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ORIGINAL ARTICLE

High LC3/Beclin Expression Correlates with Poor Survival in Glioma: a Definitive Role for Autophagy as Evidenced by In Vitro Autophagic Flux

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Abstract Recent studies suggest the role of autophagy, an evolutionarily conserved catabolic process, in determining the response of gliomas to treatment either positively or negatively. The study attempts to characterize autophagy in low and high-grade glioma by investigating the autophagic flux and clinical significance of autophagy proteins (LC3 and beclin 1) in a group of glioma patients. We evaluated the expression of autophagic markers in resected specimens of low-grade glioma (LGG) and high-grade glioma (HGG) tissues, by immunohistochemistry and Western blotting. Our results show that expression of autophagy proteins were more prominent in HGG than in LGG. Increased level of autophagic proteins in HGG can be due to an increased rate of autophagy or can be because of blockage in the final degradation step of autophagy (defective autophagy). To distinguish these possibilities, the autophagic flux assay which helps to determine the rate of degradation/synthesis of autophagic proteins (LC3-II and p62) over a period of time by blocking the final degradation step of autophagy using bafilomycin A1 was used . The assessment of autophagic flux in ex vivo culture of primary glioma cells revealed for the first time increased turnover of autophagy in high grade compared to low grade-glioma. Though autophagic markers were reduced in LGG, functionally autophagy was non defective in both grades of glioma. We then investigated whether autophagy in gliomas is regulated by nutrient sensing pathways including mTOR and promote cell survival by providing an alternate energy source in response to metabolic stress. The results depicted that the role of autophagy during stress varies with tissue and has a negative correlation with mTOR substrate phosphorylation. We also evaluated the expression of LC3 and beclin 1 with progression free survival (PFS) using Kaplan-Meier survival analysis and have found that patients with low LC3/beclin 1 expression had better PFS than those with high expression of LC3/beclin 1 in their tumors. Together, we provide evidence that autophagy is non-defective in glioma and also show that high LC3/beclin 1 expression correlates with poor PFS in both LGG and HGG.

Keywords Glioma · Autophagy · LC3 · Autophagic flux · p62 · Beclin 1

Highlights
- Increased expression of LC3/Beclin 1 was more prominent in high grade glioma as evidenced by immunohistochemistry and Western Blot.
- For the first time, autophagic flux assay done in in vitro cultured primary glioma cells revealed autophagy was non-defective in both low and high-grade gliomas.
- Patients with high expression of LC3/beclin 1 had worse PFS than patients with low expression of LC3/beclin 1 in their tumors.

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Abbreviations

LGG	low grade glioma
HGG	high grade glioma
PFS	progression free survival

Introduction

Gliomas, the tumor that affects the glial cells of brain, can be classified as low-grade (LGG) and high-grade gliomas (HGG)

depending upon the extent of aggressiveness exhibited in terms of its growth [1, 2]. Despite the multimodal standard therapies including surgery followed by radiotherapy and chemotherapy, overall survival rate of patients with HGG remains dismal [3]. These tumors are apoptosis refractory and have evolved multiple mechanisms for their survival [4–6]. Autophagy is one such biological process that helps cells to survive in various stress conditions. Recently, the net influence of autophagy, a macro process that is dialectical in nature in terms of its regulatory role in cell death, in glioma treatment is amply being discussed in literature and are still highly perplexing [7–11].

Autophagy is a highly regulated intracellular catabolic system that delivers selective cytoplasmic components into lysosomes for digestion after packing them in double membrane vesicles called autophagosomes [12]. It helps in cellular homeostasis by digesting worn out/aged organelles and toxic protein aggregates from the cytosol. However, an abnormally higher 'basal' level of autophagy occurring in cancer cells is often considered as a pro-survival process. Enhanced levels of autophagy also help the cancer cells to meet the energy demand during its phase of growth [13]. There are signals operating in cancer cells that could gauge the nutrient status and regulate autophagy accordingly [14–16]. At the centre of such coordinating act is mTOR, a serine threonine kinase activated during nutrient abundance that could suppress the level of autophagy [17, 18]. On the contrary, starved condition triggers a rapid inhibition of mTOR to relieve its inhibitory function on autophagy [19].

HGG, like any other form of aggressive tumor, is likely to face the challenges of nutrient stress [20, 21]. However, the extent to which glioma cells depends on autophagy to tide over such stress is currently unknown. Recently, studies have found that chemoradiotherapy could induce autophagy and contributes to treatment resistance in glioma [22, 23]. There are reports demonstrating the role of induced autophagy in delaying the cell death induced by radiation and in development of resistance against chemotherapeutic drug (temozolomide, TMZ) [7, 8]. Though autophagy is generally described as a process meant for the survival of tumor cells, it cannot be considered as a quintessential cell survival mechanism. Exceeding the threshold level, autophagy could hyper digest cellular components and act as a programmed cell death mechanism [21]. Drugs capable of inducing autophagy are used as a strategy to induce cell death in glioma cells [5, 24]. Anyhow, as a cell death or as a survival mechanism the importance of autophagy in modulating the outcome of glioma therapy cannot be overlooked. In this study we characterized autophagy by assessing its markers in different grades of human glioma, performing ex vivo culture of primary glioma cells for assessing autophagic flux and correlating LC3/beclin 1 expression with progression free survival (PFS).

Experimental Procedures

Materials

Antibodies against LC3-II, β-actin and all the routine chemicals used for making buffers or used in the experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Expose mouse and rabbit specific HRP/DAB detection IHC kit, antibodies to beclin 1 and p62 were procured from Abcam (Cambridge, MA, USA). Cell lysis buffer for protein extraction, antibodies to phospho-p70S6kinase, phospho–4E-BP1, and anti-rabbit and mouse IgG HRP linked secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Chemiluminiscent reagents used for Western blots and nitrocellulose membrane were purchased from Thermo Scientific (Rockford, IL, USA). DMEM/F-12 and FBS were obtained from Invitrogen (Carlsbad, CA, USA).

Methods

Patient Samples and Clinical Information

Tumor samples from different grades of glioma were collected from patients undergoing surgical tumor resection at the Department of Neurosurgery, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. The study was approved by the Institutional Ethics Committee (IEC). Glioma specimens were histopathologically classified according to the World Health Organization (WHO 2007) grading by a neuropathologist. Biopsy samples were primarily processed for immunohistochemistry, Western blotting and primary culture only, and it was not a practice to perform mutation analysis/other molecular alterations when this study (2009–13) was conducted. Fresh biopsy sample was used for the isolation and culture of primary glioma cells (LGG: $n = 5$ and HGG: $n = 5$), a portion of the sample was stored at −80 °C for protein extraction from tissues (LGG: $n = 30$ and HGG: $n = 25$) and another portion of tissue was fixed with buffered formaldehyde for immunohistochemistry (LGG: $n = 37$ and HGG: $n = 26$). LGG included grade I and grade II whereas HGG consisted of grade III as well as grade IV tumors. Follow up data were available for 51 of the total 63 patients and was obtained from medical records. The patients whose clinical outcome data was unavailable were excluded from survival analysis. PFS is defined as the time from randomisation to tumor progression or death [25]. Table 1 lists the patient characteristics.

Isolation and Culture of Primary Glioma Cells

Tumor tissues obtained after surgical resection were washed three times in HBSS, minced to small fragments

Table 1 Summary of patient characteristics

Histology criteria	WHO grade	Number of cases
Low grade glioma		37
Pilocytic astrocytoma	I	5
Astrocytoma II	II	12
Oligoastrocytoma II	II	11
Oligodendroglioma II	II	9
High grade glioma		26
Astrocytoma III	III	4
Oligoastrocytoma III	III	3
Oligodendroglioma III	III	7
GBM	IV	13

(0.5 mm) and subjected to enzymatic digestion with 0.2% papain for 15 min at 37 °C. The dissociated tumor cells were washed, subsequently resuspended and cultured in DMEM/F-12 medium with 10% fetal bovine serum (FBS). Cultures were maintained at 37 °C containing 5% CO_2 and 95% air until they reached ~80% confluence and these cells were used for further studies.

Immunohistochemical Staining of Glioma Tissue Sections Using Autophagic Markers

Biopsy tissues were fixed in 10% buffered formalin, embedded in paraffin, cut into 5 μm sections and mounted on poly-L-Lysine coated microscope slides. Later, paraffin sections of tumors were then deparaffinised in xylene, hydrated through graded alcohols, treated with heat induced epitope retrieval technique using citrate buffer (pH 6.0) at 95 °C for 5 min and allowed to gradually cool in the buffer. After that slides were immersed in 3% H_2O_2 for 10 min to block intrinsic peroxidase activity. The sections were then immunohistochemically stained with the following primary antibodies against: LC3 (rabbit polyclonal IgG; 1:250), p62 (rabbit polyclonal IgG; 1:100) and beclin 1 (rabbit polyclonal IgG; 1:50) for overnight at 4 °C. After washing in TBS containing 0.5% Tween 20 (TBST) for 15 min, the Expose mouse and rabbit specific HRP/DAB detection IHC kit (Abcam, Cambridge, MA, USA) were used as the detection system and reaction was visualised by using 3, 3′ diaminobenzidine tetrahydrochloride (DAB) as chromogen. The nuclei were counterstained with Mayer's hematoxylin and the slides were mounted using DPX as mounting medium. The immunoreactivity was scored on the basis of staining intensity and was expressed as percentage positive staining per area using ImageJ. The median values of the score were used as cut-off points to classify tumors as exhibiting low and high protein expression [26–28].

Western Blot Analysis

Protein was isolated from glioma tissues and cultured cells. For extraction of protein from tissue, frozen brain slices were powdered in liquid nitrogen and lysed in RIPA lysis buffer supplemented with protease inhibitor cocktail and phosphatase (Sigma-Aldrich, St. Louis, MO, USA; for phospho protein). Protein extraction from cultured glioma cells was done using cell lysis buffer for protein extraction (CST, Danvers, MA, USA) supplemented with protease inhibitor cocktail. The protein concentration was determined using Bradford assay with bovine serum albumin used as a standard. For Western blotting, 100 μg lysates were electrophoretically fractionated by 10% or 15% sodium dodecyl sulfate polyacrylamide gel (SDS PAGE) and transferred to a nitrocellulose membrane by a semidry blotting technique. The membranes were probed with primary antibody against the target protein followed by HRP-conjugated secondary antibody and the bands were visualized by using West Femto Chemiluminescence Detection Kit (Thermo Scientific, Rockford, IL, USA) as per the instructions of manufacturer. The bands obtained were exposed to X-ray films and documented using a documentation system (Bio-Rad Laboratories, Hercules, CA, USA). We evaluated the expression of proteins as an optical densitometry (OD) ratio by determining its densitometry relative to the densitometry of beta actin [29].

RNA Interference

mTOR siRNA was purchased from Ambion life technologies (Austin, TX, USA). Primary glioma cells were seeded onto 12 well plate at a density of 80,000 cells per well in DMEM/F12 supplemented with 10% FBS and antibiotics. Following day, the cells were treated with transfection reagent (Lipofectamine; Invitrogen, Carlsbad, CA, USA) with or without mTOR siRNA according to the manufacture's protocol. The transfection reagent alone was employed as a control. After 19 h of incubation, the cells were enriched with 10% FBS containing DMEM/F12 for another 24 h. Afterwards, the cells were lysed and immunoblotted with anti-mTOR and anti-LC3 antibodies.

Cell Staining with Acridine Orange

Autophagy is a process characterized by development of acidic vesicular organelles (AVOs), which can be detected in primary cultured glioma cells using the lysosomo-tropic agent acridine orange. Upon acridine orange staining, the cytoplasm and nucleolus fluoresce bright green and dim red respectievely, whereas acidic compartments such as AVOs fluoresce bright red. The increase in red fluorescence is proportional to the increase in the amount of acidic compartments [30, 31]. For the assay, bafilomycin A1 (autophagosome-lysosome fusion inhibitor) dissolved in DMSO was added at a 10 nM

concentration to primary cultured glioma cells for a period of 4 h. After treatment, cells were stained with acridine orange at a final concentration of 1 μg/ml for 15 min. Cells without bafilomycin A1 treatment was taken as control. Cells were then washed with PBS to remove excess stain and observed under fluorescent microscope. Quantification of acridine orange intensity was performed using ImageJ software [32].

Autophagic Flux Analysis

Autophagic flux assay was done to analyze the turnover of autophagy proteins in glioma cells [33–35]. Primary cultured glioma cells were treated with bafilomycin A1 (10 nM) for a period of 4 h and 8 h. Cells cultured in 10% DMEM/F-12 alone was taken as the control. After treatment with bafilomycin A1, Western blotting for autophagic markers such as LC3-II and p62 was performed on primary cultured glioma cells, thenceforth assessing the turnover of autophagic proteins.

Glioma Cell Culture and Amino Acid Starvation Treatment

To analyze whether autophagy can be induced in glioma cells, starvation treatment was done as reported earlier [36]. The cells plated in DMEM/F-12 with 10% FBS were allowed to grow till 75–80% confluence, after which the cells were washed twice with Hanks balanced salt solution (HBSS) and incubated in amino acid deprived HBSS, a starvation media and autophagy inducer for 4 h. The cells were lysed and Western blotting for autophagic marker LC3 II was performed.

Cell Viability Assay Using MTT

For the study, primary cultured glioma cells were seeded in a 96-well microtitre plate (10,000 cells/well) and cultured in DMEM/F-12 medium with 10% FBS (10% DMEM/F-12) at 37 °C in an incubator containing 5% CO_2 to attain 80% confluence. When the cells reached 80% confluence, they were randomly divided into experiment group and control group. Cells were treated with i) 10% DMEM/F-12 along with bafilomycin A1 (10 nM), ii) 10% DMEM/F-12 with 3-methyladenine (3-MA, 10 mM), iii) HBSS, iv) HBSS with bafilomycin A1 (10 nM) and v) HBSS with 3-MA (10 mM). After incubating the cells for 12 h at 37 °C, cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyltetrazoliumbromide (MTT) assay. During combinational treatment, 10 mM 3-MA or 10 nM bafilomycin A1 were pre-treated for 1 h followed by treatment for 12 h. Following treatment, MTT solution dissolved in the culture media at a final concentration of 1 mg/ml was added to each well and incubated till formazan crystals were formed (2.5 h). Acidified isopropanol was added to dissolve the formazan crystals and the optical density (OD) of solubilised formazan crystals was measured at 570 nm (with 630 nm as reference wavelength) using a microtitre plate ELISA reader (BioTek Instruments, Winooski, VT, USA).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5. ANOVA with Bonferroni post-hoc tests and two tailed student's t-test were used for comparisons of autophagy analysis results between low and high-grade glioma. Data were expressed as mean ± standard error of the mean (SEM). The differences in LC3-II and beclin 1 expression in low and high-grade glioma were assessed using Mann Whitney test. The Fisher's exact was used to evaluate the association of clinicopathological variables. The survival analysis was evaluated by the Kaplan-Meier method. The level for statistical significance was set at $p < 0.05$.

Results

Increased Expression of Autophagic Markers Such as LC3 and Beclin 1 in HGG Compared with LGG

The markers characteristic of autophagy (LC3-II and beclin 1) were detected in the cytoplasm of immunohistochemically stained tissue sections from both LGG (n = 37) and HGG (n = 26) (Fig. 1a–d). Both LC3-II and beclin 1 exhibited cytoplasmic staining in varying proportions in different grades of glioma. The study also evaluated the relationship between autophagic protein expression and clinicopathological variables including grade, age and sex in glioma. The data is summarised in Table 2. The high expression of autophagic proteins were more prominent in HGG than in LGG (Table 2). In the majority of LGG, autophagic markers were either not detectable or expressed a weak staining (LC3-II: 81% and beclin: 73%); higher percentages of expression were found only in a small fraction of tumors (LC3-II: 19% and beclin: 27%). Generally, the HGG exhibited higher expression of autophagic proteins (LC3-II: 65% and beclin: 62%) and only a limited number of specimens expressed weak/negative staining (LC3-II: 35% and beclin: 38%). The differences in the expression of autophagic proteins between the LGG and HGG were statistically significant (LC3-II; $p = 0.001$ and beclin 1; $p < 0.0001$) (Table 2). The age and sex was not significant with regard to expression of autophagic proteins.

Western blot analysis further showed that the expression levels of LC3-II and beclin 1 proteins were significantly higher in high grade (n = 25) than in low grade-gliomas (n = 30). The differences in expression of proteins between the low and high-grade glioma were also found to be statistically significant (LC3-II, p value = 0.0163; Beclin, p value = 0.0207). The representative results were presented in Fig. 1e–h.

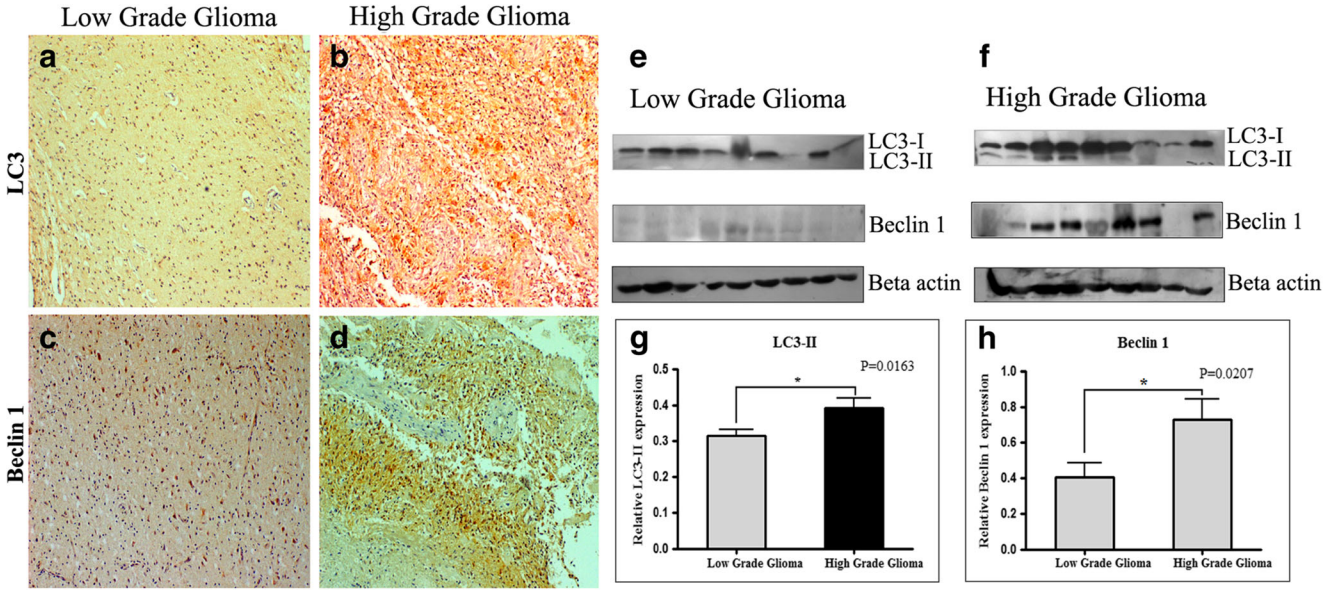

Fig. 1 Evaluation of markers of autophagic process in low and high grade glioma. **a**–**e** Representative immunohistochemistry pictures showing the expression of LC3-II and Beclin 1 (10×) in low and high-grade gliomas. Human glioma tissue sections were fixed with buffered formalin and expression of autophagic markers was examined using antibodies against LC3-II (**a** Oligodendroglioma II and **b** GBM; grade IV) and Beclin 1 (**c** Oligoastrocytoma II and **d** GBM). It was detected by HRP conjugated secondary antibody and DAB as chromogen. The sections were counterstained by hematoxylin (LGG: n = 37 and HGG: n = 26 patients). **e**–**h** Detection of autophagic proteins in glioma tissue by Western blotting. Representative blots of autophagic proteins in low and high grade gliomas (**e**) Astrocytoma II (**f**) GBM. 100 μg of protein was used for Western blotting assay with autophagic markers. β-actin antibody was used as loading control. **g**, **h** Relative expression of autophagy proteins (LC3-II and Beclin 1) was compared between the grades (LGG: n = 30 and HGG: n = 25 patients). Data represented as mean ± SEM

Autophagic Activity Is Higher in HGG Compared to LGG

In our experiments with glioma tissue, LC3-II level was significantly low or even absent in LGG, whereas the LC3-I level of LGG was more or less similar to that of HGG. Decrease in the level of LC3-II/autophagosomes can be due to blockage in the synthesis of autophagosomes (defective autophagy) or else it can be because of slow synthesis of new autophagosomes, thus the level of autophagosomes in cytosol at a given instant of time is too low to be detected by analysing the expression of endogenous autophagy proteins through Western blotting/IHC [37]. To distinguish these possibilities, the autophagic flux assay, an analysis, which helps to gauge the synthesis of new autophagosomes over a period of time by preventing autophagy at a late stage by inhibiting fusion betweeen autophagosomes and lysosomes thereby blocking its degradation using bafilomycin A1 was employed. Cells with defect in autophagosome production should not accumulate autophagosomes in the presence of bafilomycin A1, whereas those cells that synthesise autophagosomes induce autophagosome accumulation in the presence of bafilomycin A1, albeit at a slow rate. An increase in autophagosome can be demonstrated by showing an enhancement in acidic vesicular organelles (AVO) in the cytosol.

Enhanced staining of HGG over LGG by acridine orange, a stain that could fluoresce in AVO supports our observation that the level of autophagosome in LGG is too low to be detected by analysing the endogenous protein in tissue (Fig. 2a). During bafilomycin A1 treatment, autophagosomes/LC3-II and p62, a protein that clears through autophagy by degrading in autophagolysosomes, were accumulated in both low and high-grade glioma cells irrespective of its grade (Fig. 2a–d). The rate of accumulation of LC3-II and p62 proteins was low in LGG than in HGG (Fig. 2e, f), suggesting that the absence or reduced expression of autophagomes/LC3-II in LGG tissues is not due to the defect or absence of autophagosome formation but because of its reduced synthesis rate.

Table 2 Relationship between autophagy protein expression and grade of glioma

	No: of cases	LC3-II expression		p value	Beclin 1 expression		p value
		Low, % (n)	High, % (n)		Low, % (n)	High, % (n)	
LGG	37	81% (30)	19% (7)	0.0010*	73% (27)	27 (10)	<0.0001*
HGG	26	35% (9)	65% (17)		38% (10)	62% (16)	

LGG, low grade glioma; *HGG*, high grade glioma

* $p < 0.05$

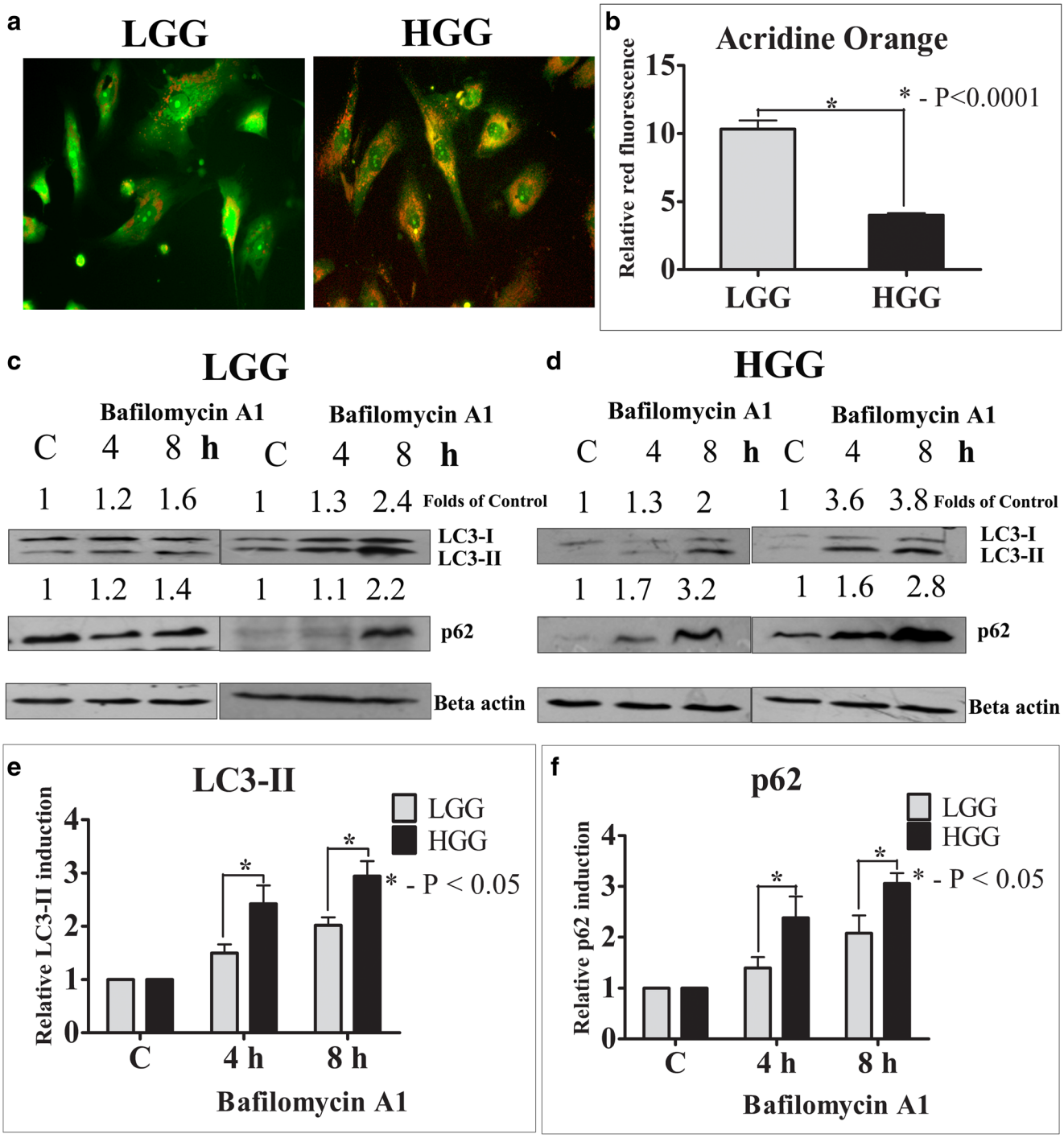

Fig. 2 Determination of autophagic flux in primary cultured glioma cells. **a**, **b** Detection of autophagy in low and high-grade glioma cell cultures before and after bafilomycin A1 treatment by acridine orange staining. **a** Glioma cells from LGG and HGG were stained with acridine orange, the bright red fluorescent spots indicating increased autophagosomes was observed under fluorescence microscope and photographed. **b** Degree of red acridine orange staining was quantified in low and high-grade glioma derived cells using ImageJ software (**c**–**f**) Autophagic flux analysis in low and high grades of glioma derived cells after bafilomycin A1 treatment. Primary cultured glioma cells treated with bafilomycin A1 were analyzed for the accumulation of autophagy markers LC3-II and p62 in LGG (**c**) and HGG (**d**). The protein levels were detected by Western blotting at 4 and 8 h following bafilomycin A1 treatment. It was normalised to β-actin. Quantification plot of autophagic induction in low (**e**) and high (**f**) grade gliomas. Bafilomycin A1 treatment increases the accumulation of LC3-II and p62 in both low and high grades of glioma cells. Values represent mean ± SEM; $n = 5$ each for LGG and HGG

Amino Acid Deprivation Induces Autophagy and its Role during Stress Varies with Tissue

It is generally assumed that autophagy is essential to tide over the nutrient stress especially that of amino acid deprivation, as one of the basal functions of autophagy is to recycle amino acids from macromolecules for cell survival when nutrients are scarce [38, 39]. In order to look at the different prospects of autophagy or to define its characteristics in glioma we have done a study with amino acid deprivation. As expected, the cells isolated from glioma tissues upon starvation induced autophagy irrespective of its grade, which was evident from the induction of LC3-II during HBSS treatment (amino acid deprived media) in all cells isolated from LGG and HGG (Fig. 3a–c). Moreover, the cells isolated from tissues of both LGG and HGG were found exhibiting 30–50% decrease in viability upon 12 h starvation (Fig. 3d, e). However, among the tissues studied (LGG: $n = 5$ and HGG: $n = 5$), the cells isolated from two different samples each from HGG and LGG, did not further alter its viability (Fig. 3d) upon autophagic inhibition during starved condition (HBSS vs HBSS + Bafilomycin A1/3-MA), whereas the glioma cells isolated from the remaining tissues further exhibited decreased viability upon autophagic blockage during starvation condition (Fig. 3e). These results suggest that the extent at which the glioma cells depends on autophagy for overcoming the starvation condition may vary among the tissues.

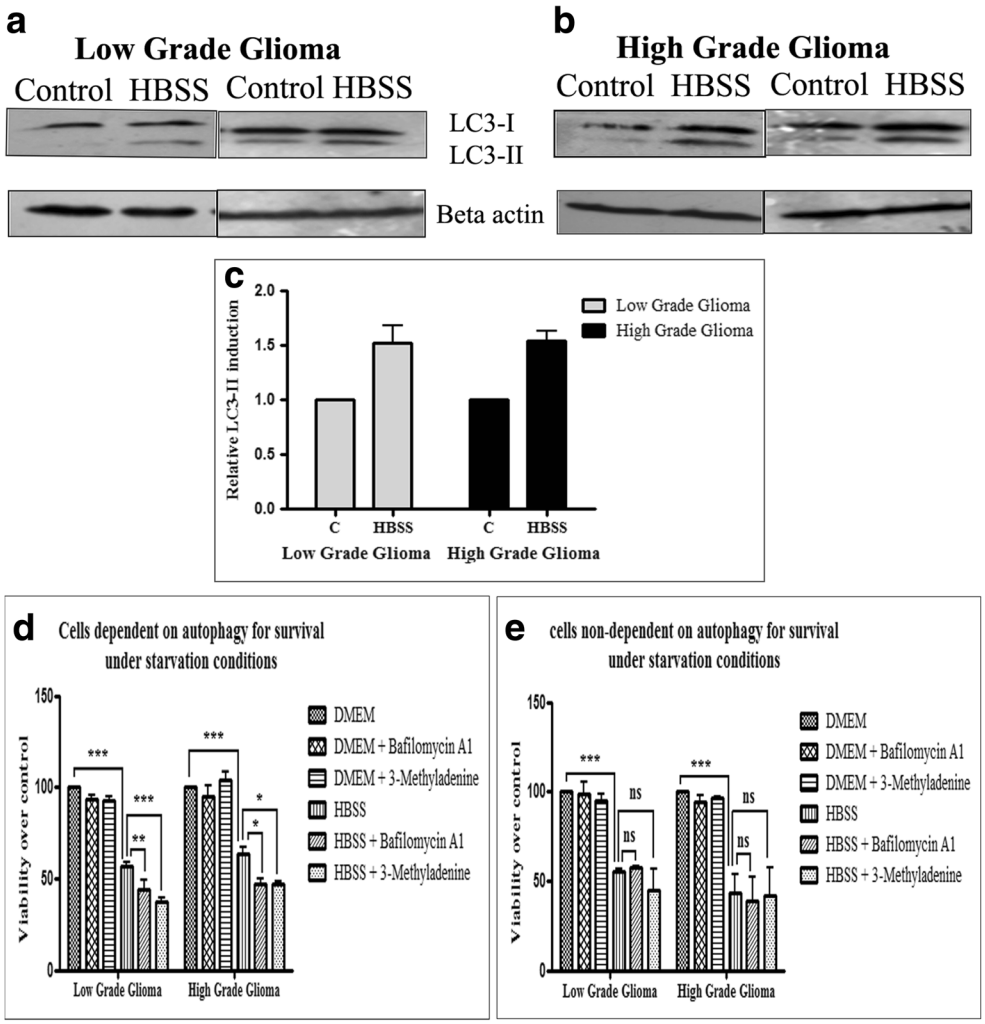

Fig. 3 Autophagy is induced under starvation conditions. **a**–**c** Starvation induces autophagy in both low and high-grades of glioma. **a** LGG **b** HGG. In both low and high-grades of glioma, LC3-II expression was more intense in cells grown in starvation condition (HBSS) when compared to control cells grown in 10% DMEM/F12 for 4 h, which was normalised to β-actin. **c** Quantification plot of LC3-II protein levels in control and HBSS treated cells in low and high-grade gliomas. **d**, **e** The viability of primary cultured glioma cells in starvation medium and upon inhibition of autophagy. **d** Cells dependent on autophagy for survival and **e** Cells non-dependent on autophagy for survival under starvation conditions. Cell viability was determined by MTT assay after a period of 12 h starvation in HBSS and also co-treatment with autophagy inhibitors, bafilomycin A1 and 3-MA. Error bars are SEM. ns means not significant, ***, **, * means significant ($p < 0.05$) in comparison of viability in cells cultured in 10% FBS containing DMEM/F12 (alone) with that in cells treated using HBSS alone or on combination of bafilomycin A1 and 3-MA ($n = 5$ each for LGG and HGG)

mTOR Shows Inverse Correlation and is a Prominent Factor in Regulating Autophagy

mTOR is a survival signaling that once activated could curb autopahgy, but it is less clear whether under normal growth conditions basal autophagy in glioma is dependent on mTOR. The mTOR activity can be detected by analyzing the phosphorylation status of its substrates such as p70S6kinase and 4E–BP1 which are sensitive to autophagy induction. Phosphorylated p70S6kinase and 4E–BP1 were detected in LGG, whereas it was undetected in all except one HGG analyzed (Fig. 4a–d). These results suggest that difference in mTOR activity is the reason for dissimilar autophagic status in HGG and LGG. To further confirm this, the expression of mTOR proteins were silenced in cells isolated from LGG using siRNA. Results showed that the mTOR inhibited cells induced LC3-II expression (Fig. 4e–f), that verify the role of mTOR activity on autopahgic rate in glioma tissues.

High LC3-II/Beclin 1 Expression Affects Progression Free Survival in Glioma

We then analyzed whether there is a relation between autophagic protein (LC3-II and beclin 1) expression and survival in low and high-grade glioma. The Kaplan-Meier survival analysis using log rank tests indicated that patients having high LC3-II and beclin 1 expression was associated with shorter progression free survival in LGG (LC3: 19%; $p = 0.0397$, Fig. 5a and beclin 1: 27%; $p = 0.0351$, Fig. 5c) and HGG (65%; $p = 0.0399$, Fig. 5b and beclin 1: 62%; $p = 0.0268$, Fig. 5d). This suggests that high LC3-II and beclin 1 expression is associated with increased malignancy in glioma.

Discussion

Recent years have seen the realization that autophagy can have an impact on the prognosis of glioma either positively [9, 40] or negatively [7, 8, 10, 41], at the same time defective autophagy has also been found to be associated with cancer. While the prognostic role of autophagy proteins in glioma following its endogenous tissue expression pattern have been well studied [42, 43], they may often not give complete information, as there may be aspects of a defective/blocked autophagy that are misinterpreted as absence/presence of autophagy, because most, if not all studies were done on immunohistochemical analysis of autophagy related proteins. Thus to be able to accurately characterize autophagy, the in situ assays should be complemented and confirmed with "autophagic rate assay" that distinguishes normal basal autophagy from blocked/defective autophagy. These aspects prompted us to investigate the prognostic role of

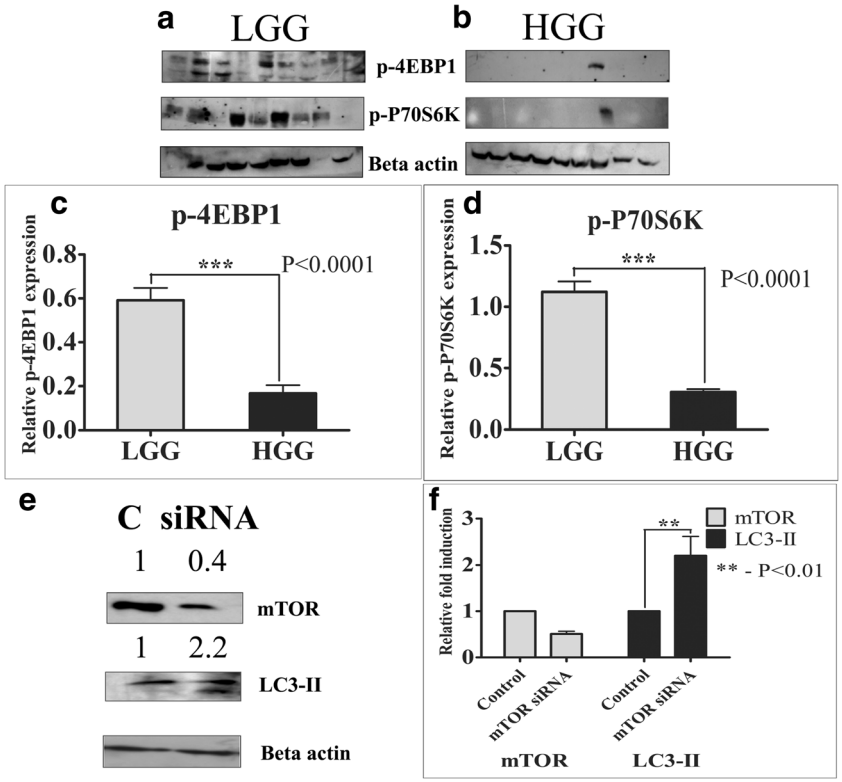

Fig. 4 mTOR expression in relation to autophagy in gliomas. **a**–**d** Western blotting of mTOR substrates p-4EBP1 and p-P70S6kinase expression. Representative blots of phosphorylation status of mTOR substrates in low and high grade gliomas **a** Astrocytoma II **b** GBM. **c**, **d** Quantitative analysis of the p-4EBP1 and p-P70S6kinase levels compared between the grades. **e**, **f** Glioma cells were treated without (control) or with siRNA targeting mTOR and analyzed by immunoblotting using anti mTOR and anti LC3-II antibodies in LGG (**e**). Anti beta actin antibody was used for confirmation of equivalence in amount of loaded proteins. **f** Quantification plot of mTOR and LC3-II protein levels in cells treated with siRNA. Data represented as mean ± SEM (*p* value - 0.0086)

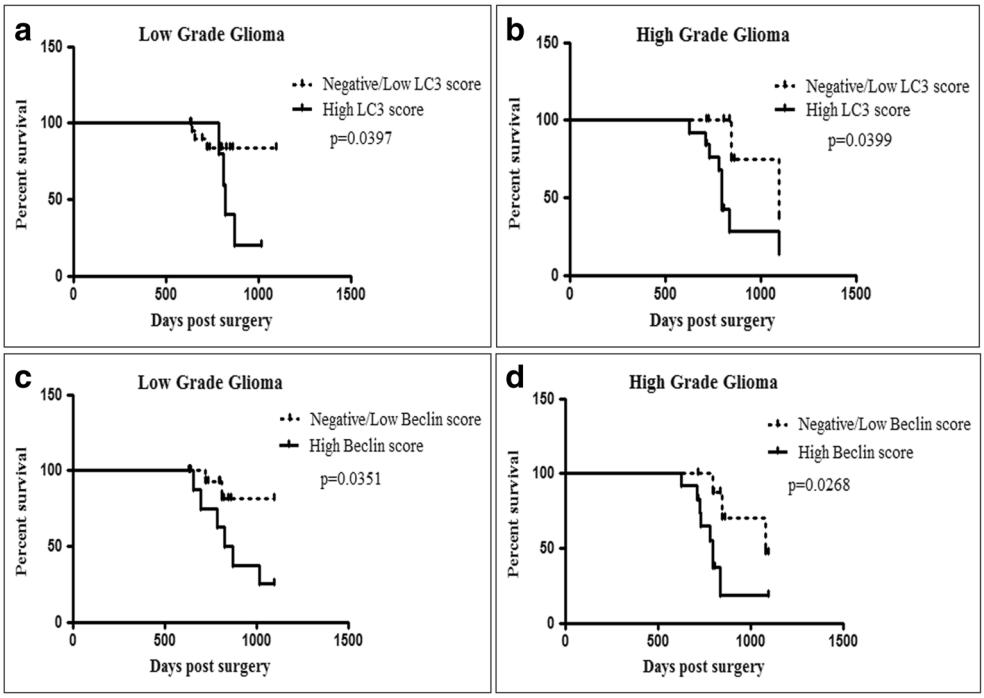

Fig. 5 Evaluation of the relationship between LC3-II and beclin 1 protein expression and progression free survival of low and high grade glioma patients using Kaplan-Meier method. **a**, **b** Plot comparing the high and negative/low LC3 expression with survival. **a** LGG **b** HGG. **c**, **d** Survival curve comparing the high and negative/low beclin 1 expression. **c** LGG **d** HGG. High LC3-II and beclin 1 expression was related to shorter progression free survival in low and high-grade glioma

autophagic proteins (LC3 and beclin 1) in low and high-grade glioma patients along with a flux assay to determine if autophagy is defective or not.

Given the dynamic nature of autophagy that involves the degradation of autophagic cargo along with the proteins involved in its machinery such as LC3-II and p62, determination of endogenous protein expression pattern may not provide useful information [33, 34]. In addition, immunohistochemical staining of LC3-II to analyze autophagy in tissues has a limitation of missing the expression of other LC3 isoforms [28]. It is because of the aforementioned controversial but important role, autophagy could be more accurately represented by assaying the rate of degradation of these proteins rather than analyzing its presence at a given instant of time [37, 44]. However such rate gauging assays are not practically possible in tissues but can be done with relative ease under in vitro conditions by assaying LC3-II and p62 accumulation in cells after blocking final autophagosomal degradation using bafilomycin A1 for a given period of time [34, 35, 45, 46]. However, this work to the best of our knowledge is the first attempt which verifies that the absence of LC3-II in LGG is due to its low autophagic flux rather than an autophagic defect by following the degradation dynamics of an autophagosome membrane marker (LC3-II) and an autophagic substrate (p62) in cells isolated from glioma tissues. Since the autophagic rate of primary glioma cells reflects the autophagic status in tissues from which it was isolated, the autophagic rate assay helps to accurately interpret the expression pattern of autophagic markers observed in glioma tissues.

In order to look at the different prospects of autophagy or to define its characteristic in glioma, we then investigated whether autophagy in gliomas is regulated by nutrient sensing pathways including mTOR and promote cell survival by providing an alternate energy source in response to metabolic stress. Recent findings show that malignant gliomas have been found to induce autophagy in response to metabolic stress that inactivates other nutrient sensing pathways including mTOR and promote cell survival by providing alternative energy sources [19]. On the premises of such previous reports and on the observation regarding induction of autophagy in response to a starvation condition in both HGG and LGG, it is tempting to assume that the autophagy induced in starvation condition is a response helping the glioma cells for its survival. However, our finding that the cells isolated from a group of tissues induce autophagy under conditions of starvation but the induced autophagy does not provide a survival advantage, suggests that at least in a fraction of glioma patients autophagy have a role other than survival. Nevertheless, this result warrants more in depth and detailed studies, which are beyond the scope of the present study.

mTOR pathway by sensing the nutrient status of the cell is thought to control autophagy through regulating the phosphorylation of ULK1, a kinase known to initiate autophagosome development [18, 47, 48]. Even though autophagy regulation through mTOR is the most understood pathway, there are recent reports that show, certain small molecules can induce autophagy in glioma cells through mTOR independent pathway [49]. We used different approaches including analysis of the phosphorylation state of mTOR substrate proteins and analysis of

LC3-II induction after silencing mTOR in glioma to show autophagy in glioma is mTOR dependent. Given the slow growth rate of LGG comparing to HGG, it is safe to assume that the nutrient stress is low in LGG comparing to that of HGG. Hence, it is not quite unexpected that the mTOR activity is found higher and autophagic rate lower in LGG comparing to HGG. These results are in line with the previous studies, which demonstrate an increased activation of mTOR in 60% of LGG [50–52]. However, there are reports demonstrating enhanced activity of mTOR in HGG, which are in contrast with our findings. Smaller sample sizes and demographic differences are the probable reason for such disparities in the observations.

The presence of non-defective basal autophagy in low and high-grade glioma has been demonstrated in our study, yet the prognostic relevance of autophagic protein expression is still a matter of debate. Several recent studies reported that, autophagy induction as well as high LC3 and beclin 1 expression are associated with poor prognosis and treatment outcome in various tumors [26, 53–56]. High LC3 expression has been associated with metastasis and vasculogenic memory in melanoma [57]. In addition, increased LC3-II and p62 expression were associated with aggressive clinicopathologic features and poor prognosis in oral squamous cell carcinoma [28]. By contrast, there are also studies that show autophagy suppresses tumor progression and contributes to good prognosis in lung tumors and human hepatocellular carcinomas [27, 58]. Decreased expression or allelic loss of beclin 1 correlates with poor clinical results in ovarian, hepatocellular and breast cancers [59]. Although there are evidences implicating the prognostic function of autophagy marker expression in HGG and GBM [40, 42, 60], the role of autophagy in glioma has not been extensively studied in both low and high grades. Even in gliomas, there were contrasting reports about autophagy as a pro and anti-survival mechanism [42, 61, 62]. Elevated cytoplasmic expression of beclin 1 (BPCE) score was associated with good prognosis in HGG [42]. Reduced expression of autophagic proteins (LC3-II and beclin 1) has been shown to be correlated with the progression of astrocytic tumors [40]. On the contrary, in a study, high LC3-II staining was associated with poor prognosis and resistance to radio-and chemotherapy in gliomas [10, 63]. Several recent studies report that, autophagy induction has been associated with tumor cell survival and adaptation to nutrient stress in GBM as well as radio resistance of glioma stem cells (GSCs) [8, 60]. Another study has shown that for GBM patients with normal karnofsky performance score (KPS); high LC3B expression was associated with poor survival and can be used for prognostic purposes. Several studies have now shown that inhibition of autophagy sensitises tumor cells to chemoradiotherapies [64–66]. Similarly, studies also revealed that inhibition of autophagy sensitized glioma cells to treatment [7, 41]. Recent studies in glioma have shown that autophagy induced in response to radio and chemotherapy (TMZ) can contribute to therapy resistance [22, 23]. The pro and anti-survival effects of autophagy is still under debate, particularly when data from clinical trials support the cytoprotective effects of an autophagy inhibitor, chloroquine, while it is still unknown whether the other inhibitors of autophagy would have the same effect. These observations were derived from studies involving TMZ treatment [23]. Our findings in this study looked at the basal autophagic status (LC3/beclin 1) at the time of diagnosis and did not evaluate the expression of these proteins after treatment. High LC3/beclin 1 expression and poor prognosis in gliomas in our study was similar to findings from various recent studies [10, 63]. It is generally accepted that starvation induced autophagy is a critical nutritional response intended to replenish cellular amino acid supplies, while basal autophagy is thought to be responsible for constitutive turnover of certain proteins or for clearance of damaged proteins or organelles [67]. Our observation of a high basal autophagy along with a non-defective autophagy in glioma, might promote tumor progression or even treatment resistance, culminating in shorter PFS in HGG, but owing to small sample size additional studies need to be performed to strengthen the relationship.

In summary, our data demonstrated an increased level of autophagic activity in HGG compared to LGG. The high expression of autophagic proteins, LC3/beclin 1 were closely correlated with a poor outcome in terms of shorter progression free survival in both low and high-grade glioma. The assessment of autophagic flux in ex vivo culture of primary glioma cells revealed autophagy was non defective in both grades of glioma. For the better understanding of the regulation of autophagy and related pathways in glioma, further study needs to be done on a large series of patients. This may provide further insights into the relevance of autophagy in glioma, leading to new therapeutic possibilities in patients.

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Compliance with Ethical Standards

Conflict of Interest All authors (PCJ, EHV, VV, GMR, SN, SG) declare no conflict of interest.

Ethical Approval All procedures performed in the above study were in accordance with the ethical standards of the Institutional Human Ethical Committee and with the 1964 Helsinki declaration and its later amendments or comparable standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

References

1. Schwartzbaum JA, Fisher JL, Aldape KD, Wrensch M (2006) Epidemiology and molecular pathology of glioma. Nat Clin Pract Neurol 2(9):494–503 quiz 1 p following 16
2. Maher EA, Furnari FB, Bachoo RM, Rowitch DH, Louis DN, Cavenee WK et al (2001) Malignant glioma: genetics and biology of a grave matter. Genes Dev 15(11):1311–1333
3. Louis DN, Holland EC, Cairncross JG (2001) Glioma classification: a molecular reappraisal. Am J Pathol 159(3):779–786
4. Lefranc F, Kiss R (2006) Autophagy, the Trojan horse to combat glioblastomas. Neurosurg Focus 20(4):E7
5. Lefranc F, Brotchi J, Kiss R (2005) Possible future issues in the treatment of glioblastomas: special emphasis on cell migration and the resistance of migrating glioblastoma cells to apoptosis. J Clin Oncol Off J Am Soc Clin Oncol 23(10):2411–2422
6. Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S (2004) Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. Cell Death Differ 11(4):448–457
7. Ito H, Daido S, Kanzawa T, Kondo S, Kondo Y (2005) Radiation-induced autophagy is associated with LC3 and its inhibition sensitizes malignant glioma cells. Int J Oncol 26(5):1401–1410
8. Lomonaco SL, Finniss S, Xiang C, Decarvalho A, Umansky F, Kalkanis SN et al (2009) The induction of autophagy by gamma-radiation contributes to the radioresistance of glioma stem cells. Int J Cancer 125(3):717–722
9. Jo GH, Bogler O, Chwae YJ, Yoo H, Lee SH, Park JB, et al (2014) Radiation-induced autophagy contributes to cell death and induces apoptosis partly in malignant glioma cells. Cancer Res Treat 47(2): 221-241
10. Ge P, Luo Y, Fu S, Ji X, Ling F (2009) Autophagy: a strategy for malignant gliomas' resistance to therapy. Med Hypotheses 73(1):45–47
11. Zhuang W, Qin Z, Liang Z (2009) The role of autophagy in sensitizing malignant glioma cells to radiation therapy. Acta Biochim Biophys Sin 41(5):341–351
12. Cuervo AM (2004) Autophagy: in sickness and in health. Trends Cell Biol 14(2):70–77
13. Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R et al (2006) Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature 441(7095):885–889
14. Chen N, Debnath J (2010) Autophagy and tumorigenesis. FEBS Lett 584(7):1427–1435
15. Hippert MM, O'Toole PS, Thorburn A (2006) Autophagy in cancer: good, bad, or both? Cancer Res 66(19):9349–9351
16. He C, Klionsky DJ (2009) Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet 43:67–93
17. Annovazzi L, Mellai M, Caldera V, Valente G, Tessitore L, Schiffer D (2009) mTOR, S6 and AKT expression in relation to proliferation and apoptosis/autophagy in glioma. Anticancer Res 29(8):3087–3094
18. Fan QW, Knight ZA, Goldenberg DD, Yu W, Mostov KE, Stokoe D et al (2006) A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma. Cancer Cell 9(5):341–349
19. Chang YY, Juhasz G, Goraksha-Hicks P, Arsham AM, Mallin DR, Muller LK et al (2009) Nutrient-dependent regulation of autophagy through the target of rapamycin pathway. Biochem Soc Trans 37(Pt 1):232–236
20. Kondo Y, Kanzawa T, Sawaya R, Kondo S (2005) The role of autophagy in cancer development and response to therapy. Nat Rev Cancer 5(9):726–734
21. Eskelinen EL (2005) Doctor Jekyll and Mister Hyde: autophagy can promote both cell survival and cell death. Cell Death Differ 12(Suppl 2):1468–1472
22. Koukourakis MI, Mitrakas AG, Giatromanolaki A (2016) Therapeutic interactions of autophagy with radiation and temozolomide in glioblastoma: evidence and issues to resolve. Br J Cancer 114(5):485–496
23. Yan Y, Xu Z, Dai S, Qian L, Sun L, Gong Z (2016) Targeting autophagy to sensitive glioma to temozolomide treatment. J Exp Clin Cancer Res 35:23
24. Lefranc F, Facchini V, Kiss R (2007) Proautophagic drugs: a novel means to combat apoptosis-resistant cancers, with a special emphasis on glioblastomas. Oncologist 12(12):1395–1403
25. Ballman KV, Buckner JC, Brown PD, Giannini C, Flynn PJ, LaPlant BR et al (2007) The relationship between six-month progression-free survival and 12-month overall survival end points for phase II trials in patients with glioblastoma multiforme. Neuro-Oncology 9(1):29–38
26. Tang JY, Hsi E, Huang YC, Hsu NC, Chu PY, Chai CY (2013) High LC3 expression correlates with poor survival in patients with oral squamous cell carcinoma. Hum Pathol 44(11):2558–2562
27. Lee YJ, Hah YJ, Kang YN, Kang KJ, Hwang JS, Chung WJ et al (2013) The autophagy-related marker LC3 can predict prognosis in human hepatocellular carcinoma. PLoS One 8(11):e81540
28. Liu JL, Chen FF, Lung J, Lo CH, Lee FH, Lu YC et al (2014) Prognostic significance of p62/SQSTM1 subcellular localization and LC3B in oral squamous cell carcinoma. Br J Cancer 111(5):944–954
29. Wang XJ, Zhou SL, Fu XD, Zhang YY, Liang B, Shou JX et al (2015) Clinical and prognostic significance of high-mobility group box-1 in human gliomas. Exp Ther Med 9(2):513–518
30. Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS et al (2008) Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy 4(2):151–175
31. Kanzawa T, Kondo Y, Ito H, Kondo S, Germano I (2003) Induction of autophagic cell death in malignant glioma cells by arsenic trioxide. Cancer Res 63(9):2103–2108
32. Carew JS, Espitia CM, Esquivel JA 2nd, Mahalingam D, Kelly KR, Reddy G et al (2011) Lucanthone is a novel inhibitor of autophagy that induces cathepsin D-mediated apoptosis. J Biol Chem 286(8):6602–6613
33. Klionsky DJ (2005) The correct way to monitor autophagy in higher eukaryotes. Autophagy 1(2):65
34. Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K et al (2012) Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 8(4):445–544
35. Rubinsztein DC, Cuervo AM, Ravikumar B, Sarkar S, Korolchuk V, Kaushik S et al (2009) In search of an "autophagomometer". Autophagy 5(5):585–589
36. Sadasivan S, Waghray A, Larner SF, Dunn WA Jr, Hayes RL, Wang KK (2006) Amino acid starvation induced autophagic cell death in PC-12 cells: evidence for activation of caspase-3 but not calpain-1. Apoptosis: Int J Programmed Cell Death 11(9):1573–1582
37. Ni HM, Bockus A, Wozniak AL, Jones K, Weinman S, Yin XM et al (2011) Dissecting the dynamic turnover of GFP-LC3 in the autolysosome. Autophagy 7(2):188–204

38. Kathryn J. Huber-Keener and Jin-Ming Yang (2011). Impact of Metabolic and Therapeutic Stresses on Glioma Progression and Therapy In: Prof. Clark Chen (Ed.), Advances in the Biology, Imaging and Therapies for Glioblastoma, InTech, Croatia
39. Kang C, Avery L (2008) To be or not to be, the level of autophagy is the question: dual roles of autophagy in the survival response to starvation. Autophagy 4(1):82–84
40. Huang X, Bai HM, Chen L, Li B, Lu YC (2010) Reduced expression of LC3B-II and Beclin 1 in glioblastoma multiforme indicates a down-regulated autophagic capacity that relates to the progression of astrocytic tumors. J Clin Neurosci: Off J Neurosurg Soc Australasia 17(12):1515–1519
41. Ge PF, Zhang JZ, Wang XF, Meng FK, Li WC, Luan YX et al (2009) Inhibition of autophagy induced by proteasome inhibition increases cell death in human SHG-44 glioma cells. Acta Pharmacol Sin 30(7):1046–1052
42. Pirtoli L, Cevenini G, Tini P, Vannini M, Oliveri G, Marsili S et al (2009) The prognostic role of Beclin 1 protein expression in high-grade gliomas. Autophagy 5(7):930–936
43. Giatromanolaki A, Sivridis E, Mitrakas A, Kalamida D, Zois CE, Haider S et al (2014) Autophagy and lysosomal related protein expression patterns in human glioblastoma. Cancer Biol Ther 15(11):1468–1478
44. Klionsky DJ, Elazar Z, Seglen PO, Rubinsztein DC (2008) Does bafilomycin A1 block the fusion of autophagosomes with lysosomes? Autophagy 4(7):849–850
45. Asanuma K, Tanida I, Shirato I, Ueno T, Takahara H, Nishitani T et al (2003) MAP-LC3, a promising autophagosomal marker, is processed during the differentiation and recovery of podocytes from PAN nephrosis. FASEB J: Off Publ Fed Am Soc Exp Biol 17(9):1165–1167
46. Tanida I, Minematsu-Ikeguchi N, Ueno T, Kominami E (2005) Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. Autophagy 1(2):84–91
47. Hosokawa N, Hara T, Kaizuka T, Kishi C, Takamura A, Miura Y et al (2009) Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. Mol Biol Cell 20(7):1981–1991
48. Kamada Y, Yoshino K, Kondo C, Kawamata T, Oshiro N, Yonezawa K et al (2010) Tor directly controls the Atg1 kinase complex to regulate autophagy. Mol Cell Biol 30(4):1049–1058
49. Hau AM, Greenwood JA, Lohr CV, Serrill JD, Proteau PJ, Ganley IG et al (2013) Coibamide a induces mTOR-independent autophagy and cell death in human glioblastoma cells. PLoS One 8(6):e65250
50. Rodriguez FJ, Raabe EH (2014) mTOR: a new therapeutic target for pediatric low-grade glioma? CNS Oncol 3(2):89–91
51. Franz DN, Leonard J, Tudor C, Chuck G, Care M, Sethuraman G et al (2006) Rapamycin causes regression of astrocytomas in tuberous sclerosis complex. Ann Neurol 59(3):490–498
52. McBride SM, Perez DA, Polley MY, Vandenberg SR, Smith JS, Zheng S et al (2010) Activation of PI3K/mTOR pathway occurs in most adult low-grade gliomas and predicts patient survival. J Neuro-Oncol 97(1):33–40
53. Hao CL, Li Y, Yang HX, Luo RZ, Zhang Y, Zhang MF et al (2014) High level of microtubule-associated protein light chain 3 predicts poor prognosis in resectable esophageal squamous cell carcinoma. Int J Clin Exp Pathol 7(7):4213–4221
54. Karpathiou G, Sivridis E, Koukourakis MI, Mikroulis D, Bouros D, Froudarakis ME et al (2011) Light-chain 3A autophagic activity and prognostic significance in non-small cell lung carcinomas. Chest 140(1):127–134
55. Wan XB, Fan XJ, Chen MY, Xiang J, Huang PY, Guo L et al (2010) Elevated Beclin 1 expression is correlated with HIF-1alpha in predicting poor prognosis of nasopharyngeal carcinoma. Autophagy 6(3):395–404
56. Fujii S, Mitsunaga S, Yamazaki M, Hasebe T, Ishii G, Kojima M et al (2008) Autophagy is activated in pancreatic cancer cells and correlates with poor patient outcome. Cancer Sci 99(9):1813–1819
57. Han C, Sun B, Wang W, Cai W, Lou D, Sun Y et al (2011) Overexpression of microtubule-associated protein-1 light chain 3 is associated with melanoma metastasis and vasculogenic mimicry. Tohoku J Exp Med 223(4):243–251
58. Guo JY, Karsli-Uzunbas G, Mathew R, Aisner SC, Kamphorst JJ, Strohecker AM et al (2013) Autophagy suppresses progression of K-ras-induced lung tumors to oncocytomas and maintains lipid homeostasis. Genes Dev 27(13):1447–1461
59. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H et al (1999) Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 402(6762):672–676
60. Hu YL, DeLay M, Jahangiri A, Molinaro AM, Rose SD, Carbonell WS et al (2012) Hypoxia-induced autophagy promotes tumor cell survival and adaptation to antiangiogenic treatment in glioblastoma. Cancer Res 72(7):1773–1783
61. Kimmelman AC (2011) The dynamic nature of autophagy in cancer. Genes Dev 25(19):1999–2010
62. Knizhnik AV, Roos WP, Nikolova T, Quiros S, Tomaszowski KH, Christmann M et al (2013) Survival and death strategies in glioma cells: autophagy, senescence and apoptosis triggered by a single type of temozolomide-induced DNA damage. PLoS One 8(1):e55665
63. Winardi D, Tsai HP, Chai CY, Chung CL, Loh JK, Chen YH et al (2014) Correlation of altered expression of the autophagy marker LC3B with poor prognosis in astrocytoma. Biomed Res Int 2014:723176
64. Karagounis IV, Kalamida D, Mitrakas A, Pouliliou S, Liousia MV, Giatromanolaki A et al (2016) Repression of the autophagic response sensitises lung cancer cells to radiation and chemotherapy. Br J Cancer 115(3):312–321
65. Koukourakis MI, Kalamida D, Mitrakas A, Pouliliou S, Kalamida S, Sivridis E et al (2015) Intensified autophagy compromises the efficacy of radiotherapy against prostate cancer. Biochem Biophys Res Commun 461(2):268–274
66. Sui X, Chen R, Wang Z, Huang Z, Kong N, Zhang M et al (2013) Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. Cell Death Dis 4:e838
67. Fimia GM, Kroemer G, Piacentini M (2013) Molecular mechanisms of selective autophagy. Cell Death Differ 20(1):1–2