes wild-type EGFR and the deleted mutant form (EGFRvIII). Original magnification x400

base-line values of the assessed determinants, the response to stimulation with glutamate, and to the modulating effects of DNQX, Gefitinib or wortmannin was indeed essentially similar (data not shown).

Discussion

The poor prognosis of GBM patient's is commonly related to the highly proliferative and invasive behavioral of the GBM tumor cells through the healthy nervous tissue [5]. Noteworthy, a number of reports associate glutamate and its receptors to GBM survival and invasion [25, 26, 28–30]. Accordingly, as GBM tumors are resistant to glutamate citotoxicity, increased extracellular glutamate levels could support tumor growth by peri-tumoral neuronal cell damage [29]. In accordance to our previous report [38] and studies from others [23, 29, 39], U-87MG GBM cell line presented high resistance to glutamate citotoxicity (IC_{50} of 56 mM).

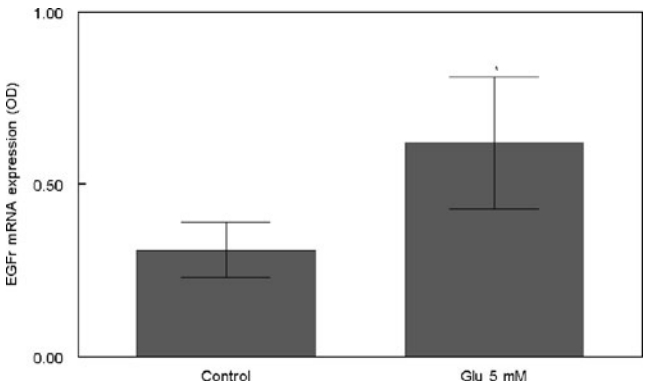

Fig. 5 Effect of glutamate on EGFR mRNA expression in U-87MG human GBM cell line. EGFR mRNA expression was calculated using Scion software by determining the optical density of EGFR corrected by GAPDH expression. Results were expressed as OD (optical density). Data was plotted as the mean ± SD of three different experiments. *Significantly different from control ($p<0.05$)

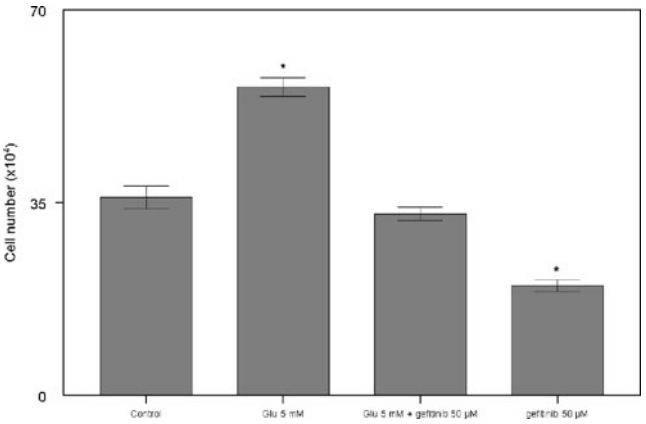

Fig. 6 Effect of glutamate and Gefitinib on U-87MG human GBM cell proliferation. Number of non-treated (control), glutamate (5 mM), glutamate (5 mM) plus Gefitinib (50 μM); and Gefitinib (50 μM) treated cells were assessed by cell count after 48 h using trypan blue exclusion. Data was plotted as the mean ± SD of three different experiments. *Significantly different from control ($p<0.01$)

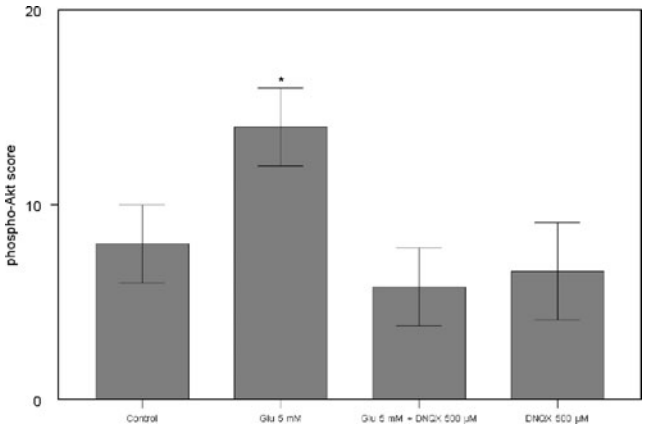

Fig. 7 Effect of glutamate and DNQX on phospho-Akt content in U-87MG human GBM cell line. Immunostaining score was calculated for non-treated (control), glutamate (5 mM), glutamate (5 mM) plus DNQX (500 μM); and DNQX (500 μM) treated cells. Data was plotted as the mean ± SD of three different experiments. *Significantly different from control ($p<0.05$)

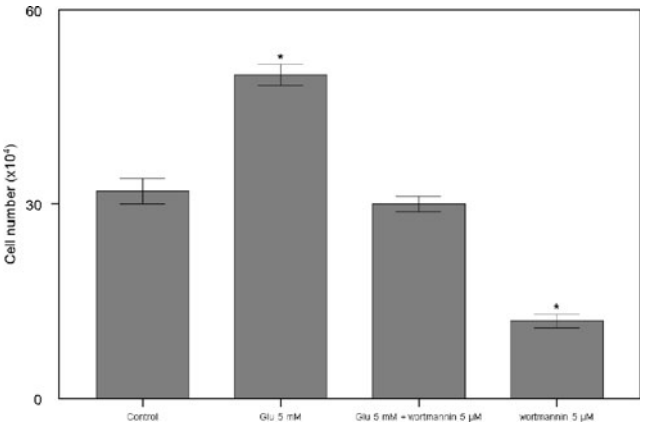

Fig. 9 Effect of glutamate and wortmannin (5 μM) on U-87MG human GBM cell proliferation. Number of non-treated (control), glutamate (5 mM), glutamate (5 mM) plus wortmannin (5 μM); and wortmannin (5 μM) treated cells were assessed by cell count after 48 h using trypan blue exclusion. Data was plotted as the mean ± SD of three different experiments. *Significantly different from control ($p<0.01$)

In fact, the relative resistance to glutamate toxicity observed on the GBM studied cell line was at least 45 times higher than the glutamate toxicity identified by others on non-tumoral glial cells [40, 41]. Our study demonstrates that treatment with non-toxic levels of glutamate leads to an increase in U-87MG cell proliferation and on the EGFR content. Although the mechanism by which glutamate promotes an increase on the EGFR content was not demonstrated in the present study, a recently report showed that GBM tumor cells could also release microvesicles containing among others EGFR-mRNA [42]. Additionally, others revealed a close functional relationship between glutamate receptors and *erbB* signaling [32, 43]. In glia cultured cells, the co-activation of AMPA and mGlu5

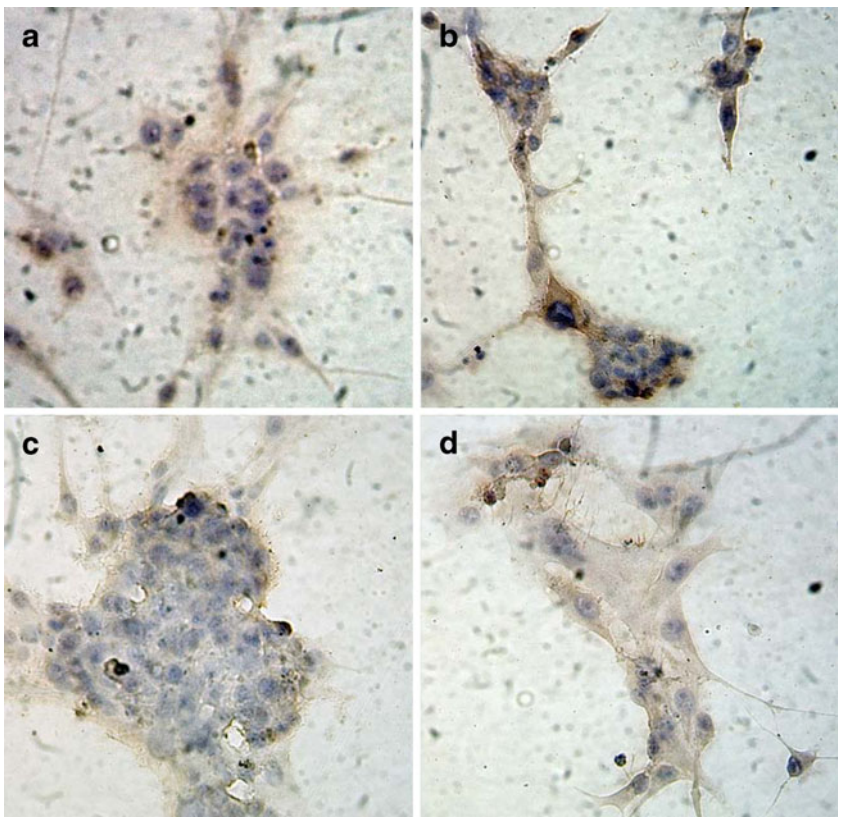

Fig. 8 Representative photomicrographs of the phospho-Akt content evaluated by immunohistochemistry in U-87MG human GBM cell line. (A) Non-treated, (B) glutamate (5 mM), (C) glutamate (5 mM) plus DNQX (500 μM); and (D) DNQX (500 μM) treated cells. Original magnification x400

glutamate receptors lead to expression of the erbB1 receptors, which include, among others, EGFR [32]. When these cell types were exposed to glutamate it was observed an increase in the expression and release of neurotrophins [44]. While, in glioma cells, the pharmacological blockade of mGlu2/3 receptors reduced cell proliferation and MAPK activation either in cell culture or in glioma implanted under the skin or inside the brain of recipient mice [26, 28].

Our findings suggest that glutamate could affect GBM signaling by activating EGFR signaling. In U87-MG cell line, glutamate treatment induced a significant increase in the contents of EGFR protein and mRNA, while the addition of DNQX restored EGFR to control levels. Besides, the further addition of the anti-EGFR-gefitinib inhibited the effect of glutamate on the U87-MG cell proliferation. EGFR plays a pivotal role in GBMs behavioral, signaling a variety of cellular responses, including: proliferation, survival, motility, invasion, differentiation, angiogenesis and cellular repair [7, 45–47]. Then, one could expect that glutamate mediated increase on EGFR levels could reflect an augment in growth rate on the U-87MG cell line studied.

Taking into account the effect of glutamate on GBM signaling, previous reports had showed that the glutamatergic system exerts a variety of effects on GBM cell behavioral. GBM cells expressing Ca^{+2} permeable AMPA receptors exposed to glutamate or AMPA agonists have enhanced motility and sustained survival [25, 31]. A study conducted by Lyons et al. showed that GBM cell lines, including U-87MG cells, can release glutamate at approximately 100–300 nmol/mg protein over a 4 h time period [30]. On the other hand, treatment of GBM cells with antagonists of metabotropic-G-coupled or NMDA receptors showed anti-proliferative effects either in vitro or in vivo [24, 26, 28]. Also, using in vitro or in vivo models the treatment with inhibitors of glutamate release decreased GBM growth [48, 49]. Thus, drugs that protect neurons against excitotoxic death (e.g. N-methyl-D-aspartate receptor antagonists) or inhibit the glutamate/cystine membrane exchanger like sulfasalazine (i.e. main system accounting for the release of glutamate) can be consider potential candidates for the treatment of malignant gliomas.

Growth factors acting through receptors, and tyrosine kinases and their signaling pathways have been the focus of therapeutic research [50]. Pathway inhibitors, including kinase inhibitors and monoclonal antibodies, have been developed [51]. Primary GBM commonly overexpress EGFR and its ligand-independent mutant EGFRvIII [14, 52]. Platelet-derived growth factors receptors (PDGFRs) are frequently overexpressed and involved in the same pathway as EGFR and EGFRvIII. Signalling for vascular endothelial growth factors and their receptors are involved in the angiogenesis of GBM and also involves the same pathway as EGFR, EGFRvIII and PDGFR [53, 54]. These growth factors finally activate the molecule AKT. A constitutively active mutant variant of EGFR, EGFRvIII, preferentially activates PI3K-Akt signaling. The tumor suppressor gene PTEN also increased AKT activity. Loss of PTEN function markedly diminished the responsiveness of EGFR inhibitors. Thus, coexpression of EGFRvIII and PTEN is associated with clinical responsiveness to EGFR inhibitors [14, 52, 55]. In the present study, the glutamate treatment that induced U-87MG proliferation promote an increase in phospho-Akt content. While, the addition of wortmannin, a specific PI3K inhibitor, suppressed the glutamate effect on the U-87MG proliferation. Thus, we can suggest that glutamate induced cell growth via Akt signaling pathway. These findings are in accordance to reports that revealed a direct relationship between EGFR content and the activation of PI3K/Akt in GBM cells [19, 37, 47, 56]. Phosphorylated Akt elicit a downstream cascade together with the Ras-MAPK pathways that ultimately activate the transcription of several promoting factors [18, 57, 58]. Moreover, Ishiuchi et al. demonstrated that Ca^{+2} signaling mediated by AMPA receptor regulates the growth and motility of GBM cells via activation of Akt [31]. Thus, activation of Akt has been shown to be essentially to GBM transformation [31, 59–61], survival, growth and motility [31, 62].

Although our results point to a relationship between glutamate and EGFR signaling, the relevance of these findings in glioma behavioral was not demonstrated in the present study. The cell culture model used can, at least in part, explain the reason for this lack of information. Since, it is well understood that monolayer model does not address the influence of environmental physiology on glutamate signaling. Given that, other culture models systems, such as multicellular tumor spheroids, which more closely resemble the tridimensional physiology of in vivo solid tumors, should be evaluated [63, 64]. Speculating a drawn for an in vivo scenario, we should consider that glutamate is released by damaged neuronal cells or glioma cells in exchange to cystine uptake [21, 23, 29, 65]. For these reason, the levels of glutamate in vivo may be continuously high in extracellular fluids [66]. Therefore, we can suggest that this high glutamate concentration can enhance sustained EGFR signaling through glutamate receptors. Finally, the sustained EGFR signaling might contribute to the glioma malignant progression, and also to the resistant phenotype against chemotherapy and radiotherapy.

In summary, the results of the current study demonstrated that EGFR signaling is involved in the response of U-87MG human GBM cell line to glutamate. Therefore, we can suggest that the activation of the glutamate- AMPA receptor- EGFR pathway may contribute to the highly proliferative and invasive behavioral of the GBM tumor

cells through the healthy nervous tissue. Although the findings of this study cannot provide a mechanistic explanation for these phenomena, we suggest that the glutamate signaling should be further investigated as a potential novel pathway to control glioma tumor progression.

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