ANALYSIS OF NEUROSPHERE FORMATION AND AUTOPHAGIC STATUS IN DIFFERENT GRADES OF GLIOMA

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

SYNOPSIS

Gliomas are by farthe most common primary brain tumors that affect the glial cells of the brain. It represents about 32% of all adult brain tumors and 81% of all malignant tumors. Despite various multimodality therapies, overall survival rate of glioma patients has not improved and there is no definitive cure or treatment for preventing the progression and recurrence of this tumor. According to CSC model, a rare sub-population of cells is responsible for the initiation and maintenance of tumor. Glioma consists of a small sub-population of tumor initiating / tumor propagating cells called glioma stem cells (GSCs) that have the ability to proliferate indefinitely, differentiate into all three main neural lineages (neurons, astrocytes and oligodendrocytes) and are more resistant to radiation and chemotherapies that may underlie the persistence and recurrence of tumors following treatment. These cells have the ability to grow as spherical clusters of cells called gliomaspheres and are the defining feature of GSCs *in vitro*. Recent reports suggest that gliomasphere formation is an indicator of tumor progression independent of grade. This study was designed to assess the gliomasphere forming ability among different grades of glioma, both low and high grade and its characterization. Possible associations between gliomasphere formation and grades of tumor were analyzed amongst 140 patients, including grade I (10), grade II (57), grade III (28) and grade IV (44) gliomas.

Growing body of literature suggests in addition to the existence of GSCs, the deadly nature/poor therapeutic response of gliomasare mainly due to its inherent resistance to apoptosis (type I programmed cell death) and are attempted to overcome by activating autophagy using pro-autophagic drugs. Autophagy is a process in which

different cellular organelles are packaged within specific vesicles called autophagosomes whose contents get digested within lysosomes and can be utilized as a cell death mechanism. The reports show that even after giving pro-autophagic drugs 60-75% of patients derive no benefit from the treatment. The reason for the therapeutic insensitiveness of certain gliomas to autophagic death inducing drugs is unclear. The studies suggest that for the effectiveness of pro-autophagic drugs, glioma cells should have non-defective autophagic machinery. It is possible that the gliomas are autophagy defective like other neurological disorders. But reports about the basal autophagic status of glioma cells are absent or rare.

This work employs previously established gliomasphere models to determine the presence of GSCs and flux analysis to determine autophagy is non-defective. Despite the frequency and severity, very little research exists that compares the relationship among gliomasphere forming ability among different grades of glioma.Because of the absence of studies and due to need for such assessment on autophagic status and the sphere formation ability in all four grades of glioma, we have now investigated for the presence of GSCs and autophagic status among different grades of glioma.

Objectives

- To assess for the presence of gliomasphere forming cells within different grades of glioma in vitro.
- To evaluate the sphere forming capacity in relation to long-term proliferation and self-renewal within different grades of glioma.
- To characterize gliomasphere forming cells with known markers of stemness and lineage and their capacity for multipotency

- To check the basal autophagic status of different grades of glioma.
- To analyze whether autophagy can be induced in glioma derived cells.

Methodology

Human gliomatissues were obtained from patients undergoing surgical treatment at the Department of Neurosurgery, Sree Chitra Tirunal Institute for Medical Sciences and Technology.Glioma specimens were histopathologically classified according to the WHO classification by neuropathologist. Fresh samples were taken for isolation and primary culture of glioma cells, a portion of sample stored at -80°C for protein extraction and yet another portion was fixed with formaldehyde for immunohistochemistry.In the current study, patient tumorsamples were enzymatically dissociated into single cellsand cultured using standard protocols and maintained in stem cell promoting serum free medium (SFM, DMEM/F12 containing supplements and growth factors) to enrich forgliomaspheres from GSCs or serum containing medium (SCM) consisting of DMEM: F12 medium with 10% fetal bovine serum (FBS) for the growth of differentiated glioma cells. All tumor cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and 95 % air.Possible associations between gliomasphere formation and grades of tumor were analyzed amongst 140 patients, including grade I (10), grade II (57), garde III (28) and grade IV (44) gliomas. The ability to form spheres at 48-72 h post plating was assessed to evaluate the presence of GSCs population within different grades of glioma. The frequency of stem cell population within different grades of glioma was determined by primary sphere formation assay. The self-renewing potential of the spheres was assayed by sub-sphere formation assay. Limiting dilution assay was done to

determine the minimal frequency of repopulating tumor stem cells within the sphere population. The gliomaspheres were tested for multipotent ability by differentiation assay and were further characterized by immunofluorescence and flowcytometry. To determine autophagic status of different grades of glioma, we performed autophagic flux assay using Bafilomycin in 10 patients.Western blot analysis was done after bafilomycin treatment in primary cultured glioma cells and also directly at the tissue level (n=63) to determine the expression status of proteins. Immunohistochemical analysis of tissue samples (n=48) was used for the detection of proteins. The role of autophagy, whether cell death or survival was analyzed by MTT assay.

Major Findings

Primary glioma tumors of different grades have the ability to form renewable neurospheresunder culture conditions that favored stem cell growth. Within 48-72 h after plating, phase-bright clones of spherical cellular clusters called neurospheres or gliomaspheres were formed from all grades of glioma irrespective of pathological subtype. The generation of neurospheres from different grades of glioma provides an indication of the clonogenicity of gliomatumors. Comparison of primary sphere forming capacity between I, II, III and IV grade gliomas resulted in higher probability of gliomasphere formation in grade II gliomas. There was no statistically significant difference in the size of spheres formed between different grades. All of the grades of glioma also exhibited secondary gliomasphere forming capacity, demonstrating an ability to self-renew. When self-renewal capacity was compared among different grades of glioma at a plating density of 10000 cells/well, high grade gliomas (III and IV) were found to generate a greater mean number of secondary

spheres compared with low grade gliomas (I and II). Our study suggests a direct relationship between self-renewal capacity and grade of the tumor which correlates with the clinical severity, as HGG (III and IV) showed increased secondary gliomasphere formation compared to LGG. When we examined gliomasphere formation as a function of age and sex, there was no significant correlation. These data show that all of the grades of glioma had a subpopulation of cells with a self-renewal capacity which in turn correlated with the clinical aggressiveness of the tumor. Different grades of glioma were further characterized for the expression of stem cell marker proteins and lineage markers. Culture in NSC conditions appears to increase the expression of stem cell markers CD133, CD15 as well as nestin and decreases the expression of mature glial and lineage markers GFAP and β III tubulin respectively, suggesting NSC conditions select for a more stem cell phenotype. The expression of stem cell markers CD133, CD15 and nestin was detected mainly in higher malignant grades of glioma whereas lineage markers GFAP and β-tubulin III protein expression was present in all grades of glioma. The multipotent ability of gliomaspheres was analysed using differentiation assay, and is found to produce progenies of different lineages.The immunohistochemical analysis of glioma tissues expressed both stem cell and lineage markers and the expression of it vary with the grade of tumor. The autophagic status of the primary cultured glioma cells isolated from the resected human tissues was analyzed using its molecular markers: LC3-II and p62.This provides an indication that tumors from which cells were isolated are capable of autophagic death.In addition to that, the studies with starvation conditions in primary cultured glioma cells have shown that autophagy can be induced in all grades of glioma.

Significance/Implication of the findings

This study throws light on the less-explored subject in glioma mainly the extensive analysis of correlation between gliomasphere formation and grade of glioma. Our results show that all grades of glioma possess a stem cell population as indicated by the gliomasphere formation. Our study also suggests that gliomasphere formation may serve as an indicator of stem cell population within the tumor thereby predicting the clinical aggressiveness/outcome of the tumor thereby indicating a direct linkbetween stem cell features (gliomasphere formation) and clinical outcome of glioma. To the best of our knowledge this is the first study that performed autophagy flux analysis in primary cultured glioma cells.For the first time, we demonstrate that autophagy is non-defective in clinical samples of glioma. In addition, autophagy is not impaired in human gliomatumors and fact that it can be induced indicates the scope of using pro-autophaguic drugs for glioma treatment but needs further exploration to check the exact role of autophagy in these patients as autophagy has found to have a dual role both survival and death.

I. INTRODUCTION

I. 1. Introduction

Gliomas are the tumors that affect the glial cells of the brain. It can arise anywhere in the central nervous system (CNS) and is most common in the cerebral hemispheres. It can also develop at any age, while most gliomas occur in adults. They are the most common primary brain tumors and make up one third of all primary brain tumors. According to the Central Brain Tumor Registry of the United States (CBTRUS), gliomas represent about 32% of all brain tumors and 81% of all malignant tumors in adults (Dolecek et al., 2012). The incidence rate of glioma in adults accounts for 6.04 per 100,000 person-years and the incidence rates were greater in men (7.17 per 100,000 person– years) than in women (5.07 per 100,000 person–years) (Dolecek et al., 2012). According to the survey of Indian council for Medical Research (ICMR), the prevalance of glioma cases for India are 5.8% in Mumbai, 6.7% in Bangalore, 3.5% in Chennai, 5.6% in Dibrugarh and 28.2% in Trivandrum among males and 6.3% in Mumbai, 5.6% in Bangalore, 7.5% in Chennai, 0% in Dibrugarh and 21.8% in Trivandrum among females (Shankarkumar and Sridharan, 2011). However, the increased incidence of glioma in Trivandrum might likely due to the study bias associated with variations in information, data entry and availability of health care access.

I.1.1. Glioma: WHO histopathology classification and grading

Gliomas are tumors of different histological types and grade. They are divided into subgroups depending on the histopathologic evidences relating to cell type, cell morphology, mitotic activity, endothelial proliferation and necrosis. Based on the

histological type, World Health Organization (WHO) has classified gliomas into different types on the basis of their resemblance in morphological appearance to normal glial cells such as astrocytes, oligodendrocytes and ependymal cells as astrocytoma, oligodendroglioma, mixed glioma (oligoastrocytoma) and ependymoma respectively (Schwartzbaum et al., 2006). These tumors are further tiered into different grades (grade I-IV) based on the degree of malignancy and prognosis (Table 1) (Louis et al., 2001). Among the different grades, the grade I and II comes under low grade glioma, whereas grade III and IV are categorized as high grade. Astrocytomas include grade I (pilocytic), II, III (anaplastic) and IV (glioblastoma multiforme [GBM]). Ependymomas consists of grade I, II and III, whereas oligodendrogliomas and oligoastrocytomas include grade II and III lesions (Table 2) (Schwartzbaum et al., 2006). Among the gliomas, GBM accounts for approximately 70%, anaplastic astrocytoma 10-15%, oligodendroglioma and oligoastrocytoma 10% and ependymoma 3-5%.

Table 1. WHO grading for glioma

Grade I	Benign, slow growing
Grade II	Increased potential for recurrence, High hypercellularity, no mitosis, no vascular proliferation, no necrosis
Grade III (Anaplastic)	High rate of recurrence, increased hypercellularity, high rate of mitosis, no vascular proliferation, no necrosis
Grade IV	Very high rate of hypercellularity, increased mitosis, presence of vascular proliferation and necrosis

WHO has developed a grading syatem for gliomas; which grades the tumor based on hypercellularity, mitosis rates, presence of necrosis and vascular proliferation.

Table 2. Classification of glioma

WHO I	Pilocytic Astrocytoma			Ependymoma
WHO II	Astrocytoma	Oligoastrocytoma	Oligodendroglioma	Ependymoma
WHO III	Anaplastic Astrocytoma	Anaplastic Oligoastrocytoma	Anaplastic Oligodendroglioma	Anaplastic Ependymoma
WHO IV	Glioblastoma multiforme (GBM)			

The WHO has classified gliomas into different types based on their malignancy and its resemblance in morphologic appearance to normal glial cells.

I.1.1.1. Gliomas: Epidemiology and Pathology

Astrocytomas are the most common type of glioma that develops from the astrocytes (glial cells). They form the largest group of glioma (76% of all gliomas) and accounts for 17% of all tumors. Low-grade astrocytomas (I and II) are slow growing whereas high grade (III and IV) are fast growing tumors. Astrocytomas are broadly divided into two major classes based on their ability for invasion and progression to malignant form as non-infiltrative astrocytomas and diffuse astrocytomas. The non-infiltrative astrocytomas are WHO grade I tumors with decreased capacity for invasion and malignant transformation which includes pilocytic astrocytoma (PA), subependymal giant cell astrocytoma (SEGA) and pleomorphic xanthoastrocytoma (PXA) (Ware et al., 2003). Diffuse astrocytomas are highly invasive tumors with increased capacity for malignant transformation and are grouped as astrocytoma (grade II), anaplastic astrocytoma (grade III), and glioblastoma multiforme (grade IV). Pilocytic astrocytomas are slow growing non-aggressive tumors with well-

defined borders. They account for 5.2% of all gliomas and occur at the first two decades of life predominantly occurring in children and young adults. Low-grade diffuse astrocytomas (grade II) are slow growing tumors with an inherent tendency for malignant progression to anaplastic astrocytomas and GBM. It accounts for 1.8% of all gliomas with a peak incidence between 25 and 50 years of age and have an average survival of approximately seven years. Anaplastic astrocytomas exhibit increased anaplasia, atypia, cellularity and proliferation along with its increased tendency to progress to GBM, have an average survival of approximately 2-5 years. GBM, the grade IV glioma is the most malignant of all brain tumors and can either arise *de novo* (primary GBM) or as a result of slow progression from low grade astrocytoma or anaplastic astrocytoma (secondary GBM). The morphological and clinical features of GBM are the same irrespective of clinical route. Primary GBM tends to occur in older patients whereas secondary GBM is mainly seen in younger patients. GBM also represents another group with an oligodendroglial component and has shown to have better prognosis and longer survival than classical GBM (Masui et al., 2012). They are characterized by increased anaplasia, cellularity, endothelial proliferation and necrosis (Louis et al., 2001; Maher et al., 2001).

Oligodendrogliomas are tumors that are believed to develop from the oligodendrocytes. It comprises grade II and grade III (anaplastic) tumors with a median survival of 8-20 and 2-10 years respectively. Anaplastic oligodendroglioma develop either *de novo* or as by progression from grade II oligodendroglioma. Malignant progression to high grade occurs less frequently in oligodendrogliomas compared to astrocytomas. Grade II oligodendrogliomas are associated with

moderate cellularity and very low proliferation index (<5%) whereas anaplastic oligodendrogliomas have high cellularity, atypia, mitotic activity, microvascular proliferation and necrosis (Maher et al., 2001).

Mixed glioma includes oligoastrocytoma with morphological characteristics of astrocytic and oligodendroglial cells. These tumors are graded as either grade II or grade III (anaplastic). The anaplastic oligoastrocytoma exhibits high cellularity, cellular pleomorphism, nuclear atypia, and increased mitotic activity compared to low grade oligoastrocytoma. Microvascular proliferation and necrosis may also be present in anaplastic oligoastrocytoma. Oligoastrocytomas respond less favourably to chemotherapy than oligodendroglioma, may be because of its astrocytic component (Sarkar et al., 2009).

Ependymomas are tumors that arise from the ependymal cells which line the ventricles of the nervous system. It has the highest incidence in children and also occurs into late middle age. It consists of a number of subtypes such as grade I, grade II ependymoma and grade III anaplastic ependymoma. The grade I subtype is biologically less aggressive and includes subependymoma and myxopapillary ependymoma. Grade II ependymoma is associated with low mitotic rate and nuclear polymorphism compared to anaplastic ependymoma (Ware et al., 2003).

The WHO classification of glioma is based on the morphological analysis by histopathology and is susceptible to interobserver variation and also does not give any information regarding survival or response of patients to therapy. Furthermore, gliomas are malignancies with high intratumoral heterogeneity showing alterations

on the cytopathological, transcriptional, genomic, and epigenetic level. The use of molecular markers along with the current histopathological classification will allow identification of gliomas with similar genetic profile thereby allowing specific targeted therapy for each patient/tumor.

I.1.2. Molecular alterations in glioma

Several molecular alterations and genetic changes in growth factor receptors, cell cycle and apoptosis regulators have found to be associated with glioma, which involves copy number changes such as amplifications, gains and deletions. Deletions and loss of heterozygosity are associated with the loss of function of tumor suppressor genes whereas amplifications and gains are connected to oncogenes leading to tumor initiation and progression. The data demonstrate that glioma has a number of signalling pathways that are constitutively activated (Lefranc et al., 2005; Van de Kelft, 1997). Understanding the molecular alterations may result in more precise knowledge of the tumor biology in relation to prognosis and prediction of tumor behavior.

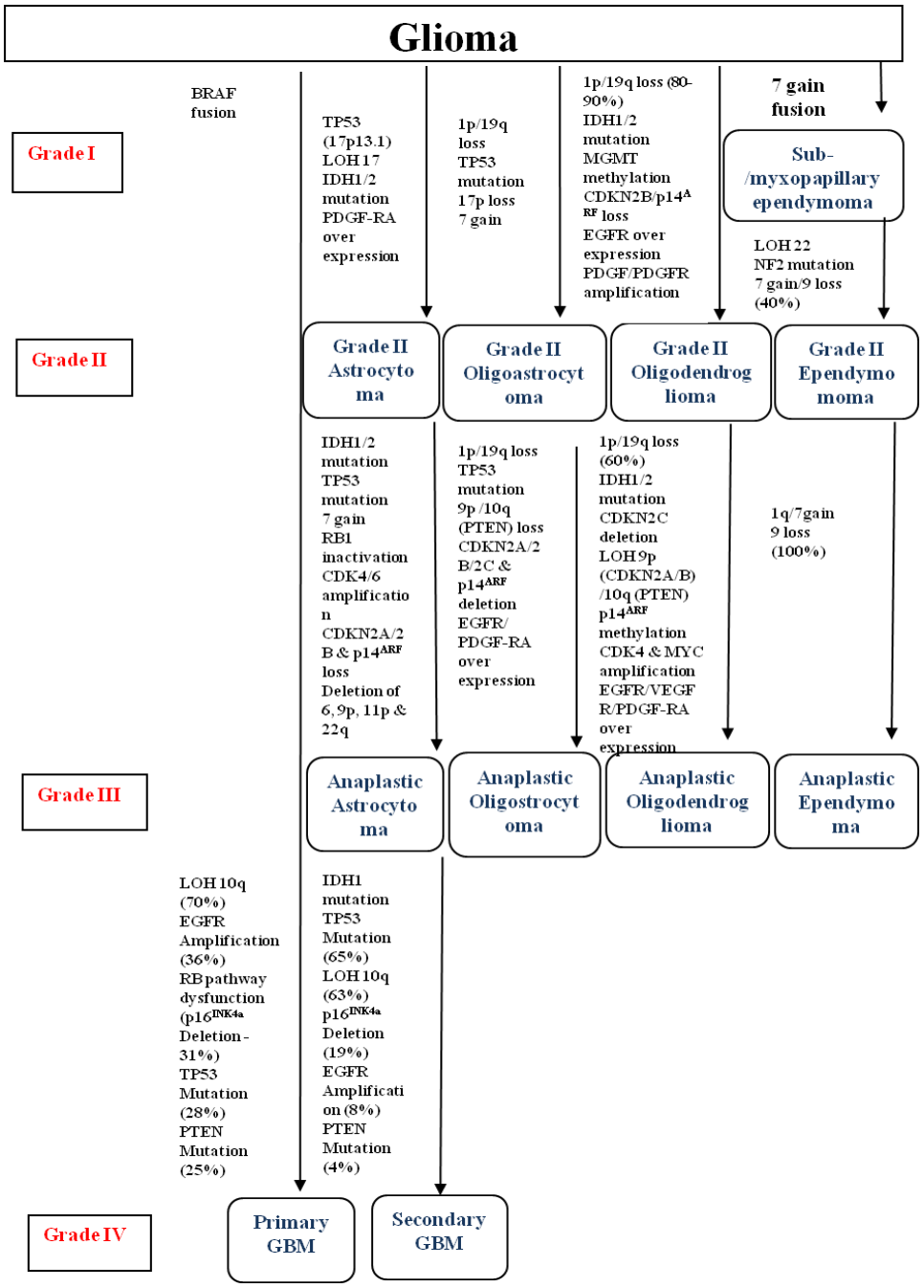
The chromosomal and genetic aberrations commonly associated with gliomas are mutations of the EGFR/PTEN/Akt/mTOR pathway; TP53/MDM2/$p14^{ARF}$ Pathway; $p16^{INK4a}$/RB1 Pathway; neurofibromin gene (NF1); mutant isocitrate dehydrogenase (IDH) 1/2 proteins; loss of heterozygosity (LOH) of chromosome 10 (commonly deleted loci include 10p14-p15, 10q23–24, and 10q25-pter), chromosome 17p, chromosome 9p (genes on 9p are cyclin dependent kinase inhibitor 2A [CDKN2A/$p16^{INK4A}$], CDKN2B and $p14^{ARF}$), or chromosome 19q; gain of the long arm of chromosome 7, amplification and rearrangement of the oncogenes N-myc, c-

myc, N-ras, Kras, and platelet-derived growth factor receptor A (PDGF-RA) (Figure 1) (Masui et al., 2012; Schwartzbaum et al., 2006; Ware et al., 2003). Phosphatase and tensin homolog deleted on chromosome 10 (PTEN, located at 10q23) deletion and epidermal growth factor receptor amplification are the frequent mutations that occurs in glioma (Nikiforova and Hamilton, 2011). IDH 1 and rarely 2 mutations have been identified in the vast majority of grade II and III astrocytoma, oligoastrocytoma, oligodendroglioma and secondary GBM but are rare in primary GBM and completely absent in pilocytic astrocytoma (Kloosterhof et al., 2011). IDH1/2 mutation usually coexists with 1p/19q deletion in oligodendroglioma and with TP53 mutation in astrocytomas (Riemenschneider et al., 2010). In addition to these markers, members of the family of matrix metalloproteases (MMP) and their endogenous tissue inhibitors (TIMPs) that play an important role in invasion and infiltration plus angiogenesis promoters such as VEGF and PDGF expression have also found to correlate with the grade of glioma (Furnari et al., 2007). The genetic alterations associated with primary GBM and secondary GBM are different (Kleihues and Ohgaki, 1999; Ohgaki, 2005; Ohgaki et al., 2004; Ohgaki and Kleihues, 2007). EGFRvIII is the most common EGFR mutation in GBM with an in frame deletion of exons 2 – 7 of the EGFR gene that leads to a truncated protein lacking an extracellular domain and remains constitutively active (Maher et al., 2001).

Mixed oligoastrocytomas and oligodendrogliomas exhibit genetic profiles that differ from astocytic tumors. The most common genetic alterations characterized by these tumors include loss of chromosome 1p and 19q and are an established genetic marker

for the prediction of chemosensitivity and survival of patients. The 1p and 19q codeletion frequency has found to vary between oligodendroglioma (80- 90%), anaplastic oligodendroglioma (60%) and oligoastrocytoma (30-50%) (Nikiforova and Hamilton, 2011). In contrary to astrocytic gliomas, distinct histological variants of ependymomas have not been associated with consistent molecular alterations. Ependymomas mainly show abnormalities of the chromosome 1p, 4q, 6q, 9p, 10, 11q, 13q, 16, 17p, 19q, 20q, 22q, as well as and neurofibromatosis type 2 (NF2) mutation; but no EGFR overexpression (Merzak and Pilkington, 1997).

Figure 1. Common genetic alterations involved in glioma



Genetic alterations are usually associated with aberrant activation in signaling pathways. Oncogenes are usually activated or over expressed whereas tumor suppressor genes are either lost or inactivated.

Glioma identification still remains as a difficult task because of the lack of specific markers that identifies and differentiates between different grades and histologies of glioma. The understanding of the pathophysiology and genetics of these tumors have allowed the identification of certain markers that predicts prognosis and survival of patients. Gliomas are highly heterogeneous in their molecular alterations thereby rendering these cells insensitive even to molecular targeted therapies. Despite progress in apprehension of molecular alterations in glioma, the clinical outcome has remained unchanged due to the ignorance of the cause or origin of glioma. The understanding of the origin of glioma will provide another route by which the resistant nature of gliomas can be addressed.

I.1.3. Origins of glioma

Glioma is a highly heterogeneous tumor with complex cellular composition. Due to the heterogeneity of glioma, it is still intriguing whether different subtypes of glioma arises from different cell types, or whether they originate from same cell type and different tumors are formed due to distinct mutations. The knowledge of cell of origin of glioma will help in precise diagnosis and therapies to this tumor. The cells giving rise to glioma is a matter of debate and is ill-defined whether the tumor arises from the transformation of an immature precursor cell or stem cell or by the dedifferentiation of mature cells. The neoplastic transformation of differentiated cells by the accumulation of oncogenic mutations is widely assumed to be the cause of gliomagenesis. The differentiated cells has to regain the ability to self renew in order to serve as the source of gliomas. An alternative possibility is those more immature neural stem cells (NSCs) or transiently amplifying committed progenitor cells as the

origin of glioma. The cell of origin has to undergo several oncogenic transformation events to get converted to a cancer stem cell (CSC) (Galderisi et al., 2006; Reya et al., 2001).

Initially, one of the widely accepted paradigms of carcinogenesis is the "somatic mutation theory" which holds that cancers are monoclonal in origin in which a differentiated somatic cell undergoes dedifferentiation/reprogramming due to mutations to form cancer. The expression of glial fibrillary acidic protein (GFAP) in astrocytomas has found to be in line with this hypothesis. This concept is supported by several *in vitro* and *in vivo* studies that have shown GFAP expressing astrocytes upon stimulation by appropriate epigenetic signaling have the ability to form tumors of astrocytic, oligoastrocytic and oligodendroglial lineage. The studies have shown that aberrations that cause over expression of growth factors such as EGFR/PDGFR or mutations in PTEN, p53 and Rb pathways can lead to glioma formation in a non-neurogenic area further supporting the possibility of dedifferentiation of glial cells in tumor formation. The major refute for differentiated cells as the cell origin of glioma is its limited proliferative ability and life span and these cells has to acquire these hallmarks to serve as rootage of glioma (Trosko, 2009).

An alternative possibility is NSCs or transiently dividing progenitor cells as the source of glioma. Stem cells are the cells that have the ability to propagate themselves through self-renewal and to generate cells of a particular tissue by multipotent differentiation. NSCs are the cells in the brain that have the ability to self-renew and multipotent differentiation into neurones, astrocytes and oligodendrocytes. According to CSC hypothesis, the tumors arise from and are

maintained by a subpopulation of rare cells with self renewal and uncontrolled proliferative ability called CSCs. CSCs are not normal cells with uncontrolled proliferative ability, but have all the functional capabilities similar to stem cells thereby mediating oncogenesis. The idea of CSCs has been extended to brain tumors also and is called brain tumor stem cells (BTSCs). BTSCs have the characteristics shared with NSCs such as self-renewal, enhanced proliferation, multilineage differentiation and ability to generate tumors recapitulating the original parental tumor. NSCs are essential for the normal physiological development of the CNS whereas the BTSCs can be attributed towards pathological conditions of brain tumor (Zaidi et al., 2009). In addition, the BTSCs have found to express signalling pathways and immature markers similar to NSCs (Prestegarden and Enger, 2010). The similarity in the characteristics between NSCs and BTSCs has led to the notion that BTSCs would have formed from NSCs that have undergone tumorigenic mutations. NSCs are the ideal candidates as they are long-lived cells with an inherent self renewal capacity, thus fewer mutations may be required to initiate tumorigenesis. The rareness of NSCs in the tissue is one limiting factor to serve as the origin of glioma. The transiently amplifying progenitor cells/committed progenitor cells derived from stem cells with a proliferative capacity for short period of time before terminally differentiating can also serve as the cell of origin of glioma. But the progenitor cells have to acquire mutations that will endow self renewal capacity for transformation. In the case of NSCs/progenitor cells, the possible explanation for the formation of morphologically heterogeneous tumors in glioma is due to difference in the nature of signaling pathways affected.

The cell of origin of glioma is highly confounding because of the presence of distinct tumor subtypes and also due to complex intratumoral heterogeneity. The recent evidences from different studies have shown that NSCs that have undergone tumorigenic mutations or transiently amplifying progenitor cells or terminally differentiated cells that had a series of mutations acquiring self-renewal and other stem cell properties could all serve as the cell of origin of glioma (Hambardzumyan et al., 2008; Martin-Villalba et al., 2008; Walker and Kaye, 2001). The relationship between different cell of origin and type of tumor formed is still unclear. The studies imply that understanding the cell of origin has clear prognostic significance and also is very important in providing highly effective targeted treatment.

I.2. Glioma stem cells

BTSCs have been isolated from different types of brain tumors and that of glioma are called glioma stem cells (GSCs). Glioma consists of a small sub-population of tumor initiating/tumor propagating cells called GSCs that have the ability to proliferate indefinitely, differentiate into rapidly proliferating progenitor cells and to differentiated cells of all three main neural lineages (neurons, astrocytes and oligodendrocytes) and are more resistant to radiation and chemotherapies that may underlie the persistence and recurrence of tumors following treatment (Dirks, 2008; Kukekov et al., 1999). GSCs can be grown *in vitro* under serum free culture conditions in minimal/defined medium in the presence of supplements and pleiotropic growth factors such as EGF and bFGF as floating cluster of cells called gliomaspheres/tumorspheres (Singh et al., 2003). The gliomaspheres are a clonal spherical cluster of cells consisting of a heterogeneous population of thousands of

cells. Among them approximately 50-100 are stem cells while the rest include progenitors and differentiated cells. Each gliomasphere is formed by the proliferation of single NSC/GSC, still heterogeneous population is maintained because a stem cell either undergo self-renewal (symmetric division) to form stem cells or asymmetric division leading to the formation of progenitors and differentiated cells. GSCs can be isolated on the basis of the immunoreactivities to certain proteins on the surface specifically on the membrane of GSC by using antibody coated magnetic columns or by fluorescence activated cell sorting (FACS) (Vescovi et al., 2006).

I.2.1. Molecular characterization of glioma stem cells

The identification of GSC is very difficult as there is no single specific reliable marker that distinguishes GSCs from the normal NSC population as well as progenitor cells. NSCs has found to express a 120 KD five transmembrane cell surface glycoprotein called CD133, which was originally known as hematopoietic stem cell marker (Uchida et al., 2000). Singh et al has shown that putative BTSCs also express the cell surface marker CD133. It was demonstrated that a small fraction of $CD133^+$ population (100 numbers) isolated from tumor mass were able to form both $CD133^+$ and $CD133^-$ cells and were capable of recapitulating the original tumor in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice, whereas even after injecting 5×10^4 to 1×10^5 $CD133^-$ cells they failed to form tumors (Wang et al., 2008). They have shown the expression of $CD133^+$ BTSCs in different brain tumors such as glioma, meduloblastoma, ganglioglioma and ependymoma. But growing body of evidence also suggests that not only $CD133^+$ but $CD133^-$ cells also possess tumorigenic capacities. The expression of the stem cell

marker may vary from tumor to tumor and also depends on the grade of the tumor and culture conditions such as availability of nutrients. The CD133 is considered as a putative stem cell marker and its expression has found to vary from 1-60% in GBM patients itself (Uchida et al., 2000; Wang et al., 2008; Yuan et al., 2004). There are contradictory reports about whether $CD133^+$ cells or $CD133^-$ cells are tumorigenic. Chen et al has demonstrated the presence of three different types of GSCs: $CD133^+$ cells with ability to generate $CD133^-$ cells, $CD133^-$ cells that have the capacity to form $CD133^+$ cells and $CD133^-$ cells that produce only $CD133^-$ cells (Mizrak et al., 2008). The other putative NSC and BTSC markers include nestin, a cytoplasmic intermediate filament protein and most recently CD15, a transmembrane protein and the expression of these markers were found to vanish upon differentiation of these cells (Mao et al., 2009). CD15 is another marker associated with GSCs and is found to be tumorigenic and expression varies from 2.4-70%, even $CD15^+/CD133^-$ cells were tumorigenic (Son et al., 2009). There are also other stem cell markers such as Oct-4, Musashi-1, SOX-2, MELK, nanog, and L1CAM (Gilbert and Ross, 2009). A2B5 is another marker associated with GSCs and studies has reported the existence of two populations including $A2B5^+/CD133^+$ and $A2B5^+/CD133^-$ and both cells were found to be tumorigenic. The other markers associated with GSCs include podoplanin and integrin α 6 (Table 3) (Brescia et al., 2012; Friedman et al., 2013; Gilbert and Ross, 2009).

Table 3. Markers of Glioma stem cells

Attribute	Marker
Cell surface proteins	CD133, CD15, CD44, CXCR-4, integrin α 6, A2B5, LICAM
Cytoplasmic and nuclear proteins	Nestin, musashi-1, bmi-1
Transcription factors	Sox-2, nanog, Oct-4,
Enzymes	ALDH1
Functional	Neurosphere formation, side population, tumor initiation ability

(Table adopted from Gregory K. Friedman; Pediatric glioma stem cells: biologic strategies for oncolytic HSV virotherapy, *Frontiers in oncology, 3, 2013)*

The studies suggest the use of combination of markers to identify and isolate GSCs rather than sticking on to a specific marker. Due to the lack of reliable markers that specifically identifies the GSC population and investigates its tumorigenic process, the targeting of GSCs remains a major challenge.

I.3. Therapeutic resistance of glioma

Gliomas have been found to be highly resistant to existing conventional therapies and get spared due to some idiosyncratic properties thereby contributing to the relapse of tumor. The current standard of care for malignant glioma includes surgical resection followed by radiation therapy (external beam radiation) and chemotherapy. Malignant gliomas have found to show distinct mechanisms that may effectively resist current treatments. The highly infiltrative nature of glioma making the total

surgical excision impossible and the non-specificity of adjuvant treatment after surgery are the main factors of therapeutic resistance. Glioma cells also have a mechanism of immune suppression by releasing a lot of immunosuppressive factors such as TGFβ, VEGF, PGE2, B7-H1, galectin-3, CCL-2, IL-10 and Fas-ligand that helps to evade the immune system (Wei et al., 2010). The studies have shown that the conditioned medium from glioma cells has found to inhibit T cell activity and induces T-reg cells which inhibit T cells, B cells, natural killer cells, monocytes, dendritic cells and macrophages (Humphries et al., 2010).

Glioma has been found to have a variety of signalling pathways activated such as notch pathway and sonic hedgehog which may contribute to the enhanced survival and proliferation of tumor (Clement et al., 2007; Fan et al., 2010). The notch pathway inhibitors such as γ-secretase inhibitors (GSI) and sonic hedgehog inhibitor cyclopamine have found to sensitize the glioma to treatment. Phosphtaidylionositol-3-kinase (PI3K)/receptor tyrosine kinase (RTK) mainly via EGFRs and PDGFRs is a survival signalling network that is frequently deregulated in gliomas (Li et al., 2009). The vascular endothelial growth factor (VEGF) signalling cascade and hypoxia-inducible factor 1α (HIF-1α) are the key molecules associated with tumor cell angiogenesis and have found to be strongly expressed in malignant gliomas (Chakravarti and Palanichamy, 2008). Glioma has found to have increased vasculature, secretes VEGF and the stem cell markers such as CD133 and CD15 has found to be in the proximity of vascular niche, thus treatment with angiogenesis inhibitors such as bevacizumab that targets the VEGF has found to sensitize to treatment.

The main attributes that help malignant gliomas evade therapies include mechanisms such as increased expression of growth factors and stem cell maintenance pathways, increased expression of drug transporters, ability to evade apoptosis, increased DNA damage response and repair activity leading to resistance to most of the therapies. Glioma cells have an inherent resistance towards apoptosis because of increased expression of anti-apoptotic BCL-2 family proteins such as bcl2, bcl-XL and high level expression of inhibitors of apoptosis (IAPs) proteins (Dolecek et al., 2012; Gilbert and Ross, 2009). Recent reports suggests that glioma has a subpopulation of cells called GSCs that contributes to the resistance of these tumors to existing therapies. GSCs have increased DNA repair activity and a variety of drug transporters that help to evade therapies (Van Meir et al., 2010). The drugs such as temozolomide (TMZ) that are used for glioma treatment have shown to induce quiescence of GSCs and thus escaping the treatment and later repopulating the tumor. Elevated levels of O^6-methylguanine-DNA-methyltransferase (MGMT) have been associated with resistance to TMZ and are the strongest predictor of outcome from therapy (Brescia et al., 2012; Patel and Rameshwar, 2013).

The studies over the past decade support the hypothesis that treatment resistance of glioma tumors is in large part due to the presence of GSCs and the inherent resistance of glioma to apoptotic cell death.

I.4. Major modes of cell death in glioma

Glioma has found to have a number of cellular alterations that helps to evade cell death and the goal of therapeutics is to evoke a tumor selective cell death. Apoptosis (type I programmed cell death) is the principle mechanism by which cells are removed both physiologically and also by anti-cancer therapies. Glioma has an inherent reistance to apoptosis and is the basic factor that affects the standard therapies. Apoptosis resistance is closely linked to drug resistance and therapy failure which is interlinked with tumorigenesis. There exist other non-apoptotic mechanisms of cell death including necrosis, autophagy and also other mechanisms such as senescence, mitotic catastrophe, paraptosis and autoschizis. Necrosis could represent an alternative pathway for cells to die and is an unregulated passive energy independent form of cell death that involves spilling of contents and causing a potentially damaging inflammatory response. Necrosis surrounded by hypercellular regions known as pseudopalisades is the most common hallmark of GBM that differentiates from other low grade gliomas. The presence of necrosis has a negative prognostic value and is inversely related to patient survival. Resistance to apoptosis and other forms of cell death has driven the search for novel targets in glioma treatment. Autophagy is one such target and exploring the same will open up promising new avenues for the development of new therapeutic strategies for glioma treatment.

I.4.1 Autophagy in glioma

Autophagy is a self eating process that occurs by the sequestration of cargo such as a portion of the cytosol and organelles and proteins inside a double membrane

autophagosome which fuses with lysosome and degrades the contents using lysosomal hydrolases. It is a protein degradation process that has been activated in various conditions such as nutrient deprivation, aging, transformation and cancer (Cuervo, 2004). The effect of autophagy may vary depending on the context. Gliomas are nowadays treated using pro-autophagic drugs (Jiang et al., 2011). Temozolomide (TMZ) is the drug of choice used for the treatment of glioma and is found to induce autophagy (type II programmed cell death) (Hirose et al., 2001). This prompted the analysis of gliomasphere forming ability and autophagic status among different grades of glioma. Before presenting the study objectives, an overview of currently available knowledge about GSCs and autophagy in glioma is attempted through a literature review.

II. LITERATURE REVIEW

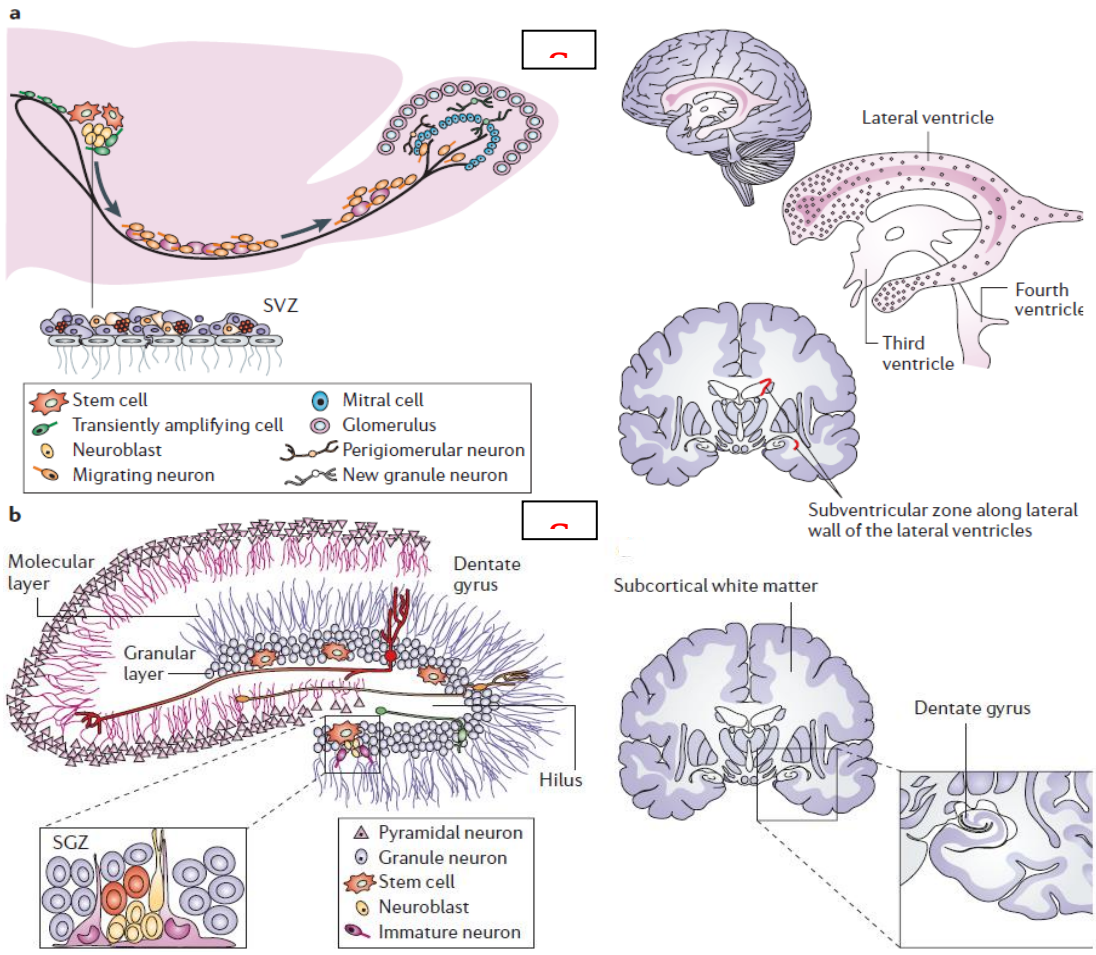
II.I. Introduction

It has long been thought that adult brain is an organ where no significant cell turnover occurs. The concept known as "no neuron dogma" was overruled, and reports show that neurogenesis occurs and neural stem cells (NSCs) exist throughout life in adult brain. Neurogenesis in adult brain was initially claimed by Altman (1962) but was ignored at that time (Altman and Das, 1965). The pioneering work of Fernando Nottebohm (1990s) in adult song birds has showed the relevance of neurogenesis in post natal brain. Great deal of studies that followed have isolated NSCs from other non-mammalian and mammalian species; concurrent studies also reported that neurogenesis is crucial for learning, memory, maintenance of brain integrity and function (Vescovi et al., 2006).

II.1.1. Neurogenesis in adult brain

Neurogenesis mainly occurs in discrete regions in brain called subventricular zone (SVZ) lining the lateral ventricle and subgranular zone (SGZ) in the dentate gyrus of hippocampus, thenceforth the newly formed neurons will get integrated into the neuronal circuitry (Gage, 2002; Kukekov et al., 1999). Neurogenesis was first shown in the forebrain SVZ and then in the dentate gyrus (Figure 2). SVZ is the largest neurogenetic region in the adult brain and includes the lateral wall of the lateral ventricle and traverses through the rostral migratory stream (RMS) to the olfactory bulb. There exists a hierarchical organization among the three main cell types in SVZ; among them type B astrocytes that express GFAP and are relatively quiescent

Figure 2. The neurogenesis in subventricular zone and subgranular zone in brain



(A) *Subventricular zone (SVZ), the largest area of adult neurogenesis lines the lateral ventricles of the forebrain and is comprised of three main cell types: the multipotent, type B astrocytes, that have been identified as the bona fide SVZ stem cells, give rise to fast-cycling transiently proliferating precursor cells that are called type C precursors and that, in turn, generate mitotically active type A neuroblasts which gets integrated as new interneurons.* ***(B)*** *An additional adult neurogenetic region is found in the subgranular zone (SGZ), which is located within the dentate gyrus of the hippocampus. A cellular hierarchy, somewhat similar to that of the SVZ, is seen in the SGZ in which the true stem cell is probably the type B astrocyte, which produces the intermediate type D precursor that eventually gives rise to the type G granule neurons. These neurons integrate functionally into the granule cell layer.*

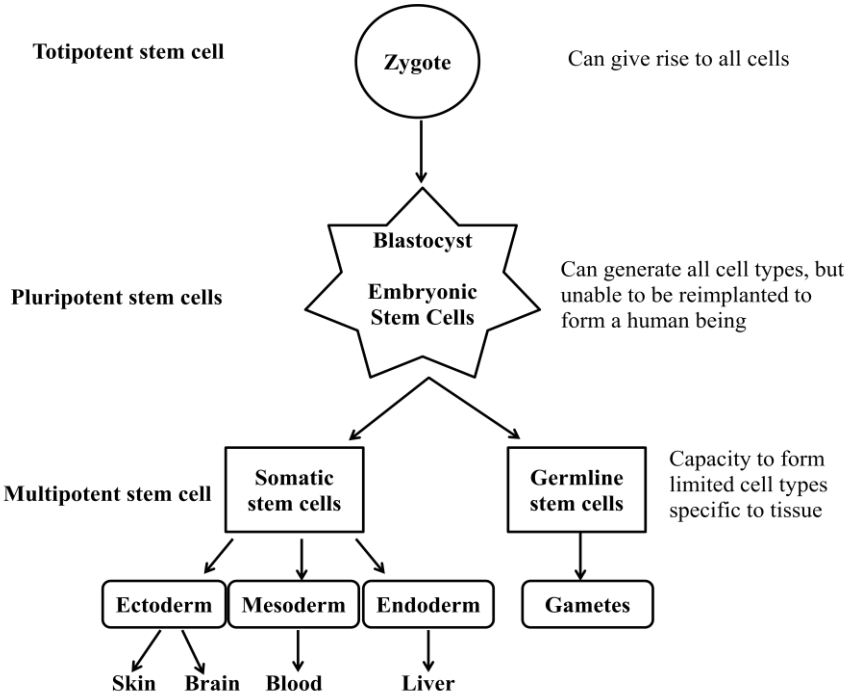
(Figure and its description adapted from Angelo L. Vescovi; Brain tumor stem cells, Nature reviews)

with a cell cycle time of approximately 28 days have been identified as the stem cell, which give rise to fast cycling transiently dividing multipotent progenitor cells called type C cells with a proposed cell cycle time of 12 hours which in turn generate type A cells that mature into neurons. The germinal zone in SGZ also shows a similar cellular hierarchy in which type B cells are the stem cells and produces type D precursors that give rise to type G neurons. The molecular mechanism that regulates cell fate decisions, whether to differentiate to neurons or glia remains unidentified.

II.1.2. Stem cells: A classical view

Stem cells are cells that have the ability to propagate themselves through self renewal and exhibit multilineage differentiation capacities. Under physiologic conditions, stem cells are usually quiescent thus undergo self renewal and proliferation only when triggered during tissue homeostasis and injury fixing. All tissue in adults has a pool of stem cells, the development of which is highly regulated and play an important role in tissue ontogenesis, replacement, and repair (Figure 3). Unlike embryonic and fetal stem cells which are pluripotent with the ability to divide and differentiate into all cell types, the adult stem cells are multipotent with only a limited capacity to differentiate and give rise to cell types specific to a particular tissue.

Figure 3. The developmental hierarchy of stem cells

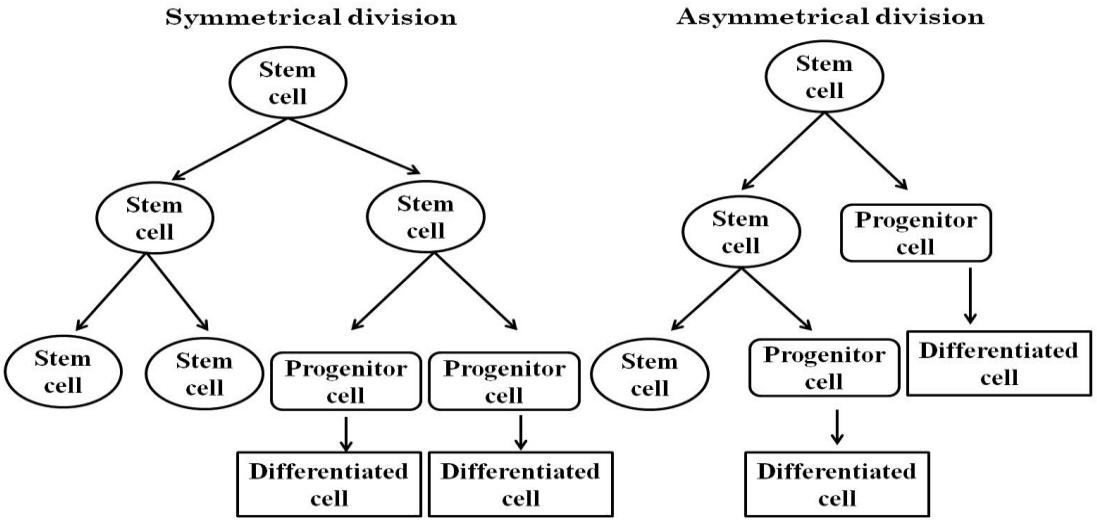


The successive differentiation potential of stem cells which has the unique ability to self-renew to generate themselves and also give rise to lineage-committed progenitors that differentiate into more differentiated progeny.

Stem cells can proliferate either symmetrically or asymmetrically, symmetric division can give rise to two stem cells whereas asymmetric division produces a daughter cell that maintains stemness like its mother cell and a progenitor cell that form differentiated cell (Figure 4). The asymmetric division ensures cell diversity and even cells generated by symmetric division as a result of their exposure to varying environmental factors can have different fates (Trosko, 2009) (Berger et al., 2004). The stem cell function is regulated by its intrinsic genetic program and also the niche signal. Stem cells are found in well maintained environment called niche

where they constantly interact with each other and also with the environment that provides specific cues to regulate its behavior thereby maintaining stemness. The niche comprises of heterologous including the extracellular components and other non stem cells but its function as well as molecular regulation between niche and stem cells remains unexplored (Scadden, 2006).

Figure 4. Models of stem cell division



Symmetric division of stem cells helps to replenish and expand the stem cell population through self-renewal and also a stem cell can divide symmetrically to produce two progenitor populations that lead to extinction of stem cells. Asymmetric division helps to generate a stem cell and a progenitor cell.

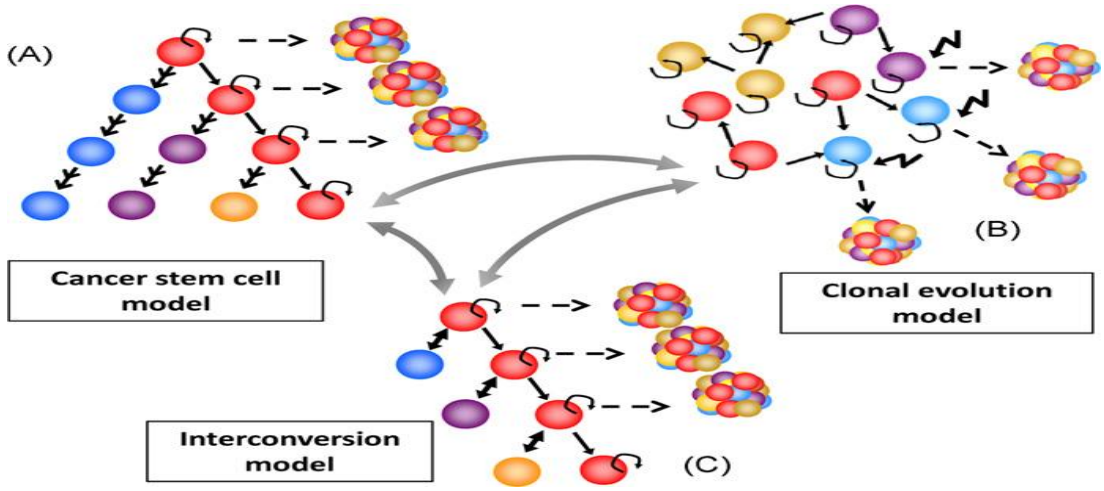
II.1.3. Normal stem cells and the concept of cancer stem cells

Normal tissue have a heterogeneous or hierarchical combination of cells with different proliferative and phenotypic characteristics comprising stem cells, transiently amplifying progenitor cells and differentiated cells. Stem cells are a rare population of cells in a tissue endowed with three fundamental properties that are

tightly regulated mainly extensive self renewal, proliferation potential and multipotent differentiation capacity. In a normal tissue, stem cells due to its self renewal and wide differentiation ability give rise to a heterogeneous population of cells; whereas progenitor cells proliferate for a shorter period of time to produce cells of a particular lineage and undergo terminal differentiation. The properties attributed to normal cells were also present in cancer cells, but in a deregulated manner (Tan et al., 2006).

Cancer is a disease that arises as a result of series of mutations and epigenetic alterations that bestow the cells with indefinite and deregulated proliferative potential. Different models have been put forward to explain the origin of cancer that includes cancer stem cell (CSC) model, clonal evolution (stochastic) model and interconversion model (Figure 5). According to the CSC theory, not all cells in a tumor have the ability to grow and sustain a cancer and are driven by only a small portion of the tumor cells (Fabian et al., 2009). In analogy to normal tissues, a tumor as a result of accumulated and continuing mutations also have phenotypically diverse population of cells including those with features of stem cells called cancer stem cells (CSCs) and aberrantly differentiated cells. The clonal evolution model states that all cells in a tumor have tumorigenic potential which is activated asynchronously and by acquiring additional mutations certain cells gain increased malignant potential. The interconversion model holds that the cells with high proliferative malignant capacity can interconvert into quiescent state and vice versa. All these models of cancer propagation are not mutually exclusive, but recent observations support the CSC theory of carcinogenesis (Shackleton, 2010).

Figure 5. Models of cancer propogation



(A) *In the cancer stem cell (CSC) model, a small population of infrequent tumorigenic cells (colored red, renewal of malignant potential indicated by circular arrow) generate more tumorigenic cells and also non-tumorigenic cells (non-red cells) in hierarchical pattern (indicated by double-head arrows).* ***(B)*** *In the clonal evolution model, many phenotypically distinct cancer cells have malignant potential and some cells gain an advantage in disease-propagating ability by acquiring additional genetic mutations (indicated by jagged arrows).* ***(C)*** *In the interconversion model, although many cells have intrinsic malignant potential, cells can interconvert (indicated by two-way arrows) between actively malignant and relatively quiescent states which may be associated with phenotypic differences between cells. The large, central, two-way arrows depict the notion that these models are not mutually exclusive.*

(figure and its description adapted from Mark Shackleton; Normal stem cells and cancer stem cells: similar and different, Seminars in Cancer Biology)

In the CSC model, CSCs are minority of cells in a tumor having self renewal and indefinite proliferative potential thereby driving cancer growth; whereas rest of the population in a cancer represents non-tumorigenic cells with limited or no proliferative potential (Tan et al., 2006). Thus tumor consists of a heterogeneous population of cancer cells comprising of tumorigenic CSCs as well as non-

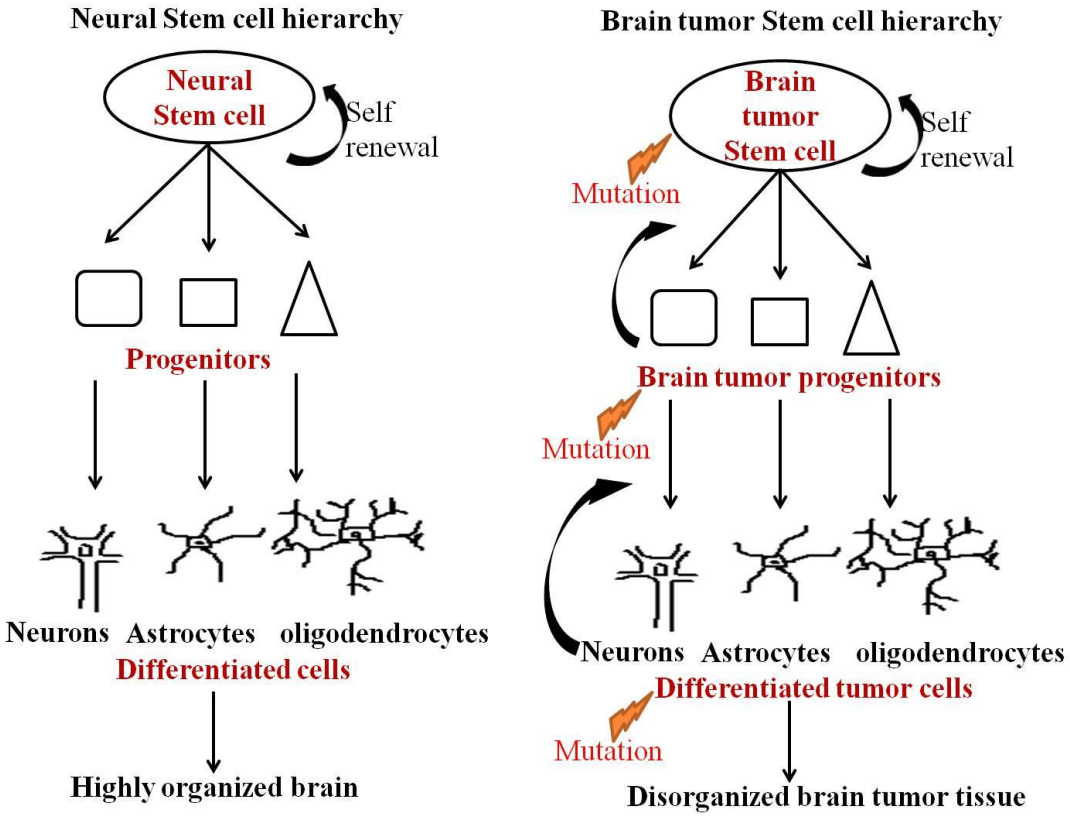
tumorigenic cells including transiently amplifying progenitor cells and differentiated cells, both carrying oncogenic mutations. CSCs can give rise to both CSCs (by self renewal) as well as non-tumorigenic cancer cells that constitute the bulk of tumor. In contrast to the CSCs that are bestowed with tumorigenic potential, non-tumorigenic cells lack self renewal potential and have either no or diminished capacity to form tumors. There is evidence that in cancers, a minority cell population with cancer-initiating ability is responsible for the genesis and growth of the tumor. The signalling pathways associated with normal stem cell development and that of CSCs are same, whereas these pathways are highly deregulated in CSCs in contrary to normal stem cells (Reya et al., 2001).

The stem cell theory of carcinogenesis was put forward since 1997, after the identification of CSCs in hematopoietic system (Bonnet and Dick, 1997). The seminal finding for the existence of CSCs came from studies in hematopoietic malignancy, acute myeloid leukemia (AML); among the cell population leukemia stem cells were characterised as $CD34^{+}CD38^{-}Thy\text{-}1^{-}$ cells at the same time $CD34^{+}CD38^{+}$ cells were found to be non-tumorigenic (Bonnet and Dick, 1997). The first direct evidence for CSCs in solid tumors came from studies in breast cancer and was identified as $CD44^{+}CD24^{-/low}Lineage^{-}$ cells (Al-Hajj et al., 2003). Thereafter the idea of CSCs has been applied to all tumors such as prostate, colon, pancreas etc. This led to the extension of the idea of CSCs to tumors of the brain, similar to hematopoietic and breast cancers; only a few atypical cells within the cancerous mass might be responsible for the growth and recurrence of some brain tumors (Zaidi et al., 2009).

II.1.4. Cell of origin of glioma

Neural stem cells (NSCs) are the cells endowed with proliferative capacity in brain hence they are highly sensitive to transformation than other non-proliferative or cells with low proportion of proliferation. GSCs have the characteristics shared with NSCs such as self-renewal, enhanced proliferation, multilineage differentiation and ability to generate tumors recapitulating the original parental tumor (Figure 6) (Dirks, 2008; Kukekov et al., 1999; Zaidi et al., 2009). The similarity in the characteristics between GSCs and NSCs has led to the notion that GSCs would have formed from NSCs that have undergone tumorigenic mutations or else from transiently amplifying progenitor cells or by dedifferentiation of terminally differentiated cells that had a series of mutations acquiring self-renewal and other stem cell properties (Hambardzumyan et al., 2008; Martin-Villalba et al., 2008; Walker and Kaye, 2001). The greater frequency of tumor formation in germinal regions of brain when compared to other non proliferating areas upon administration of carcinogens or exposure to oncogenic viruses has led to the notion that NSCs are most likely candidate for transformation. In addition, the transformed cells undergo migration thus the place of glioma origin and the site where tumor eventually develops might be distinct (Dirks, 2008).

Figure 6. Hierarchical organization of neural stem cell and brain tumor stem cell



In a brain, normal NSCs undergo highly regulated divisions to generate themselves through self-renewal and also the progenitor cells which then gets differentiate into lineage cells. On the other hand, due to accumulated mutations the BTSCs with malignant potential also undergo self-renewal to generate themselves and also proliferate to produce progenitor population in a highly uncontrolled and aberrant fashion.

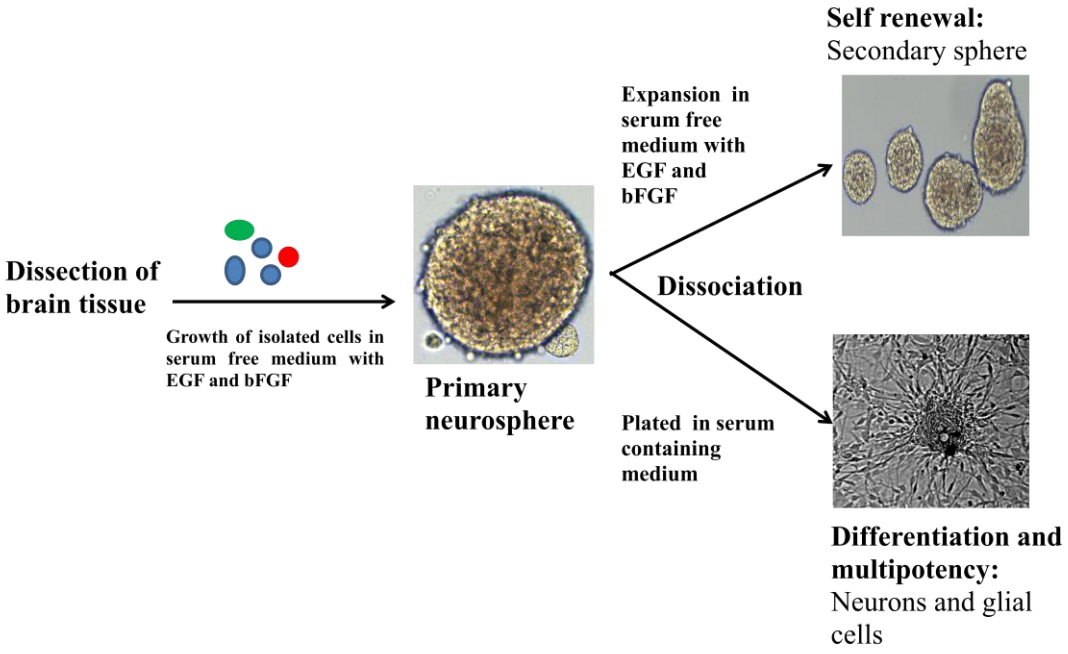
Reynolds and Weiss (1992) came with the initial evidence that NSCs can be grown as spherical colonies termed neurospheres in a serum free supplemented medium with mitogens and the assay is termed as neurosphere assay and became the method for identification of NSCs. They isolated NSCs from the SVZ to perform neurosphere assay and were found to express nestin, a cytoplasmic intermediate filament protein that identifies primitive undifferentiated neural precursor cells.

Using the same, Ignatova et al. has shown that cells isolated from GBM also has the ability to grow as spheres and Galli et al. established that neurospheres isolated from GBM has the capacity to differentiate into different lineages of brain tissue (Galli et al., 2004; Vescovi et al., 2006). Uchida et al. (2000) has found out that human NSCs can be isolated using CD133 marker. Singh et al. has identified that CD133 can be used as the marker for in vitro characterization and identification of Brain tumor stem cells (BTSC) (Singh et al., 2003). GSCs can be grown in vitro under serum free culture conditions in minimal/defined medium in the presence of supplements and pleiotropic growth factors such as EGF and bFGF as floating cluster of cells called gliomaspheres/tumorspheres (Singh et al., 2003). Glioma tumors following differentiation has found to produce multiple lineages of cells such as glia and neurons as well as upon orthotopic transplantation they have the ability to recapitulate the original parent tumor with heterogeneous population of cells thereby providing further evidence that cells with stem cell properties are the source of transformation. These experimental findings clearly supports the potential of the neurogenetically active NSCs to be a target for oncogenic transformation but warrants further studies to confirm the exact cellular origin of glioma.

Neurosphere assay is the commonly used method for the isolation, identification and propagation of NSCs (Figure 7). Neurosphere is a spherical cluster that comprises a heterogeneous population of thousands of cells and among them only 50-100 are stem cells along with various progenitors and differentiated cells. The limiting dilution assay or clonogenic assay can be used to determine the number of stem cells in a neurosphere. The neurospheres can be extensively propagated as they form

secondary spheres following dissociation and replating of cells, which allows measuring its self renewal potential. Neurospheres are multipotent and when plated in a serum containing medium, they differentiate into glial and neuronal cells (Tropepe et al., 1999).

Figure 7. Neurosphere assay of neural stem cells



Neurosphere assay is an in vitro method to study neural stem as well as progenitor cells. Isolated cells from brain when grown in a stem cell promoting serum free medium supplemented with growth factors generate floating cluster of cells called neurospheres. Glioma can also be similarly cultured in neurosphere conditions to generate gliomaspheres. The neurospheres / gliomaspheres can be plated in serum containing medium to generate differentiated cells.

Neurospheres has found to harbour a fraction of cells that express CD133 surface marker protein. CD133+ cells were initially used for the enrichment of both NSCs as well as BTSCs from dissociated brain tissue (Tamaki et al., 2002). CD133, a 120 kDa cell surface protein was initially identified as the putative stem cell marker, but

plethora of recent observations suggests not all CD133+ population have stem cell properties, in addition even CD133- cells have shown to possess stem cell characteristics. NSCs as well as GSCs are heterogeneous and has found to express a variety of markers mainly CD133, CD15, nestin, an array of other proteins associated with neural precursors such as Sox2, musashi, Notch, jagged1 and BMI1 and also expresses different lineage specific markers (Colin et al., 2006; Dirks, 2008). There is no definitive marker that differentiates NSC from BTSC and progenitor population.

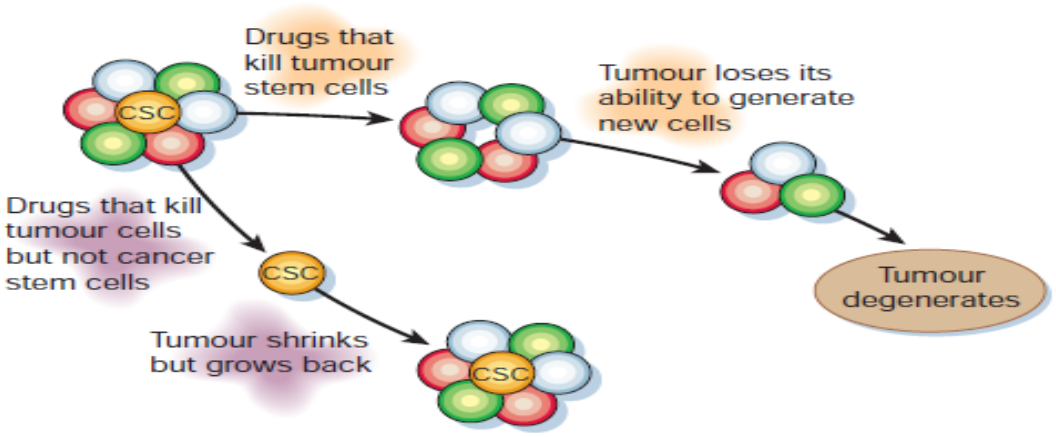
II.1.5. Implications of glioma stem cells

GSCs have shown to have chemoresistance and radioresistance that helps to survive therapies (Bao et al., 2006). These GSCs have found to arrest cell cycle and become quiescent thereby escaping radiotherapy and other treatments (Mellor et al., 2005). Most of the conventional therapies that targets the proliferating cells will spare the GSC and contribute towards the relapse. The conventional therapy using radiotherapy and chemotherapy has found to target the whole tumor which targets the proliferating population that forms the bulk of the tumor and sparing the GSCs which is present as a small population and are usually quiescent thereby contributing to tumor recurrence following therapy (Figure 8) (Brescia et al., 2012; Lefranc et al., 2005).

In a model proposed by Berger et al., the major factor that contributes for the resistance of gliomas to therapies is the separate locations for the tumor mass and the site of its origin which is explained by the asymmetric division of NSCs or BTSCs producing a stem cell that reside in the neurogenetic area whereas the progenitor cell

with mutation will migrate to a distant site where they undergo numerous cell divisions or migrate specifically towards the tumor mass (Berger et al., 2004).

Figure 8. Implications of conventional and cancer stem cells specific therapy



Conventional therapies may shrink tumours by killing mainly cells with limited proliferative potential. If the putative cancer stem cells are less sensitive to these therapies, then they will remain viable after therapy and re-establish the tumour. By contrast, if therapies can be targeted against cancer stem cells, then they might more effectively kill the cancer stem cells, rendering the tumours unable to maintain themselves or grow.

(figure and its description adapted from Tannishtha Reya et al.; Stem cells, cancer, and cancer stem cells, Nature)

II.2. Autophagy: Molecular mechanism and its role in tumor cells

Autophagy (Greek, self eating) is a process in which macromolecules such as long lived proteins and damaged organelles (cargo) are sequestered inside a double membrane autophagosome (that is formed by the expansion of isolation membrane called phagophore) which then fuses with the lysosome forming single membrane autophagolysosome thereby degrading the cargo using lysosomal enzymes acting at low pH. Autophagy occurs at basal level in all cells to perform physiological

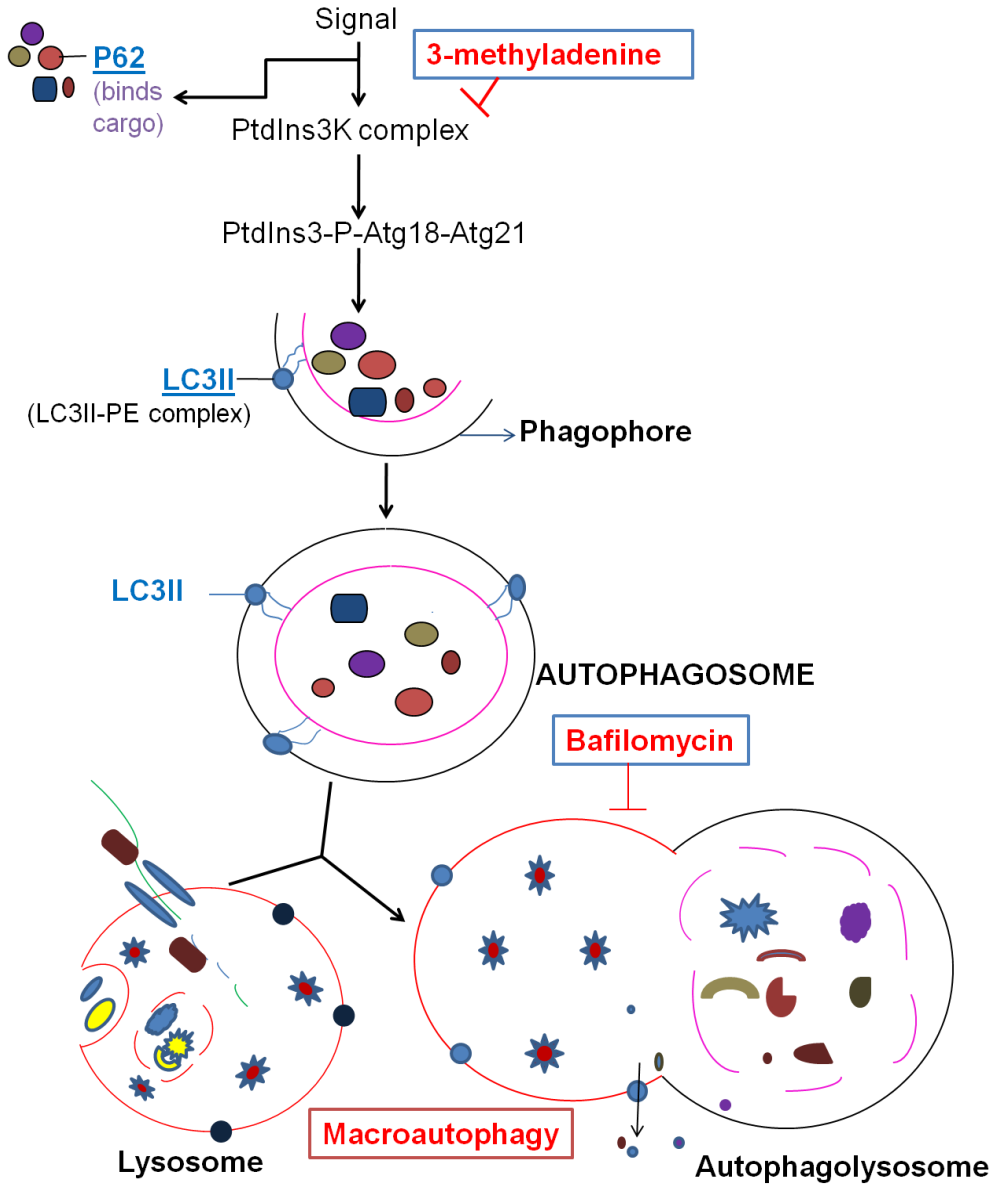
functions such as turnover of organelle and nutrients, whereas it gets enhanced during starvation or other physiological developmental processes. Autophagy was initially described as a survival mechanism during stresses such as mitochondrial damage, protein aggregation, pathogens and nutrient deprivation. During nutrient limitation, the autophagy degrades the macromolecules and other organelles thereby providing substrates and energy for the maintenance of homeostasis. Autophagy has also found to play another role, cell death. The mechanism of autophagy to play a dual role by performing seemingly opposite functions: pro-survival and pro-death remains elusive (Kang and Avery, 2008; Kang et al., 2007). Levine and colleagues has shown that the role of autophagy whether survival or death is decided by the level of autophagy i.e. physiological autophagy promotes survival and insufficient or excess autophagy leads to cell death (Levine and Yuan, 2005).

II.2.1. Process of autophagy

Autophagy detection is critical to understand its role and the molecular markers that are mainly used include: p62/sequestosome (SQSTM1) protein – which tags the cargo that has to be degraded by autophagy and LC3 protein – it exists in two isoforms, LC3-I and LC3-II (LC3B). LC3 is a very good marker for autophagy and is initially synthesized as pre-form which gets converted to LC3-I by the Atg4B mediated cleavage of 14 aminoacids at the N-terminal exposing the glycine residue which in turn forms LC3-II by the addition of phospholipid phosphatidylethanolamine (PE) gets integrated in the inner and outer membrane of autophagosome (Asanuma et al., 2003). During autophagy LC3-II in the outer membrane of autolysosome gets delipidated by Atg4B and gets converted to LC3-I

in the cytosol, whereas the LC3-II in the inner membrane gets degraded along with other contents. The LC3-II formation is associated with autophagosome formation and in turn autophagy (Ni et al., 2011). p62 is another marker can also be used along with LC3-II for autophagy detection,. P62 interacts with LC3-II and gets degraded inside autophagolysosome, thus decrease in p62 is an induction of autophagy, and has the autophagy gets suppressed there will be accumulation of p62 indication it is a marker of autophagy suppression (Figure 9). But p62 can't be used alone as an autophagy marker as the p62 is associated with conditions other than autophagy (Mizushima and Yoshimori, 2007).

Figure 9. Schematic model demonstrating the molecular mechanisms of autophagy



Autophagy involves the formation of phagophore and it expands to form autophagosome which in turn fuses with lysosome to form autophagolysosome and thereby degrading the contents. Bafilomycin is the most commonly used autophagosome degradation inhibitor that was used to study autophagic turn over, and helps in detecting whether autophagy is defective or a complete autophagic flux is present.

II.2.2. Methods to characterize autophagy

One of the main problems associated with autophagy study is the absence of robust and efficient methods to detect autophagy and was studied by analysis of autophagic vacuoles, LC3-II and p62 degradation or accumulation. Electron microscopic analysis of autophagolysosomes and autophagosomes is a gold standard in the assessment of autophagy, but it requires expertise and is susceptible to observer bias. The acridine orange staining is not specific to autohagy. LC3-II and p62 are markers of autophagy that can be analysed using techniques such as western blot, immunofluorescence, live cell imaging using GFP tagged LC3-II or p62 and also pH sensitive GFP-mCherry double tagged LC3B or p62 (Bjorkoy et al., 2009). The detection of autophagy doesn't mean just showing an increase or decrease in autophagy markers, because the decrease in LC3-II and p62 might be due to the over activity of autophagy leading to enhanced degradation whereas its increase may be due to the suppression of degradation. This indicates that it is essential to analyze autphagy in the presence and absence of inhibitors which gives the lysosomal turnover of LC3-II rather than measuring absolute autophagy as typically done (Barth et al., 2010). It is essential to do a autophagic flux analysis after treatment with autophagosome-lysosome fusion inhibitors such as bafilomycin or lysosomal enzyme inhibitors such as pepstatin and E64D to get the exact picture (Tanida and Waguri, 2010). Bafilomycin is the widely used autophagic inhibitor that stalls autophagy at the pre-lysosomal stage, which prevents degradation of contents and thereby accumulating LC3 and other autophagy substrates. Bafilomycin is a V-ATPase inhibitor and exerts its action by increasing the pH of the organelle

containing V-ATPase such as endosomes and lysosomes. The increase in pH has found to inhibit the autophagy because the lysosomal enzymes won't be active in higher pH. There are reports that the inhibition of organelle acidification has found to disturb the autophagosome-lysosome fusion and prevents autophagy. It has also reported that the internal acidification of organelle is essential for vacuolar transport, maturation of endosome and its fusion with lysosome (Kawai et al., 2007).

Autophagy regulation is a complex process and one of the most studied signalling pathways is phosphatidyl inositol 3-kinase (PI3-K) and its downstream effector molecule mammalian target of rapamycin (mTOR) (Fan et al., 2006). mTOR is a nutrient sensor that regulates autopahgy in response to various upstream signals such as nutrients, growth factors, energy and cytotoxicity which turns on autophagy during nutrient depletion and shuts off on nutrient abundance. mTOR when activated has found to phosphorylate S6-Kinase ribosomal protein and 4E binding protein (4EBP1) thereby promoting protein synthesis and cell growth. At the same time, mTOR suppresses protein turnover by inhibiting autophagy (Nyfeler et al., 2012). mTOR has found to play an important role in regulation of autophagy (Kamada et al., 2010). One of the most common hallmark aberration associated with cancers is the alteration in PI3K/Akt/mTOR pathway. The activation of mTOR pathway results in the suppression of autophagy, thus inhibition of phosphorylation of p70s6kinase downstream effectors of mTOR can be used to determine whether autophagy is activated or not (Jiang et al., 2009).

Autophagy has found to play a role in both physiological and pathological conditions. Under physiologic condition, autophagy plays an important role in

maintenance of cellular homeostasis and developmental process (Lefranc and Kiss, 2006). Autophagy has found to be associated with a lot of pathological conditions such as cancer, neurodegenerative disorders such as parkinsons disease, alzhiemers and huntingtons disease (Ventruti and Cuervo, 2007). In the cancer scenario, autophagy has found to play a dual role both pro-survival and pro-death.

II.2.3. Role of autophagy in glioma

Gliomas are resistant to apoptosis, the type I programmed cell death used by most of the chemotherapeutic drugs to kill the cancer cells; as an alternative type II programmed cell death autophagy has been used for treatment of glioma cells (Furnari et al., 2007; Lefranc and Kiss, 2006). Glioma has therefore been treated using pro-autophagic drugs. Pro-autophagic drugs including rapamycin (mTOR inhibitor), arsenic trioxide, ceramide, temozolomide (TMZ), dopamine, endostatin , histone deacetylase (HDAC) inhibitors butyrate and suberoylanilide hydroxamic acid, neodymium oxide, the chemotherapeutic vitamin D analogue EB1089, saponins and resveratrol have been reported to induce autophagy *in vitro* and *in vivo* in cancer cells (Kanzawa et al., 2004; Lefranc et al., 2007). The role of autophagy in glioma, whether pro-survival or cell death remains uncertain and is a topic of intense debate. Different studies have reported the contrasting role of autophagy as an inducer of cell death and also as a survival mechanism in glioma. There are reports which showed that inhibition of autophagy sensitized glioma to therapies. Autophagy by recycling the molecules to produce macromolecules and ATP act as a survival mechanism during stress conditions in response to chemotherapy and radiation. In contrast, however, Stupp et al has reported that treatment of glioma patients with radiotherapy

and adjuvant chemotherapy using TMZ has increased the survival of patients (Stupp et al., 2002; Stupp et al., 2005). GSCs are resistant to pro-autophagic drugs; as the autophagy related proteins were found to be down regulated, which might contribute for the increased resistance and recurrence of tumors following therapy. Even after giving pro-autophagic drugs 60-75% of patients derive no benefit from the treatment.

II.3. Treatment strategies for glioma

The standard treatment for glioma includes surgical resection of the tumor followed by either radiotherapy alone or plus concomitant and adjuvant chemotherapy. The new targeted therapies include immunotherapies, anti-angiogenic therapy with bevacizumab or anti-vascular endothelial growth factor (anti-VEGF) antibodies, targeted toxins and receptor tyrosine kinase inhibitors. Despite various multimodality therapies, overall survival rate of glioma patients has not improved and there is no definitive cure or treatment for preventing the progression and recurrence of this tumor (Stupp et al., 2010). Even after multimodal therapies, the median survival of a patient affected with grade IV glioma, GBM is approximately 12 months.

II.3.1. Limitations of current therapies in glioma

Glioma is a highly infiltrative and aggressive malignant tumor with and because of its diffuse nature, even after multimodal treatment strategies including surgical resection followed by radiotherapy and chemotherapy the median survival of patients has not increased in an apparent level. The key feature that contributes towards aggressiveness of glioma includes excessive proliferation, high invasiveness and suppression of immune surveillance. Glioma has a variety of signalling pathways activated that may contribute to the enhanced survival and proliferation of tumor.

Phosphatide inositide 3-kinase (PI3-K) is a signalling pathway that plays an important role in cell survival, proliferation and growth. The activated PI3-K/Akt/mTOR pathway is a hallmark of high grade gliomas. These protein kinases have shown to play an important role in glioma spread and growth. The studies have shown that inhibitors of kinases play an important role in curbing cancer growth. But the problem with this approach is that a PI3-kinase itself has 15 related kinases in their family. Thus a single inhibitor won't be able to inhibit cancer cell growth and there is need of inhibitors that affect multiple steps. A broad spectrum protein kinase has shown to have inevitable toxicity and thereby producing other effects (Fan and Weiss, 2010). The other factors attributed for the tumor therapy resistance include increased expression of transporters in the membrane that effluxes drugs, ability to evade apoptosis, enhanced expression of growth factors and DNA repair activity. The membrane transporters that efflux drug has found to be highly expressed in glioma tumors thereby contributing to the therapy resistance (Schmalz et al., 2011). mTOR is the most important molecule involved in autophagy regulation and its inhibitor rapamycin or derivative RAD001 (Everolimus) has been shown to radiosensitize the cancer cells due to its anti-angiogenic effect (Doherty et al., 2006). But the clinical trials with the mTOR inhibitors did not give telling results (Cloughesy et al., 2008).

The growing body of literature indicates that the glioma growth, propagation and oncogenic potential are maintained by a small sub-population of cells GSCs. One of the main reasons for the resistance of glioma cells to therapies is the presence of a small population of cells called GSCs responsible for the maintenance, propagation

and recurrence of tumor. GSCs are well spared by conventional therapies that target the whole tumor due to some idiosyncratic properties such as ability to efflux drug, altered expression of anti-apoptotic proteins and DNA repair proteins such as MGMT thereby contributing to the relapse of tumor (Gilbert and Ross, 2009). Transforming growth factor β (TGF β) has found to maintain the growth and proliferation of GSCs and inhibit its differentiation. TGF β has also found to influence the pro-invasive factors such as matrix metalloproteinases (MMPs), integrin α5 and β3 (Zhang et al., 2011).

II.4. Rationale of the study

Gliomas are the most common primary brain tumors with high morbidity and mortality despite multimodality therapies. An important advancement in glioma with therapeutic implication is the identification of cells with stem cell properties called as GSCs which are responsible for the initiation, propagation, recurrence and underlying therapeutic resistance of this tumor (Galli et al., 2004). GSCs are an elite subset of cells in a tumor mass which have the ability to proliferate indefinitely and differentiate into other cell lineages. GSCs are originally characterized and identified by their ability to form loosely attached spherical cluster of cells known as gliomasphere *in vitro* under the influence of defined stem cell promoting medium and also have the ability to serially recapitulate the original tumour *in vivo* (Singh et al., 2003). Thus gliomasphere formation is a specifying characteristic of GSCs (Reynolds et al., 1992). Gliomaspheres have the ability to self-renew and form different cell lineages such as neuronal cells, glial cells and oligodendrocytes upon differentiation in a serum containing medium. Neurosphere assays are most

commonly used to study GSCs from primary brain tumors. There are reports that neurosphere formation is a predictor of glioma tumor progression and clinical outcome independent of grade. To date, different studies have indicated the presence of GSCs in glioma thus, it is critical for glioma therapy that treatments must target and eliminate GSCs. The clinical relevance of GSCs has not been fully elucidated and therefore, the study of GSCs is extremely pertinent for the development of novel therapies. Although, the presence of stem cells has been described in glioma, but an extensive study on all the four grades of glioma has not yet been reported. This study was designed to assess the gliomasphere forming ability among different grades of glioma, both low and high grade.

Growing body of literature suggests in addition to the existence of GSCs, the deadly nature/therapeurtic insensitivity of gliomas can be attributed to its inherent resistance to apoptosis (type I programmed cell death) and are attempted to overcome by activating autophagy using pro-autophagic drugs (Hambardzumyan et al., 2008; Lefranc et al., 2005). Recent studies suggest that autophagy plays an important role in the regulation and response of glioma cells to treatment (Lefranc and Kiss, 2006). Despite treatment with pro-autophagic drugs, the overall survival rate of glioma patients remains dismal. However, the precise role of autophagy in glioma is still under debate even though, it is the main response of glioma to radiation and chemotherapy. For the effectiveness of pro-autophagic drugs, glioma should have a non-defective autophagy. However, the traditional approach of assaying autophagy by analyzing the autophagosomal increase in cells could not detect such an autophagic defect, if exists. The analysis of basal autophagic status by performing

autophagic flux assay using inhibitors that block autophagy at the specific stages will gives an idea of the real autophagic activity. Although, the induction of autophgy after treatment with certain drugs have been described in glioma, but the basal autophagic status of different grades of glioma has not been yet reported. In our study, we examined the basal autophagic status of different grades of glioma by performing an autophagic flux analysis using Bafilomycin A1. The autophagic status of the primary cultured glioma cells isolated from the resected human tissues and at the tissue level was analysed using its molecular markers such as LC3-II and p62. To our knowledge, this is the first study focusing on the rate of autophagy in primary cells isolated from glioma tissues.

This work employs previously established gliomasphere models to determine the presence of GSCs and flux analysis to determine autophagy is non-defective. Focus will be on gliomasphere forming ability among different grades of glioma and its basal autophagic status. Additionally, any previous and current work on gliomaspheres has been restricted to high grade gliomas mainly GBM, suggesting that extensive study including low grade gliomas have yet to be explored. Furthermore, the analysis of basal autophagic status is also a very new area not much studied.

II.5. Broad objective of the study

Against this backdrop, this study aims at analyzing the gliomasphere forming ability and autophagic status among different grades of glioma.

- Assessing the gliomasphere formation ability among low grade gliomas (LGG) and high grade gliomas (HGG)

- To evaluate the sphere forming capacity in relation to long-term proliferation and self-renewal within different grades of glioma.

- To characterize gliomasphere forming cells and diffrentiated glioma cells with known markers of stemness and lineage and their capacity for multipotency.

- Relationship between gliomasphere formation and progression free survival (PFS) in low- and high-grade glioma

- Characterization of autophagy by assessing its markers (LC3 and beclin 1) in LGG and HGG

- Flux assay to determine autophagy is defective or not in *ex vivo* culture of primary glioma cells

- To analyze whether autophagy is capable of induction in glioma and its role during starvation.

- Correlating autophagy proteins (LC3 and beclin 1) expression with PFS in low and high-grade glioma

III. MATERIALS AND METHODS

III.1. Materials

III.1.1. Chemicals and reagents

Hanks balanced salt solution (HBSS), papain, Dulbecco's modified eagle's medium (DMEM/F-12), L-glutamine, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), bovine serum albumin (BSA), calcium chloride, disodium hydrogen phosphate, magnesium chloride, potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate, sodium chloride, sodium dihydrogen phosphate, sodium hydroxide, sodium fluoride, sodium orthovandate, isopropanol, hydrochloric acid (HCl), ethanol, glycerol, skim milk, Tris HCl, EDTA, triton X 100, sodium dodecyl sulphate (SDS), sodium deoxycholate, trizma base, glycine, sodium acetate, acrylamide, bis-acrylamide, β-mercaptoethanol, TEMED, APS, ponceau S, acridine orange, Bafilomycin A1 A1, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3-methyladenine (3-MA), trypan blue stain, LC3 II antibody and β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the routine chemicals used for making buffers or used in the experiments were also purchased from Sigma-Aldrich unless otherwise specified. Expose mouse and rabbit specific HRP/DAB detection IHC kit, CD133, nestin, β-tubulin III, GFAP, beclin 1 and p62 antibodieswere from Abcam (Cambridge, MA, USA). CD15 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).Cell lysis buffer for protein extraction, phospho-p70 S6 kinase, phospho–4E-BP1 and secondary anti-rabbit and mouse IgG HRP linked antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Bradford reagent was from Bio-Rad laboratories (Hercules, USA). Chemiluminiscent reagents used for western blots, nitrocellulose membrane, Goat Anti-Rabbit IgG DyLight^{TM} 488 and were purchased from Thermo

Scientific (Rockford, IL, USA). DMEM/F-12, FBS, TrypLE, L-Glutamine and B27 supplement were obtained from Invitrogen (Carlsbad, CA).

III.1.2. Cell culture wares

T25 flask, 15 ml and 50 ml falcon tubes, 96 well microtitre plate, 24 well plate, 12 well plate, 6 well plate, cell scrapers, 35 mm, 60 mm and 100 mm cell culture treated dishes were from Becton Dickinson (USA). Cryovials and pipette tips were obtained from Axygen (CA). Cell culture filter ware was procured from Millipore (USA).

III.1.3. Equipments used

Weighing balance (Sartorius, Germany), water bath (Julabo, Germany), pH meter (Eutech, USA), centrifuge (Eppendorf, Germany), CO_2 incubator (Sanyo, Japan), laminar air flow (Micro Filt, India), ice flaker (Hoshizaki, Japan), ELISA reader (Bio-Tek Instruments, USA), electrophoresis unit (Bio-Rad laboratories, USA), mini-blot apparatus (Bio-Rad Laboratories, USA), semidry transblot apparatus (Bio-Rad), gel documentation system (Bio-Rad laboratories, USA), BD FACS Aria bench top flow cytometer (Becton and Dickinson, USA), Phase-contrast and fluorescent microscope (Olympus, Melville, NY), magnetic stirrer (Schott, Germany).

III.1.4. Media, buffers and reagents

III.1.4.1. Phosphate Buffered Saline (PBS)

137 mM sodium chloride, 2.7 mM potassium chloride, 10.14 mM disodium hydrogen phosphate, 1.76 mM potassium dihydrogen phosphate, pH 7.4.

III.1.4.2. Sample collection and washing buffer

Hanks Balanced Salt Solution (HBSS) without calcium (Ca^{2+}) and magnesium (Mg^{2+}) supplemented with antibiotics, 175 Units/ml penicillin and 1260 µg/ml streptomycin.

III.1.4.3. Stem cell culturing serum free medium (SFM)

Dulbecco's Modified Eagle Medium: F-12 medium (DMEM/F-12; Invitrogen) supplemented with L-Glutamine (2 mM; Invitrogen), basic fibroblast growth factor (bFGF; 20 ng/ml, Sigma), epidermal growth factor (EGF; 20 ng/ml, Sigma), B27 supplement (1X, Invitrogen) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin).

III.1.4.4. Differentiation promoting serum containing medium (SCM)

10 % Fetal Bovine Serum (FBS), 100 mg/L streptomycin and 100 mg/L penicillin in DMEM//F-12.

III.1.4.5. Tissue digestion solution

0.2% Papain (Sigma) in HBSS

III.1.4.6. TPVG solution

0.1 % trypsin, 0.2 % EDTA, 0.5 % glucose in PBS

III.1.4.7. MTT stock solution

5 mg/ml MTT in PBS

III.1.4.8. MTT working solution

1 mg/ml MTT in DMEM/F-12

III.1.4.9. Tris Buffered Saline (TBS) (10 X)

24.2 g Tris base, 80 g sodium chloride in 1L deionized water, pH 7.6

III.1.4.10. Tris buffered saline with Tween 20 (1 X TBST)

1 X TBS containing 0.5% Tween 20

III.1.4.11. Blocking and permeabilization solution for immunofluorescence

5% FBS and triton X 100 (0.2% for cytoplasmic proteins, 0.4% - nuclear, 0.1% - membrane) in 1 X PBS

III.1.4.12. Antibody dilution buffer for immunofluorescence

1% BSA in TBS

III.1.4.13. Formaldehyde solution for cell fixation

4% paraformaldehyde in PBS

III.1.4.14. Propidium iodide staining solution

Stock solution: 1 mg/ml Propidium iodide in 1 X PBS.

Working solution: 1 µg/ml stock in 1 X PBS

III.1.4.16. RIPA lysis buffer for protein extraction from tissue

50 mM Tris HCl (78.8 mg), 150 mM NaCl (87.66 mg), 1 mM EDTA (3.7224 mg), 1% triton X 100 (1 ml), 1% sodium deoxycholate (1 ml) in 10 ml deionized water

III.1.4.17. RIPA working solution

1 ml RIPA buffers supplemented with 10 µl 0.1% SDS, 10 µl 0.1 M freshly prepared PMSF and 142.9 µl 1 X protease inhibitor cocktail‘Complete, Mini’(Roche Applied Science, Indianapolis, IN).For extraction of phospho proteins, 1 µl 0.2 mM sodium orthovandate and 100 µl 50 mM sodium fluoride were added.

III.1.4.18. Acrylamide/Bis-acrylamide Solution (39:1)

39 % (w/v) acrylamide and 1% (w/v) N,N'-Methylene Bis-acrylamide in deionized water

III.1.4.19. Resolving gel buffer for SDS PAGE (8 X)

3 M Tris base and 0 .4 % SDS in deionized water pH 8.8

III.1.4.20. Stacking gel buffer for SDS PAGE (4 X)

5 M Tris Base and 0.2 % SDS in deionized water pH 6.8

III.1.4.21. Resolving gel for SDS PAGE (6%)

Acrylamide/Bis-acrylamide - 1.5 ml, Resolving gel buffer - 1.25 ml, deionized water - 7.25 ml, TEMED - 10 µl, 0.02 % APS - 20 µl

III.1.4.22. Resolving gel for SDS PAGE (10%)

Acrylamide/Bis-acrylamide - 2.5 ml, Resolving gel buffer - 1.25 ml, deionized water - 6.25 ml, Tetramethylethylenediamine (TEMED) - 10 µl, 0.02% Ammonium persulfate (APS) -20 µl

III.1.4.23. Resolving gel for SDS PAGE (15%)

Acrylamide / Bis-acrylamide - 3.75 ml, Resolving gel buffer - 1.25 ml, Deionized water - 5 ml, TEMED - 10 µl, 0.02% APS - 20 µl

III.1.4.24. Stacking gel for SDS PAGE (5%)

Acrylamide/Bis-acrylamide solution - 0.625 ml, Stalking gel buffer – 1.25 ml, deionizedwater- 3.75 ml, TEMED – 10 µl, 0.02 % APS - 20 µl

III.1.4.25. Gel loading dye for western blotting

2% (W/v) SDS, 0.03 % Bromophenol blue, 0.3 % β - mercaptoethanol, 10 % glycerol (v/v) in 0.067 M Tris, pH 6.8

III.1.4.26. TBST for antibody dilution

3 % BSA in TBST

III.1.4.27. Running buffer for SDS PAGE (10X)

25 mM Tris Base, 192 mM glycine and 1% SDS in deionized water

III.1.4.28. Running buffer for SDS PAGE (1X)

100 ml 10X running buffer in 900 ml of deionized water

III.1.4.29. Blotting buffer for SDS PAGE (10X)

0.25 M Tris Base and 1.92 M glycine in deionized water

III.1.4.30. Towbin's buffer (Transfer buffer)

100 ml 10 X transfer buffer, 200 ml methanol made up to 1L with deionized water

III.1.4.31. Blocking solution for western blotting

5 % of skim milk in TBST containing 0.5% tween-20

III.2. Methods

III.2.1.Patient samples and Clinical information

Human glioma tissues were collected from patients undergoing surgical tumor resection at the Department of Neurosurgery, Sree Chitra Tirunal Institute for Medical Sciences and Technology as approved by the Institutional Ethics Committee (IEC). Glioma specimens were histopathologically classified according to the world

health organization (WHO) classification by neuropathologist.Fresh samples were taken for isolation and primary culture of glioma cells, samples stored at -80°C for protein extraction from tissues and samples fixed with formaldehyde for immunohistochemistry. The tissue samples were obtained from 140 glioma patients and consisted of 67 low- (LGG) and 73 high- grade gliomas (HGG). LGG included grade I (10 pilocytic astrocytome) and grade II (16 astrocytoma, 27 oligoastrocytoma and 14 oligodendroglioma) whereas HGG consisted of grade III (5 anaplastic astrocytoma, 9 anaplastic oligoastrocytoma and 15 anaplastic oligodendroglioma) as well as grade IV (44 GBM) tumors. The gliomasphere formation ability was studied in all the glioma samples whereas autophagic status were assessed in 63 glioma tissues (9 grade I, 27 grade II, 9 grade III and 18 grade IV).

III.2.2. Isolation, culture and identification of cancer stem cells in human gliomas

III.2.2.1. Isolation and culture of primary glioma cells and spheres

Tumor tissues obtained within of surgical resection were washed with, minced to small fragments and subjected to enzymatic digestion with 0.2% papain. The dissociated tumor cells were washed, subsequently resuspended and cultured in stem cell promoting serum free medium (SFM) consisting of Dulbecco's Modified Eagle Medium: F-12 medium (DMEM/F-12) supplemented with L-Glutamine (2 mM), basic fibroblast growth factor (bFGF; 20 ng/ml), epidermal growth factor (EGF; 20 ng/ml), B27 supplement (1X) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) for enrichment of gliomaspheres from GSCs in or serum containing medium (SCM) consisting of DMEM/F-12 medium with 10% fetal bovine serum (FBS) for the growth of differentiated glioma cells. Cultures were maintained at

37°C containing 5% CO_2 and 95% air. The use of the term 'SFM' in this section represents DMEM/F-12 supplemented with L-Glutamine and growth factors whereas 'SCM' corresponds DMEM/F-12 with 10 % FBS unless otherwise stated.

III.2.2.1.1. Sub-culture of gliomaspheres and adherent monolayer culture cells

For passaging, the gliomaspheres were treated with TrypLE for 20 min at 37°C and seeded into culture plates containing SFM for gliomasphere culture or SCM for differentiation of gliomaspheres. For experiments with adherent monolayer culture, the culture supernatant was removed from the flask containing cells at 80% confluence and was trypsinized in TPVG solution for 2 - 5 min at 37°C. Detached cells were suspended in SCM and a known amount of cells were transferred to appropriate culture plates for further experiments.

III.2.2.1.2. Primary sphere formation assay

To determine the presence and frequency of stem cell population within different grades of human glioma primary sphere formation assay was performed. The primary sphere formation assay was performed on entire acutely dissociated tumor tissue to determine the stem cell frequency within the tumor. Tumor cells were plated in stem cell promoting SFM at a clonal density of 5 X 10^3 cells per cm^2 in 25 cm^2 tissue culture flasks. The cultures were fed every 2 days until day 27. The formation of tumor spheres was observed and the wells were scored for sphere formation and photographed under a phase contrast microscope on days 3, 7, 14, 21 and 27 post plating (Singh et al., 2003).

III.2.2.1.3. Sphere size determination

The tumor cells were seeded at 1.25 X 10^5 in T25 flask in a neurosphere promoting medium. Sphere diameters from10 visual fields were measured after 3, 7, 14, 21 and 27 days using ProgRes® CapturePro Software (Jenoptik). The average size of the spheres and standard deviation were calculated (Varghese et al., 2008)

III.2.2.2. Characterization of glioma derived cells *in culture*

III.2.2.2.1. Morphological characterization of glioma cells

The morphologies of SFM grown gliomaspheres and differentiated glioma cells were studied using an Olympus inverted microscope along with ProgRes® CapturePro Software (Jenoptik, Germany). The cells were studied at a fixed time period by analyzing the morphology as well as size and number of gliomaspheres after 3, 7, 14, 21 and 28 days.

III.2.2.2.2. Immunoflourescence staining

Immunostaining of gliomaspheres and differentiated glioma cells were performed to detect the expression of stem cell and lineage markers. To investigate the immunoreactivity, the cultured cells were fixed with ice cold 4% paraformaldehyde (Ref. III.1.4.13) for 15 min at 4°C and then were permeabilized and blocked with Triton X 100 and FBS (Ref. III.1.4.11). The cells were then incubated with antibodies against CD133 (rabbit polyclonal IgG; 1:50), CD15 (mouse monoclonal IgG; 1:50), nestin (mouse monoclonal IgG; 1:1000), β-tubulin III (rabbit polyclonal IgG; 1:200) and GFAP (rabbit polyclonal IgG; 1:1000) overnight at 4°C. After rinsing in PBS, for detecting primary antibody, cells were incubated for 1.5 h at room temperature in dark in the DyLight488 conjugated secondary antibody (goat

polyclonal IgG; 1:600). The cells were then counterstained with propidium iodide (PI, Ref. III.1.4.14) to reveal the nuclei. For imunostaining of differentiated tumor spheres after differentiation assay, the tumorspheres were cultured on SCM for seven days and immunofluorescence was performed.

.III.2.2.2.3. Secondary sphere formation assay

Sub-sphere formation assay was performed as described previously for analyzing the self-renewal potential of gliomaspheres. Gliomaspheres were harvested and dissociated into single cells using 2 ml TrypLE for 20 min at 37°C. For quantitative determination of the frequency of secondary spheres, cells were plated at a density of 5000cells/200 µl per well in a 96-well plate. Cultures were kept at 37°C, 5% CO_2. After seven days, the number of spheres formed was counted and was used to estimate the mean number of spheres per 5000 cells. This assay estimated secondary sphere formation in different grades of glioma (Singh et al., 2003).

III.2.2.2.4. Differentiation assay and sphere reforming assay of gliomaspheres

Gliomaspheres were grown in the absence of growth factors and in the presence of serum containing differentiation medium for seven days and cells were processed for immunocytochemistry. For aberrant differentiation assay, the spheres grown in differentiation promoting SCM for seven days was washed with HBSS and switched back to SFM. After seven days, immunofluorescence was performed to detect the expression of stem cell and differentiation markers (Yuan et al., 2004).

III.2.2.2.5. Flowcytometry

The neurospheres were dissociated using trypLE, fixed with ice cold 4% paraformaldehyde for 20 min at 4°C and blocked with 3% BSA containing PBS. The

cells were incubated with anti-CD133 (rabbit polyclonal IgG; 1:50) in 3% BSA containing PBS for overnight at 4°C. Negative controls were kept in 3% BSA containing PBS and incubated for overnight at 4°C. After washing with PBS, cells were incubated with DyLight488 conjugated secondary antibody (1 in 600 dilutions) for 1.5 h at room temperature. At least 10000 cells were assayed and the cells were passed through a mesh before analysis to remove the cellular clumps in it. BD FACS Aria beanchtop flow cytometer was used for the flow cytometry analysis.

III.2.2.3. Immunohistochemical staining of glioma sections using stem cell and lineage markers

The expression levels of stem cell and lineage markers in different grades of glioma tissue were analyzed by immunohistochemical staining. Tissues were fixed in 10% buffered formalin, embedded in paraffin, cut into 3-5 µm sections and mounted on positive charged microscope slides. Briefly, paraffin sections of tumors were then deparaffinized/dewaxed in xylene, hydrated through graded alcohols, treated with heat induced epitope retrieval technique using citrate buffer (pH 6.0) at 95°C for 5-20 min and allowed to gradually cool in the buffer. The tissue sections were taken out and washed with double distilled water, 3% H_2O_2 for 10 min was used to block intrinsic peroxidise activities. The slides were again washed in distilled water for 15 min and blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS, pH 7.6) for 10 min. The sections were immunohistochemically stained with the following primary antibodies against: CD133 (rabbit polyclonal IgG; 1:50), CD15 (mouse monoclonal IgG; 1:50), nestin (mouse monoclonal IgG; 1:100), β-tubulin III (rabbit polyclonal IgG; 1:250) and GFAP (rabbit polyclonal IgG; 1:5000) 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 was 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. 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.

III.2.3. Monitoring autophagic status among different grades of glioma

III.2.3.1. Immunohistochemical staining of glioma tissue sections using autophagy markers

Immunohistochemistry of autophagy markers was performed as described above in paraffin sections and stained using antibodies against LC3 (rabbit polyclonal IgG; 1:250), P62 (rabbit polyclonal IgG; 1:100) and beclin 1 (rabbit polyclonal IgG; 1:50). The primary antibodies were detected as described previously.

III.2.3.2. Western blot analysis for autophagy markers in different grades of human glioma

III.2.3.2.1. Protein sample preparation

Protein sample preparation for western blotting was done as per standard protocol. Western blotting was performed on different grades of glioma tissues and also on cell culture lysates. For extraction of protein from tissue, frozen brain slices were minced and pasted in a mortar and pestle after dipping in liquid nitrogen. Afterwards, the tissue powder were dispensed in RIPA lysis buffer using a total volume of 100 µl per 50 mg of tissue and incubated for 45 min in intermittent quick vortex at 5 or 10 min interval. The solubilised tissue was centrifuged at 12000 rpm for 15 min at 4°C and supernatant was aliquoted and stored at -80°C until use.

Protein extraction from cultured glioma cells was done using cell lysis buffer for protein extraction (CST). Briefly, glioma cells at 80% confluence were exposed to desired treatment before protein isolation. After treatment, cells were washed with PBS and lysed in cell lysis buffer followed by 30 min incubation at 4^{o} C. The lysate was then centrifuged for 30 min at 13000 rpm for removing the cell debris. Supernatant with the protein content was aliquoted and stored at – 80°C for further use. Proteins extracted from tissue and lysates were quantified using the Bradford reagent following standard protocols.

III.2.3.2.2. Protein quantification for western blotting

2.5 µl of protein sample to be quantified was diluted with 122.5 µl of deionized water and 50 µl of this mixture was added to a 96 well plate. Standards for protein estimation were prepared using BSA taken at a concentration of 1.5 mg/ml. 1, 2, 5 and 10 µl of 1.5 mg/ml BSA solution (which amounts to 1.5, 3, 7.5 and 15 µg protein) were diluted with 121.5, 120, 117.5, and 112.5 µl of deionized water respectively along with 2.5 µl of cell lysis buffer added to each of the diluted standard samples. The blank was prepared by diluting 2.5 µl of cell lysis buffer with 122.5 µl of deionized water. 50 µl of blank, diluted standard and protein samples were then transferred to the 96 well plates. 200 µl of Bradford reagent were added to 50 µl of blank, diluted standards and diluted protein samples and kept in dark for 10 min. Colour developed in the sample was quantified using a spectrophotometer at 570 nm. The OD values obtained for the protein standards (after negating with the blank values) were plotted against its concentration in a graph and the unknown concentration of the protein samples were calculated.

III.2.3.2.3. Fractionation and transfer of proteins to nitrocellulose membrane

For western blotting, the proteins were denatured by mixing with gel loading buffer at a 6:1 ratio and heated at 95ºC for 10 min. 100 µg of proteins was electrophoretically fractionated using 10% and 15% sodium dodecyl sulfate polyacrylamide gel (SDS PAGE) depending upon the protein to be detected. The proteins after loading in gel were run at125 V over 2 h or 3 h in 1 X Tris Glycine SDS (TGS) running buffer. After that, the proteins were transferred to a 0.45 micron nitrocellulose membrane by a semidry blotting technique at 15 V for 20 min or 20 V for 45 min with blotting buffer. The transfer of protein to the nitrocellulose membrane was checked by Ponceau S staining.

III.2.3.2.4. Development of protein bands

The nitrocellulose membrane after transfer were blocked in 5% skim milk containing blocking buffer (Ref. III.1.4.30) over 1 h at room temperature. The primary antibodies against primary antibodies LC3 (rabbit polyclonal IgG; 1: 10000), P62 (rabbit polyclonal IgG; 1:3000), Beclin 1 (rabbit polyclonal IgG; 1: 5000), phospho-p70 S6 kinase (rabbit polyclonal IgG; 1: 1000) and phospho-4E-BP1 (rabbit polyclonal IgG; 1: 1000) were diluted in 3% BSA containing TBST (Ref. III.1.4.26) and incubated overnight at 4°C. After washing using TBST (3 X 5 min) the nitrocellulose membrane with bound antibodies were labelled by applying a HRP linked secondary antibody (anti-rabbit polyclonal IgG; 1: 2000) for 1 h at room temperature. Following a washing step with 1 X TBST, the antigen-antibody complexes were detected using west femto chemiluminiscent detection kit and bands obtained were exposed to x-ray film. The membrane was then stripped by washing

with TBST, re-probed with β-actin antibody (mouse monoclonal; 1:5000) and was developed as described above.

III.2.3.3. Cell Staining with Acridine Orange for autophagy detection

Acridine orange is a supravital dye that stains cytoplasm green, nucleolus bright red and acidic compartments bright red. The increase in red fluorescence is proportional to the increase in the amount of acidic compartments. Autophagy is characterized with the formation of acidic vascular organelles. For autophagy detection, cell staining with Acridine orange was performed in primary cultured glioma cells from resected human tissue. Bafilomycin A1 (Sigma), an autophagic inhibitor dissolved in DMSO at a 10 nM concentration was added for a period of 4h. After treatment with indicated reagent, cells were stained with acridine orange at a final concentration of l μg/ml for 15 min. Cells were then washed with PBS to remove excess stain and observed under fluorescent microscope (Kanzawa et al., 2003; Klionsky et al., 2008a).

III.2.3.4. Autophagy flux analysis

Autophagic flux assay was done to analyze the autophagic status of glioma cells (2 grade I, 3 grade II, 3 grade III and 2 grade IV). It was performed in the presence of an inhibitor that prevents the fusion of autophagosoma and lysosome thereby preventing autophagic degradation, thenceforth assessing the turnover of autophagic proteins. Bafilomycin A1 is a proton pump (V^+ ATPase) inhibitor that prevents final stage of autophagy (autophagosome-lysosome fusion) and was dissolved in DMSO and added to the medium. To analyze the autophagic status, primary cultured glioma cells were treated with Bafilomycin A1 (10 nM) for a period of 4 h. Cells cultured in

10% DMEM/F-12 was taken as the control. After treatment with Bafilomycin A1, western blotting for autophagy markers such as LC3-II and p62 was performed on primary cultured glioma cells (Klionsky, 2005; Klionsky et al., 2012; Rubinsztein et al., 2009).

III.2.3.5. Glioma cell culture and aminoacid starvation treatment

To analyze whether autophagy is capable of induction in glioma starvation treatment was done. For experimental purpose, the cells plated in DMEM/F-12 with 10% FBS at 75–80% confluency were washed twice with HBSS and incubated in amino acid deprived medium Hanks balanced salt solution (HBSS, a starvation media andautophagy inducer) and western blotting for autophagy marker LC3-II has been performed (Sadasivan et al., 2006).

III.2.3.6. MTT–Cell Viability Assay

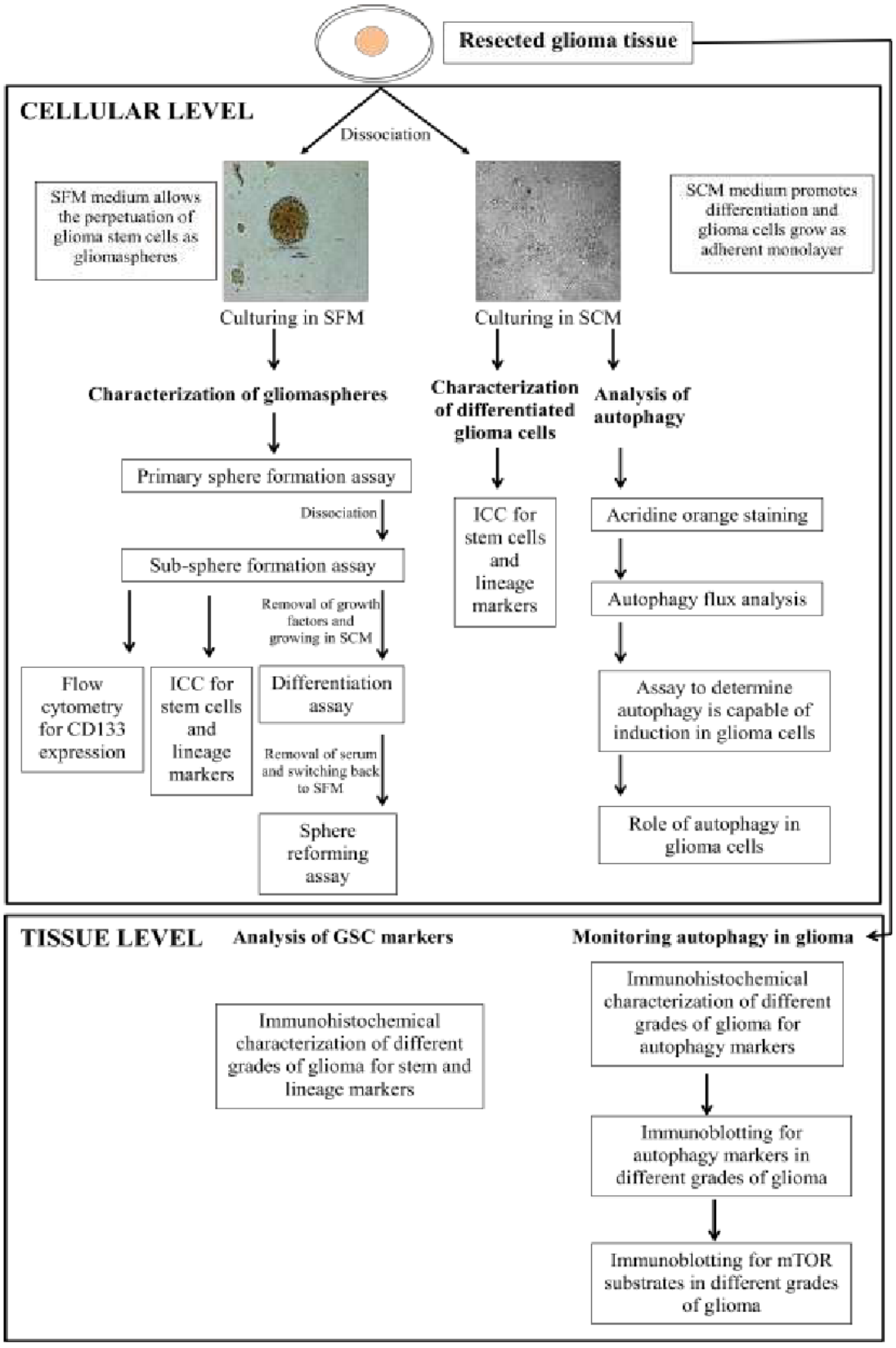
MTT assay was performed to determine the role of autophagy (whether cell death or survival) in glioma cells upon induction by starvation stimulus. For the study, primary cultured glioma cells were seeded in a 96-well microtitre plate (10,000 cells/well) and cultured in DMEM/F-12 medium with 10% FBS (10% DMEM/F-12) at 37°C in an incubator containing 5% CO_2 to attain 80% confluence. When the cells reached 80% confluence, they will be randomly divided into experiment group and control group. Control cells were re-suspended in 10% DMEM/F-12. Experiment group consisted of cells treated with 10% DMEM/F-12 along with Bafilomycin A1 (10 nM), 10% DMEM/F-12 with 3-methyladenine (3-MA, 10 mM), HBSS, HBSS with Bafilomycin A1 (10 nM) and HBSS with 3-MA (10 mM). After incubating the cells for 12 h at 37^0C, cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-

2,5,diphenyltetrazoliumbromide (MTT) assay. After treatment, the medium from each well is removed, 100 µl fresh medium with 20 µl of MTT solution dissolved in the culture media at a final concentration of 1mg/ml was added to each well and incubated till formazan crystals are formed (2.5 h). Acidified isopropanol was added to dissolve the formazan crystals and the optical density (OD) of solubilized formazan crystals was measured at 570 nm with 630 nm reference using a microtitre plate ELISA reader. The OD in control group was taken as 100% of viability (Sadasivan et al., 2006).

III.2.4. Statistical analyses

Statistical analyses were performed using GraphPad Prism 5. ANOVA with Bonferroni post-hoc tests and two tailed student's t-test was used for comparisons of protein expression results between LGG and HGG. 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 of statistical significance was set at $P < 0.05$. Cumulative incidence function (CIF) for competing risk analysis was done using R and statistical significance assessed using Gray's test.

Figure 10. Flowchart outlining design and critical experimental steps



IV. RESULTS

IV.1. Clinical Data

Human tumor specimens were acquired from patients undergoing surgery for different grades of glioma. A total of 140 samples were included in the study and it consisted of 67 low- (LGG) and 73 high- grade gliomas (HGG). LGG included grade I and grade II whereas HGG consisted of grade III as well as grade IV tumors. The gliomasphere formation ability was studied in all the glioma samples whereas autophagic status were assessed in 63 glioma tissues (grade I (9), grade II (27), grade III (9) and grade IV (18)). For detailed analysis of gliomaspheres in terms of marker expression and autophagic flux, we studied a sub group of eight and nine tumors respectively. Table 4 lists the patient characteristics.

Table 4. Summary of patient characteristics

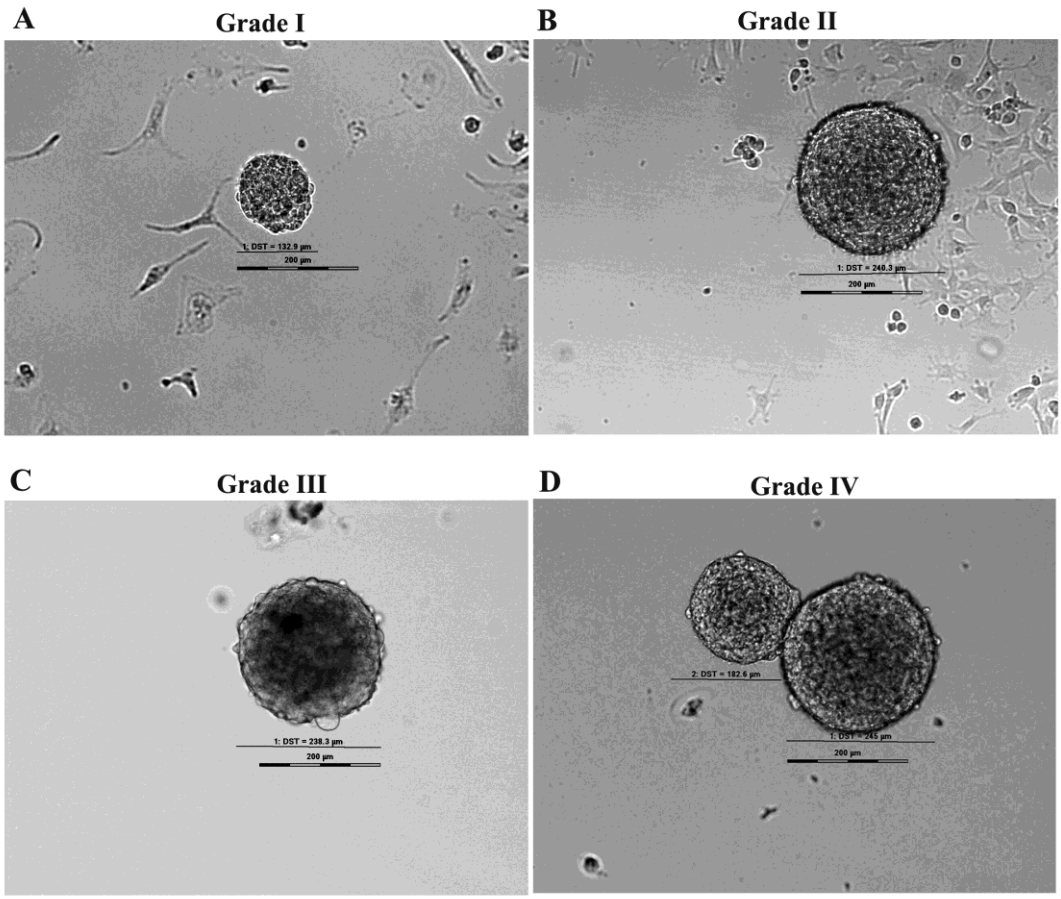
Histology criteria	**WHO Grade**	**Number of cases**	**Sex (M/F)**	**Age (Mean ± SD)**
LOW GRADE GLIOMA		**67**		
Pilocytic Astrocytoma	I	10	5/5	22.4 ± 14.3
Astrocytoma II	II	16	9/7	33.8 ± 11
Oligoastrocytoma II	II	27	18/9	39.2 ± 12.7
Oligodendroglioma II	II	14	8/6	40.7 ± 10.6
HIGH GRADE GLIOMA		**73**		
Astrocytoma III	III	5	2/3	44.7 ± 17
Oligoastrocytoma III	III	9	5/4	37.6 ± 16.3
Oligodendroglioma III	III	15	7/8	44.2 ± 10
GBM	IV	44	27/17	45.9 ± 14.6

Abbreviations: M – male, F – female, Y – years

IV.2. Gliomas of different grades has the ability to form neurospheres

It has previously been shown that primary brain tumours, including glioma possess a GSC population (Singh et al., 2003) and gliomasphere formation corresponds to the frequency of stem cell population within the tumor. To assess for the presence of glioma stem cells (GSCs) within different grades of glioma, we established cultures from 140 gliomas including grade I (10), grade II (57), grade III (28) and grade IV (44) tumors. The tissues were acutely dissociated into single cells and plated in a low attachment plate at clonal density of 5 X 10^3 cells/cm^2 in culture conditions that favored stem cell growth (neurosphere medium). Within 48-72 h after plating, phase-bright clones of spherical cellular clusters called gliomaspheres were formed from all grades of glioma irrespective of pathological subtype. Large numbers of small spheres of approximately 3-10 cells in diameter were observed between 3 and 7 days after plating and within 14 days, spheres had increased their diameters approximately 50 to 100 cells (Figure 11).

Figure 11. Phase-contrast microphotographs showing examples of gliomasphere forming ability among different grades of glioma.



(A) Pilocytic astrocytoma (B) Astrocytoma II (C) Anaplastic astrocytoma and (D) GBM. (100X); Scale bar = 200 µm. Glioma cells isolated from all the four grades when grown in SFM supplemented with EGF and bFGF have the ability to form gliomaspheres.

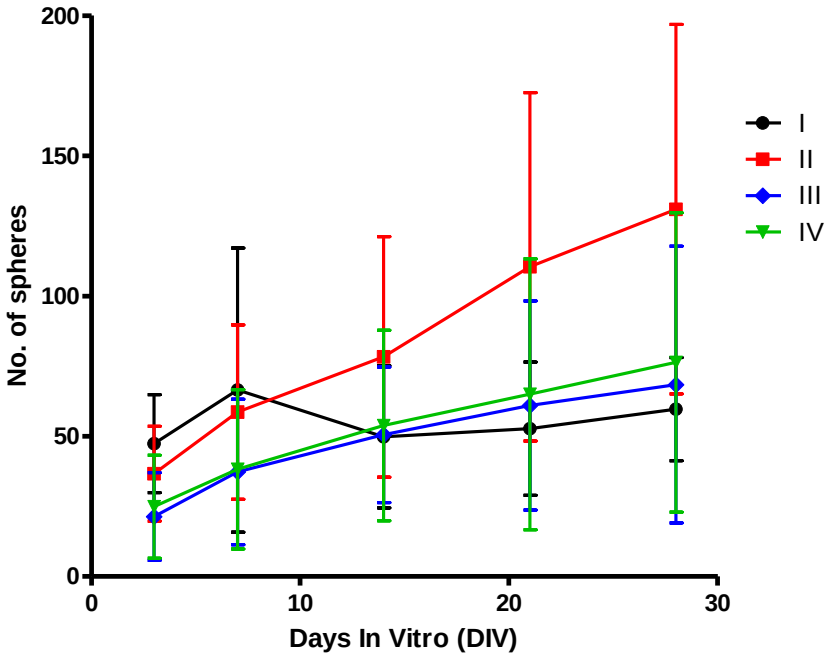
IV.2.1. Grade II gliomas were found to have greater primary sphere forming capacity compared with other grades of glioma

Primary sphere formation analysis was performed on to quantify the frequency of stem cell population within the tumor. The generation of neurospheres from different grades of glioma provides an indication of the clonogenicity of tumors. Tumor samples from 140 patients with glioma, including a subpopulation of 67 patients with low grade glioma (10 grade I and 57 grade II) and 72 with high grade glioma (29 grade III and 44 grade IV) were cultured in neurosphere conditions (Table 5). For primary sphere formation analysis, the cells from acutely dissociated tumor tissue was plated at a density of 5 X 10^3 cells/cm^2 on to a 25-cm^2 tissue culture flask with serum free medium (SFM) containing growth factors and assayed for their ability to form neurospheres after 48-72 h. The number of gliomaspheres formed was scored on days 3, 7, 14, 21 and 28, post plating. Possible associations between gliomasphere formation and grades of tumor were analyzed (Figure 12).

Table 5. Neurosphere formation rate among different grades of glioma

Days in vitro (DIV)	**Number of neurospheres**			
	Grade I (n=10)	**Grade II (n=57)**	**Grade III (n=28)**	**Grade IV (n=44)**
3	47.4	36.7	21.4	24.9
7	66.5	58.7	37.3	38.2
14	49.8	78.3	50.5	53.9
21	52.7	110.4	61	65
28	59.7	131	68.5	76.3

Figure 12. Graph showing the primary sphere formation rate of different grades of glioma



Comparison of primary sphere forming capacity between I, II, III and IV grade gliomas revealed that grade II gliomas has a higher probability of gliomasphere formation (Table 5). The high grade gliomas, grade III and IV did not match up to the sphere forming capacity of grade II, whereas grade I glioma had the lowest (Table 6). Association among gliomasphere formation and grades of the glioma were examined using Spearman's rho correlation analysis. When different grades of glial tumors were compared to sphere forming capacity, spearman's correlation analysis (correlation coefficient = -0.254, $P = 0.000$) resulted in a significant negative correlation. The negative correlation is because of the increased capability of grade II gliomas in generating gliomaspheres.

Table 6. Primary sphere formation ability

Glioma tumor type	Means (no: of spheres) ± SEM	Number of samples
Grdae I	59.7 ± 5.8	10
Grade II	131 ± 8.7	57
Grade III	68.5 ± 9.2	29
Grade IV	76.3 ± 8.1	44

The number of spheres generated at day 28 were 59.7 ± 5.8 for grade I, n = 10; 131 ± 8.7 for grade II, n = 57; 68.5 ± 9.2 for grade III, n = 29; 76.3 ± 8.1 for grade IV, n = 44 (means± SEM per 1.25 X 10^5 cells).

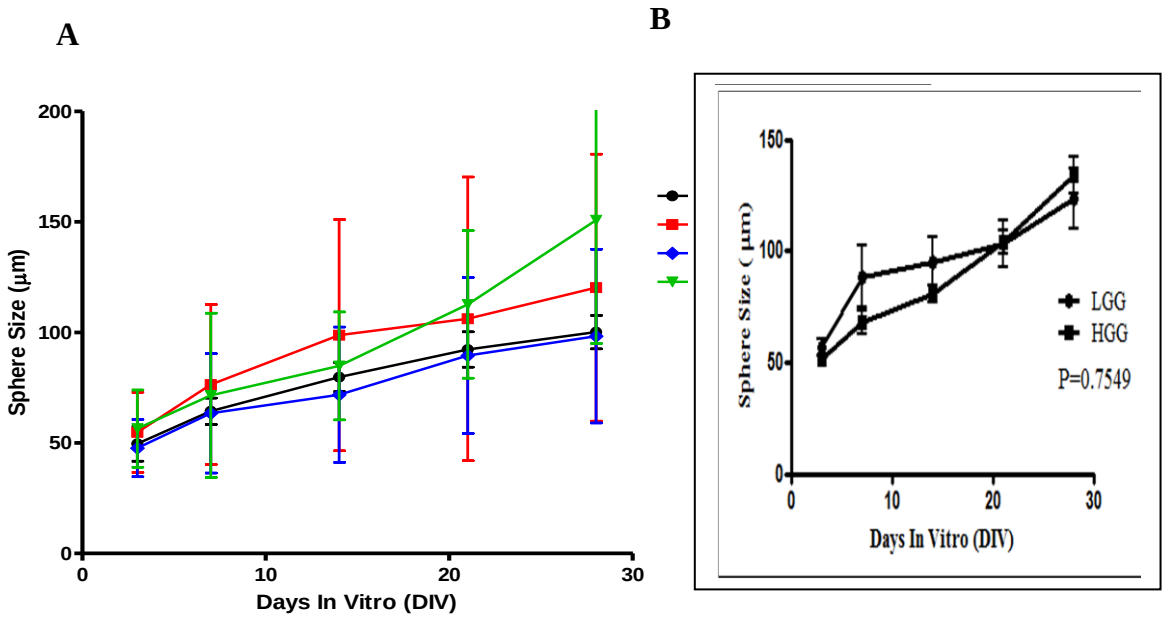
IV.2.2. Gliomasphere growth rate shows no significant difference between grades of glioma

To obtain an estimate of the growth rate of spheres among different grades of glioma, the sphere diameter was measured. It was found to increase linearly. The size of gliomaspheres was measured on day 3, 7, 14, 21 and 28 (Table 7) after plating was found to increase linearily (Figure 13a-b). Even though the size of gliomaspheres was increasing, but when compared between grades there exists no significant correlation. When compared between HGG and LGG also there existed no significant correlation (P = 0.7549) between the size and the grades, the size of neurospheres was found to increase linearly.

Table 7. Comparison of sphere size among different grades of glioma

Days in vitro (DIV)	Sphere diameter (µm)			
	Grade I (n=10)	Grade II (n=57)	Grade III (n=28)	Grade IV (n=44)
3	49.6	54.8	47.7	56.5
7	64.3	76.4	63.4	71.5
14	79.7	98.8	71.8	84.9
21	92.3	106.1	89.6	112.7
28	100.1	120.2	98.3	150.8

Figure 13. Graph showing the growth rate of different grades of glioma

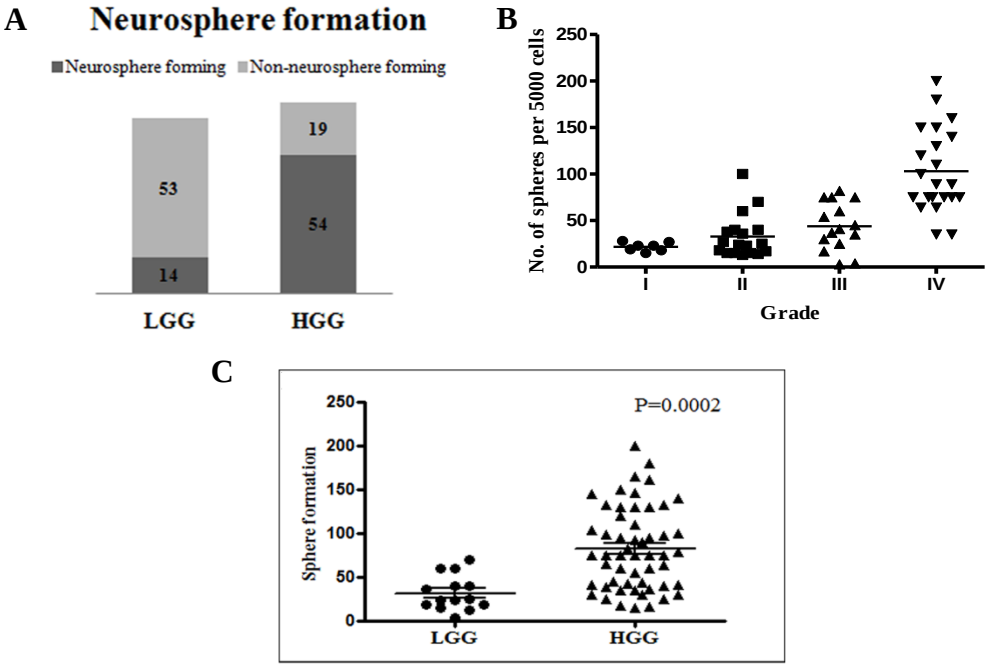


Association among sphere size and grades of the glioma were also examined, there was no significant correlation between the grade of glial tumors and sphere size. Furthermore, when we examined gliomasphere formation as a function of age and sex, there was no significant correlation.

IV.2.3. Glioma of different grades contain cells with increased potential for secondary sphere formation

Tumor tissue samples that formed gliomaspheres for a minimum of three passages in culture were categorized as having the potential of renewable gliomasphere formation. Numbers of gliomasphere forming and non-neurosphere forming tumors were calculated in both LGGs and HGGs. Among the 140 lesions, 67 samples (49%) formed neurosphere in vitro. Twenty-one percent LGGs (20%—grade I and 21%—II) and 74% of HGGs formed gliomaspheres; in the HGG group, 77% of grade III and 72% of GBM formed gliomaspheres. The number of HGG samples forming gliomaspheres was higher compared to the number of LGG samples that formed gliomaspheres ($P < 0.0001$, Figure 14A). To evaluate the sphere forming capacity in relation to long term proliferation and self-renewal within the LGG and HGG, a sub sphere formation rate assay was performed. After seven days of plating, (which involved dissociation of spheres and culturing at a density of 5000 cells/well) counting the number of neurospheres was performed. All the four grades of glioma have shown the capacity to form secondary tumor spheres, suggesting that gliomaspheres contain stem like cells with the ability to self-renew. However, the capacity for secondary sphere formation was much higher for grade IV glioma than the other grades of (I, II and III) glioma (Figure 14B and Table 8). When grade I, II and III was compared with that of grade IV there was a significant difference in secondary sphere formation with a $p<0.05$, but no statistically significant difference was seen when compared between grade I, II and III. The number of gliomaspheres formed was much higher for HGGs than LGGs ($P = 0.0002$) further confirming the presence of enhanced stem cell compartment in HGGs (Figure 14C).

Figure 14. Secondary sphere formation of glioma



A. Gliomasphere formation in 140 different grades of glioma patients. First bar stand for LGG, and last bar represents renewable gliomasphere formation by HGG. Renewable gliomasphere forming tumors are represented in dark bars and non-sphere forming tumors in light bars. The number of tumors is represented in each bar. B. Secondary sphere formation of different grades of glioma was plotted and grade IV has the highest gliomasphere forming ability. C. Number of gliomaspheres formed was much higher for HGGs than LGGs. Each dot is representative of one patient sample; bar represents mean of biological replicates.

Table 8. Secondary sphere formation capacity of gliomaspheres

Glioma tumor type	Means (no: of spheres/5000 cells) ± SEM	Number of samples
Grade I	29.9 ± 2.5	2
Grade II	30.2 ± 2.4	12
Grade III	59.9 ± 10	23
Grade IV	122.7 ± 11.1	31

The number of secondary spheres generated were compared among different grades of glioma (I, II, III and IV) and among them grade IV has shown the highest secondary sphere generation/self-renewal capacity.

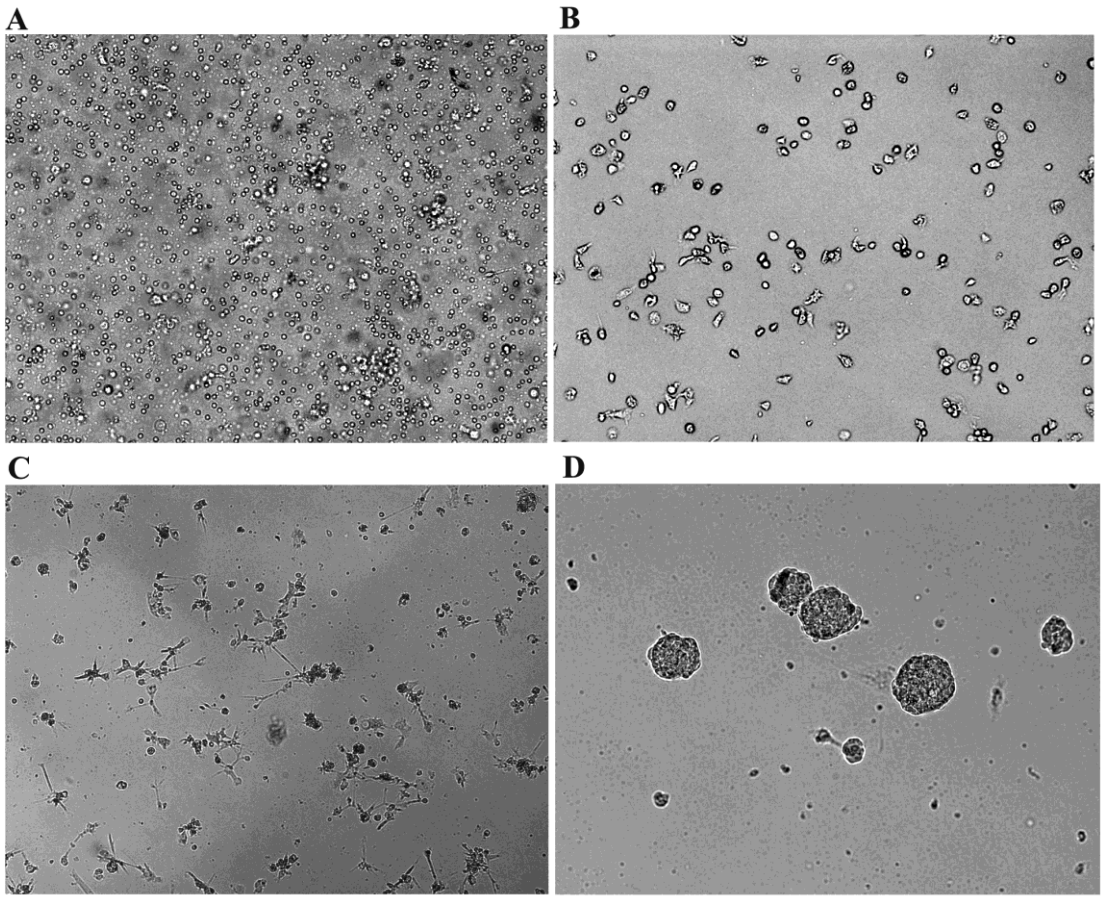
IV.3. Characterization of gliomaspheres and differentiated glioma cells isolated from different grades of glioma

Morphological analysis and immunocytochemical staining was performed for characterization of gliomaspheres and differentiated glioma cells from adherent monolayer culture. Morphological analysis was done an all the samples (140 gliomas) collected, whereas immunocytochemical analysis using stem cell and progenitor markers was done on eight (1 grade I, 3 grade II (including astrocytoma II, oligoastrocytoma II and oligodendroglioma II), 3 grade III (including astrocytoma III, oligoastrocytoma III and oligodendroglioma III) and 1 grade IV) glioma cultures.

IV.3.1. Morphological analysis

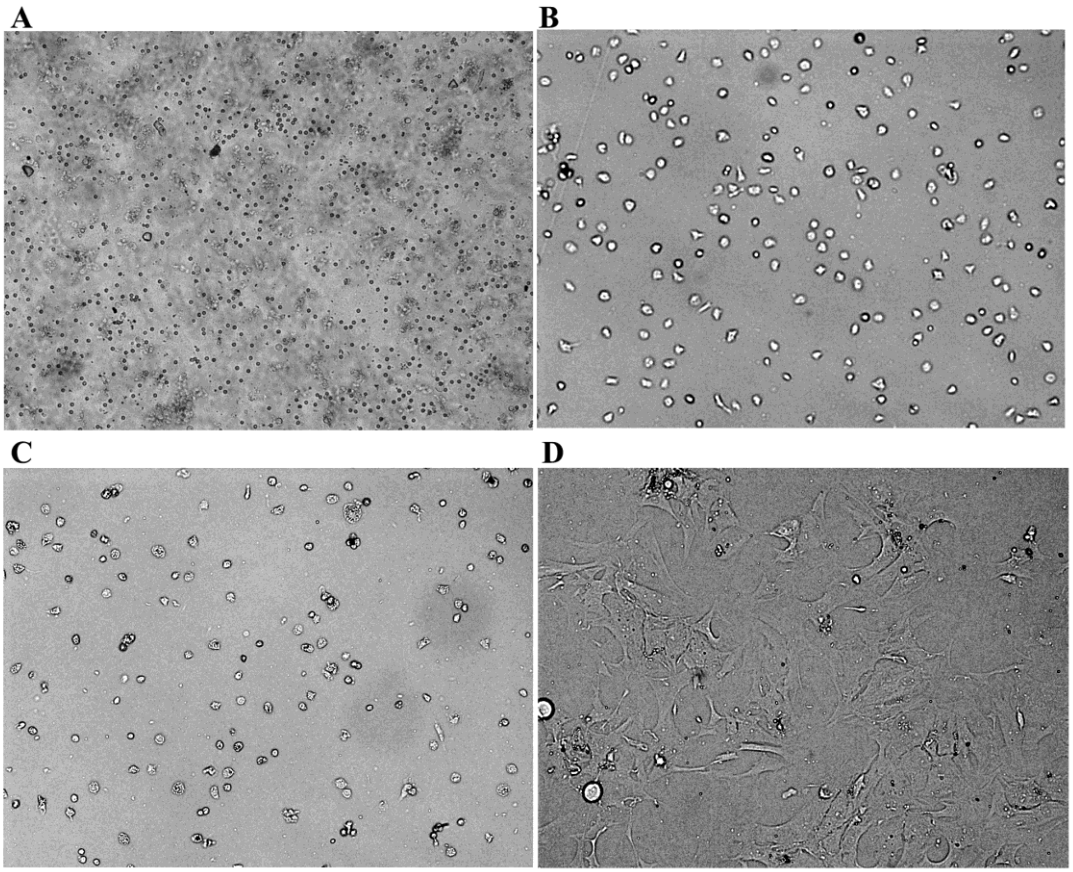
Glioma tissue was dissociated into single cells and cultivated in a defined stem cell promoting serum free medium (SFM) to enrich for gliomaspheres from GSCs or serum containing medium (SCM) for the growth of differentiated glioma cells. SFM provides a stringent system that allows for the maintenance, proliferation and exponential expansion of undifferentiated stem cells, concurrently selects away differentiated cells that form the majority of tumor cells. The sphere formation corresponds to the frequency of stem cell population. In SFM, cells from all the four grades of glioma formed spherical clusters of non-adherent free floating phase-bright clones called neurospheres or gliomaspheres (Figure 15). The gliomaspheres formed are of two types: with well defined spherical shape or with uneven irregular shape. Under serum containing culture conditions, glioma cells were found to grow as adherent monolayer (Figure 16).

Figure 15. Phase-contrast microphotographs showing glioma derived cells cultured under neural stem cell conditions at different time periods.



(A) Day 0 (B) Day 1 (C) Day 2 and (D) Day 3; Gliomaspheres (100X). Glioma cells, isolated as described under 'methods' and were grown in stem cell promoting SFM consisting of DMEM/F-12 with growth factors and supplements to form gliomaspheres.

Figure 16. Phase-bright micrographs of glioma derived cells cultured in SCM at different time points.



(A) Day 0 (B) Day 1 (C) Day 2 and (D) Day 3 at confluence (100X). Tumor cells isolated as described in 'methods' were grown in DMEM/F-12 medium containing 10% FBS for adherent monolayer primary glioma cell cultivation.

IV.3.2. Immunocytochemical staining

To characterize the undifferentiated gliomaspheres and differentiated cells from adherent monolayer culture immunocytochemistry was performed (1 grade I, 3 grade II, 3 grade III and 1 grade IV glioma) with a panel of antibodies: CD133, CD15, nestin, β-tubulin III (lineage marker for neurons) and GFAP (for glial cells). CD133 and CD15 are cell surface markers associated with NSCs and progenitors (Patru et al., 2010; Zeppernick et al., 2008). Nestin is a cytoplasmic intermediate filament protein that is universally accepted as the NSC/progenitor marker (Osterberg and Roussa, 2009). GFAP and β-tubulin III are seen in the cytoplasm of cells (Colin et al., 2006). Gliomaspheres generated from all the four grades of glioma exhibited immunoreactivity for CD133, CD15 and nestin. Furthermore, some cells within gliomaspheres were also found to be positive for lineage markers GFAP and β-tubulin III (Figure 17). $CD133^+$ and $CD15^+$ cells were relatively small populations, 1.88% ± 2.43 and 1.89% ± 1.03, respectively; nestin on the other hand, selected for an average of 32.16% ± 4.45 of cells. When looking at the same populations, with lineage markers, only 4.04% ± 0.64 and 0.36% ± 0.05 of cells were positive for GFAP and β-tubulin III respectively. It was visualized using Zeiss LSM 510 meta confocal microscope at 10x magnification under the settings of 543 for PI and 488 for FITC.

However, cells in non-sphere forming monolayer cultures grown in SCM stained positive for the lineage markers of CNS; GFAP (65.6 % ± 12.93) and β-tubulin III (58.65% ± 4.43), and had minimal, weak expression of NSC and progenitor markers such as CD133 (0.36% ± 0.06), CD15 (0.81% ± 0.45) and nestin (25.77% ± 1.84)

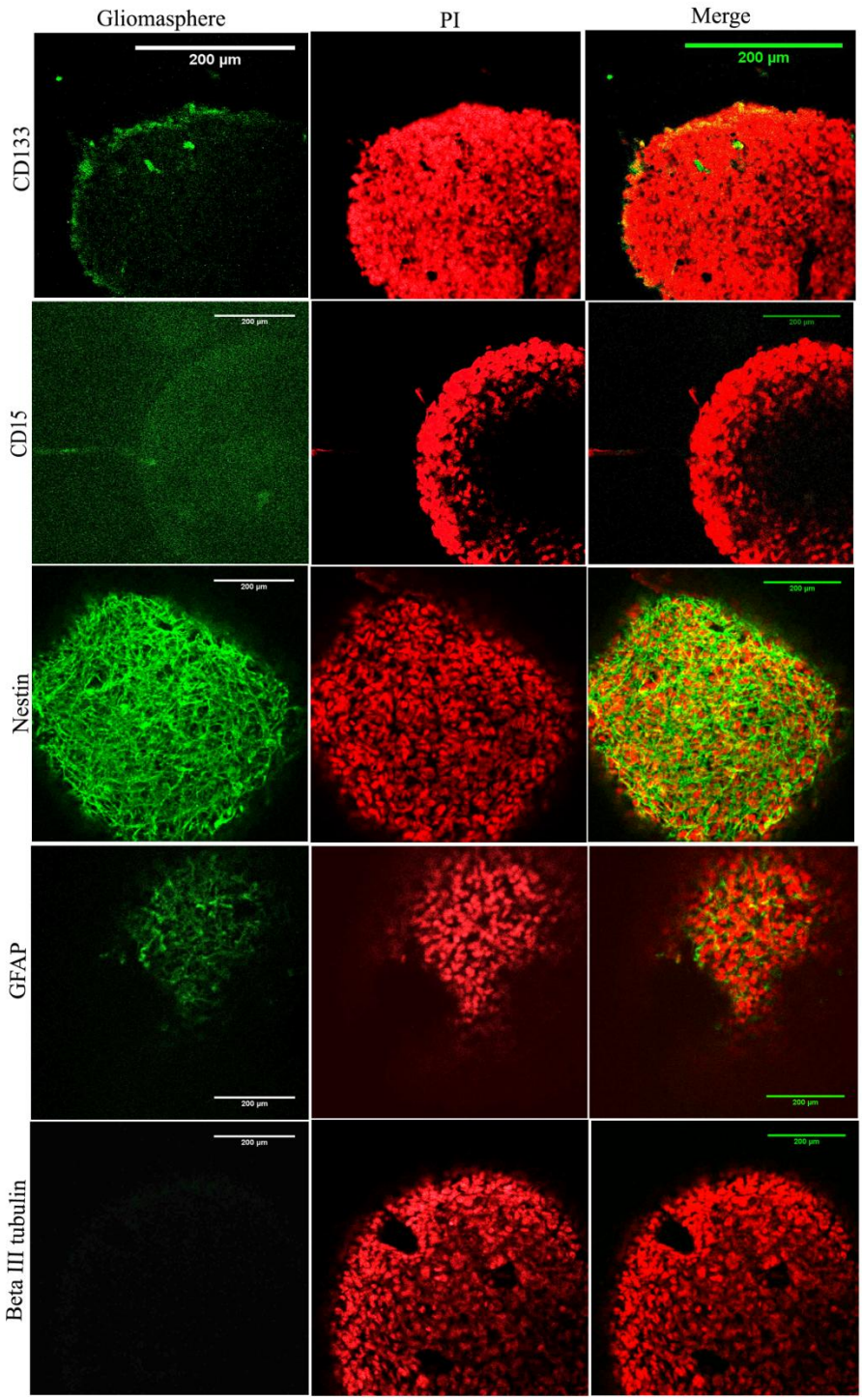
(Figure 18). It was visualized using an Olympus fluorescence microscope and at 10x magnification. The images were quantified using Image J software (Table 9).

Table 9. Quantitative analysis of gliomaspheres and differentiated cells grown as adherent monolayer using neural stem cell and lineage marker

	% Immunostained cells	
	Gliomaspheres	**Differentiated cells from monolayer culture**
CD133	1.88 ± 2.43%	0.36 ± 0.06%
CD15	1.89 ± 1.03%	0.81 ± 0.45%
Nestin	32.16 ± 4.45%	25.77 ± 1.84%
GFAP	4.04 ± 0.64%	65.6 ± 12.93%
Beta III tubulin	0.36 ± 0.05%	58.65 ± 4.43%

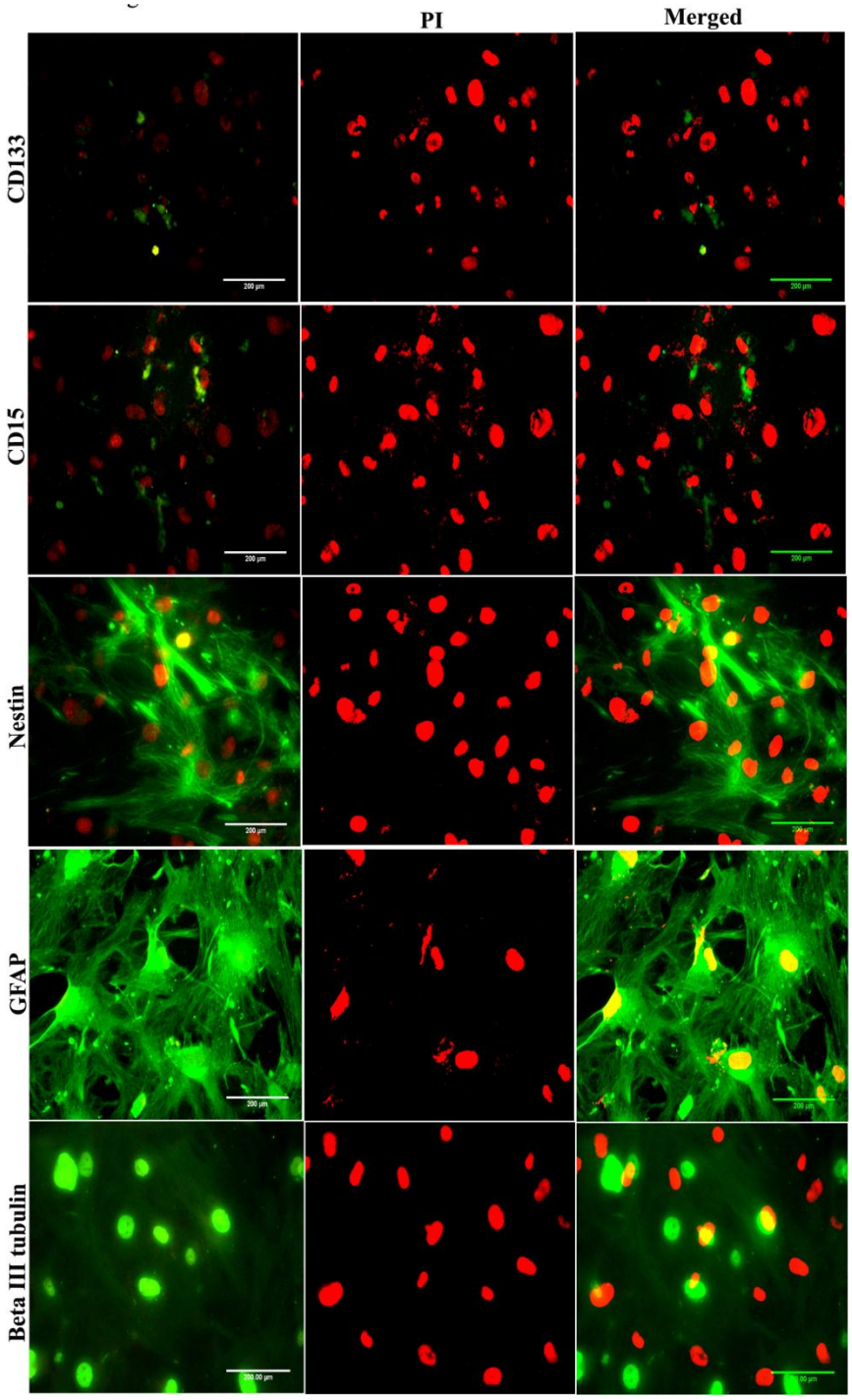
The table shows the quantitative analysis of the presence of neural stem cells and lineage markers in gliomaspheres and differentiated glioma cells grown as adherent monolayers.

Figure 17. Fluorescence immunocytochemistry of gliomaspheres showed expression of NSC as well as lineage markers



Gliomaspheres were stained by NSC markers CD133, CD15 and nestin as well as lineage markers for glial cells, GFAP and neuron β-tubulin III. Nuclei were counterstained with propidium iodide (PI).

Figure 18. Expression of stem cell and lineage markers in differentiated monolayer cultured glioma cells

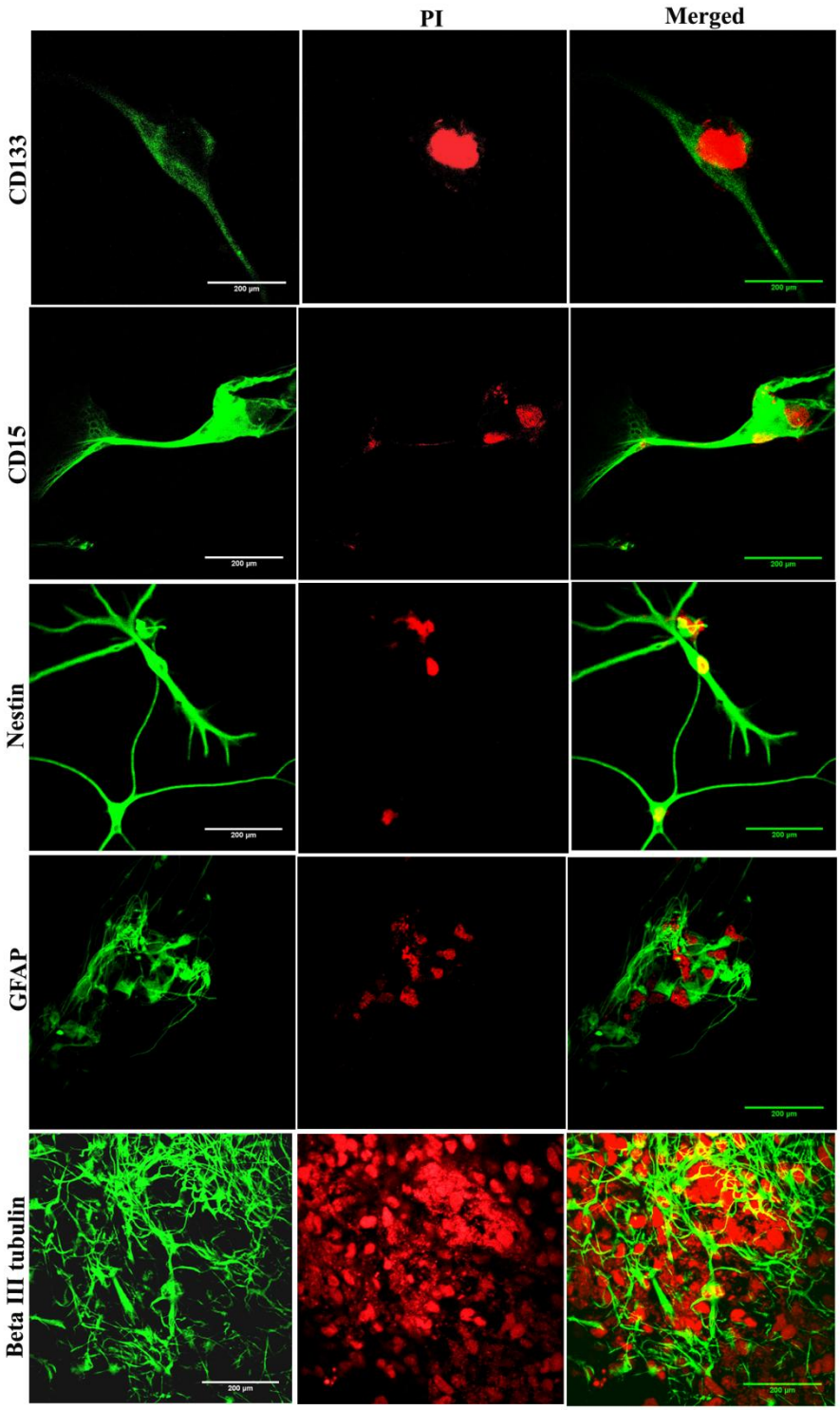


Adherent monolayer culture of differentiated glioma cells grown in DMEM/F-12 containing 10% FBS were incubated with CD133, CD15, nestin, GFAP and β-tubulin III antibody followed by staining with DyLight 488 conjugated secondary antibody. Nuclei were counterstained using propidium iodide.

IV.3.3. Gliomaspheres are multipotent

We next assessed whether tumor derived gliomaspheres maintained the multilineage differentiative capacity of NSCs by examining the types of molecular markers expressed by neurospheres grown under differentiating conditions. To test the multipotent ability, the spheres were subjected to a differentiation assay and thereafter fluorescence immunocytochemistry was performed (grade I (1), grade II (3), grade III (3) and grade IV (1)). Gliomaspheres like NSCs has the capacity to differentiate in serum containing medium without growth factors. After growing in SCM, the floating spheres adhered to the medium and cells migrated from spheres centrifugally. Gliomaspheres grown in 10% FBS containing medium for seven days were stained for stem cell (CD133, CD15 and nestin) and lineage markers (GFAP for astrocytes and β-tubulin III for neurons). Immunofluorescence staining showed that cells differentiated from gliomaspheres were positive for GFAP and β-tubulin III (Figure 19). Furthermore, a few cells were positive for stem cell markers. These findings show that gliomaspheres are multipotent in their capacity to generate multiple lineage daughter cells. It was visualized using Zeiss LSM 510 meta confocal microscope at 10x magnification under the settings of 543 for PI and 488 for FITC and % immunoreactivity was measured using Image J software (Table 10).

Figure 19. Differentiation potential of gliomaspheres in serum containing medium



Analysis of the multipotency of the gliomaspheres by immunofluorescence after seven days of differentiation, many cells expressed markers for neuronal (β-tubulin III) and glial (GFAP) lineage (100X).

IV.3.4. Gliomaspheres show an intrinsic potential to resist differentiation as they possess the ability to retrieve stem cell characteristics after differentiation

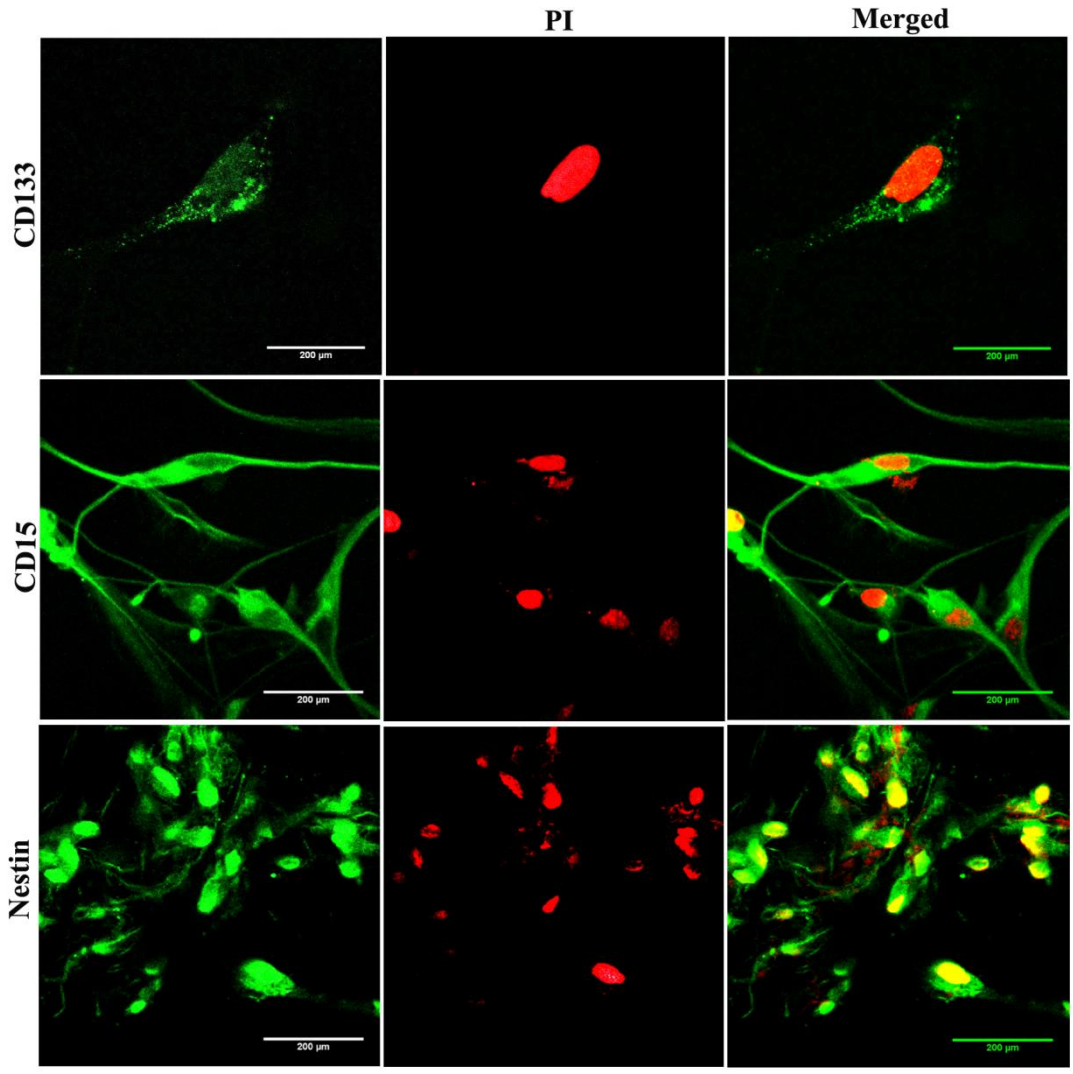
The gliomasphere's (1 grade I, 3 grade II, 3 grade III and 1 grade IV) potential to maintain their immature state even after growing under differentiating conditions for two weeks was analyzed. For the study, gliomaspheres were grown in a serum containing differentiation promoting DMEM/F12 medium (SCM) for two weeks. After two weeks of differentiation, the cells were then switched into NSC promoting medium. After one week of growth in NSC medium, the cells were immunostained for stem cell markers. These cells stained positive for stem cell markers (Figure 20) showing that gliomaspheres from different grades of glioma have GSCs with an intrinsic potential to resist differentiation and maintain their immature state. It was visualized using an Olympus fluorescence microscope and at 10x magnification. The images were quantified using Image J software (Table 10).

Table 10. Quantitative analysis of multipotent differentiation ability of gliomaspheres and its ability to resist differentiation using neural stem cell and lineage markers.

	% Immunostained cells	
	Gliomaspheres after growing in SCM for differentiation	**Gliomaspheres grown in SCM for differentiation cues and subsequently switched into NSC medium**
CD133	0.65 ± 0.08%	3.59 ± 0.86%
CD15	0.34 ± 0.23%	5.96 ± 4.85%
Nestin	27.14 ± 6.81%	31.61 ± 11.28%
GFAP	55.42 ± 2.92%	18.04 ± 0.96%
Beta III tubulin	57.77 ± 2.39%	10.06 ± 1.29%

The table shows the quantitative analysis of the differentiation capacity of the gliomaspheres with respect to their ability to generate glial (GFAP) and neuronal (Beta III tubulin) lineages and also its inherent resistance to differentiation by the expression the stem cell markers (CD133, CD15 and nestin) even after subjecting to differentiation cues.

Figure 20. Sphere reforming ability of gliomaspheres after exposing to differentiation cues



Gliomaspheres after growing in 10 % FBS containing DMEM/F12 differentiation medium for seven days was switched to neural stem cell medium to check for its potential to resist differentiation. Gliomaspheres has depicted an inherent ability to resist differentiation and was shown by the expression of NSC markers even after growing in a differentiation promoting 10 % FBS containing DMEM/F12 medium.

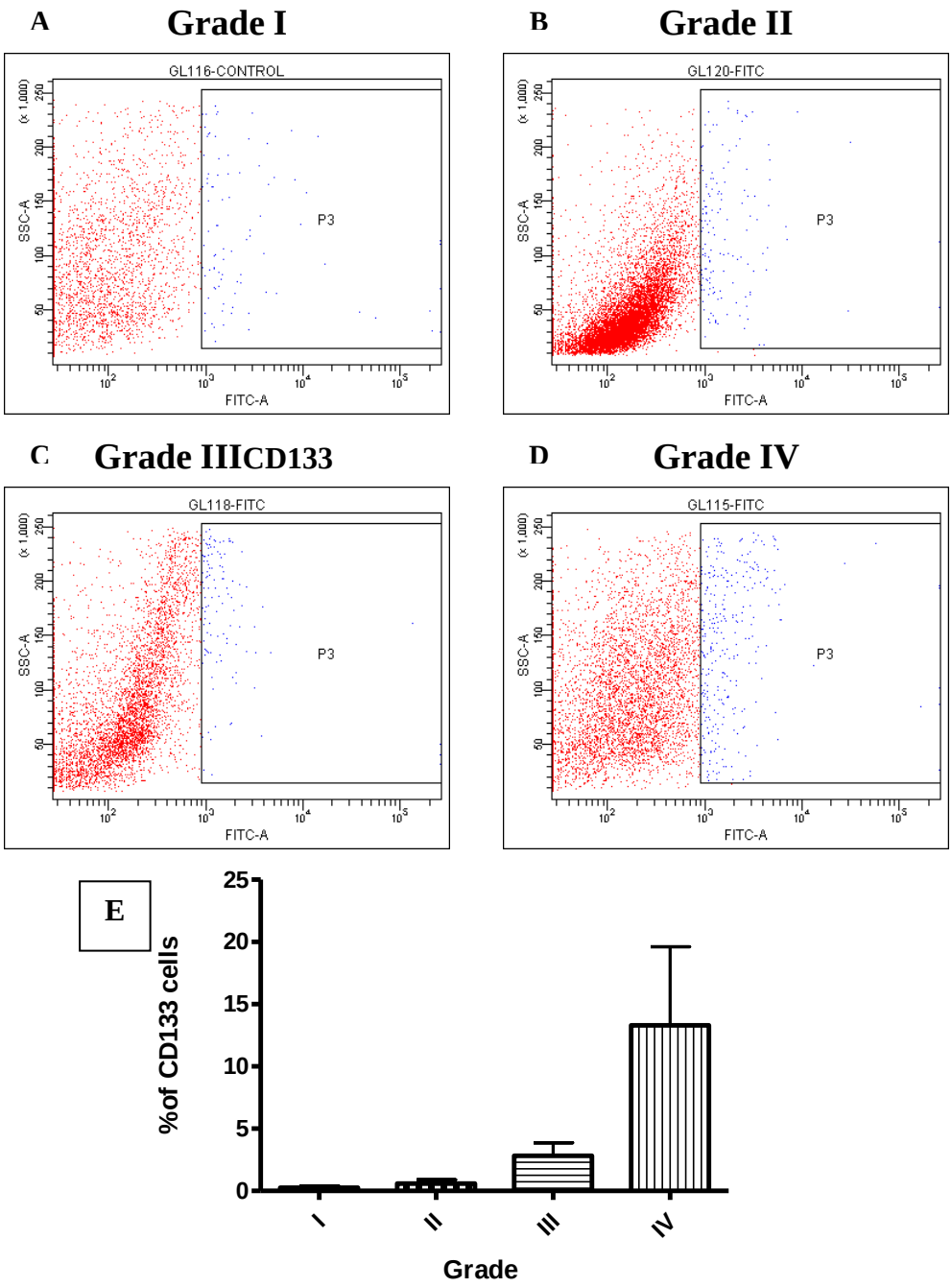
IV.3.5. CD133 expression in human glioma cultures

Since, CD133 is a putative marker that has been frequently used for the identification of and characterization of NSC/GSCs. Gliomaspheres from all the four grades of glioma were analyzed for their expression of CD133 marker using flowcytometry. The CD133 expression in different grades of human glioma ranged from 0.1 to 20% (Figure 21). Low grade gliomas contained the lowest proportion of $CD133^+$ cells, as grade I contained 0.1-0.4% and grade II with 0.3-0.9% of $CD133^+$ cells. On the otherhand, we detected a strong expression of CD133 in most high grade gliomas, as 1-5.4% of grade III and 0.7-20% of the cells of grade IV gliomas showing CD133 positivity (Table 11). On average, CD133 was expressed in a comparable proportion of cells in all the four grades of glioma. The lower grade gliomas displayed a relatively small population of $CD133^+$ cells compared with high grade gliomas.

Table 11. Percentage of CD133 positive cells in different grades of glioma by flow cytometry

Tumor type	% CD133 positive cells
Pilocytic Astrocytoma (n = 4)	0.1 – 0.4%
Astrocytoma II (n = 3)	0.3 - 0.9%
Anaplastic Astrocytoma (n = 3)	1 – 5.4%
GBM (n = 4)	0.7 – 20%

Figure 21. Representative images showing flow cytometry for CD133 in different grades of human glioma



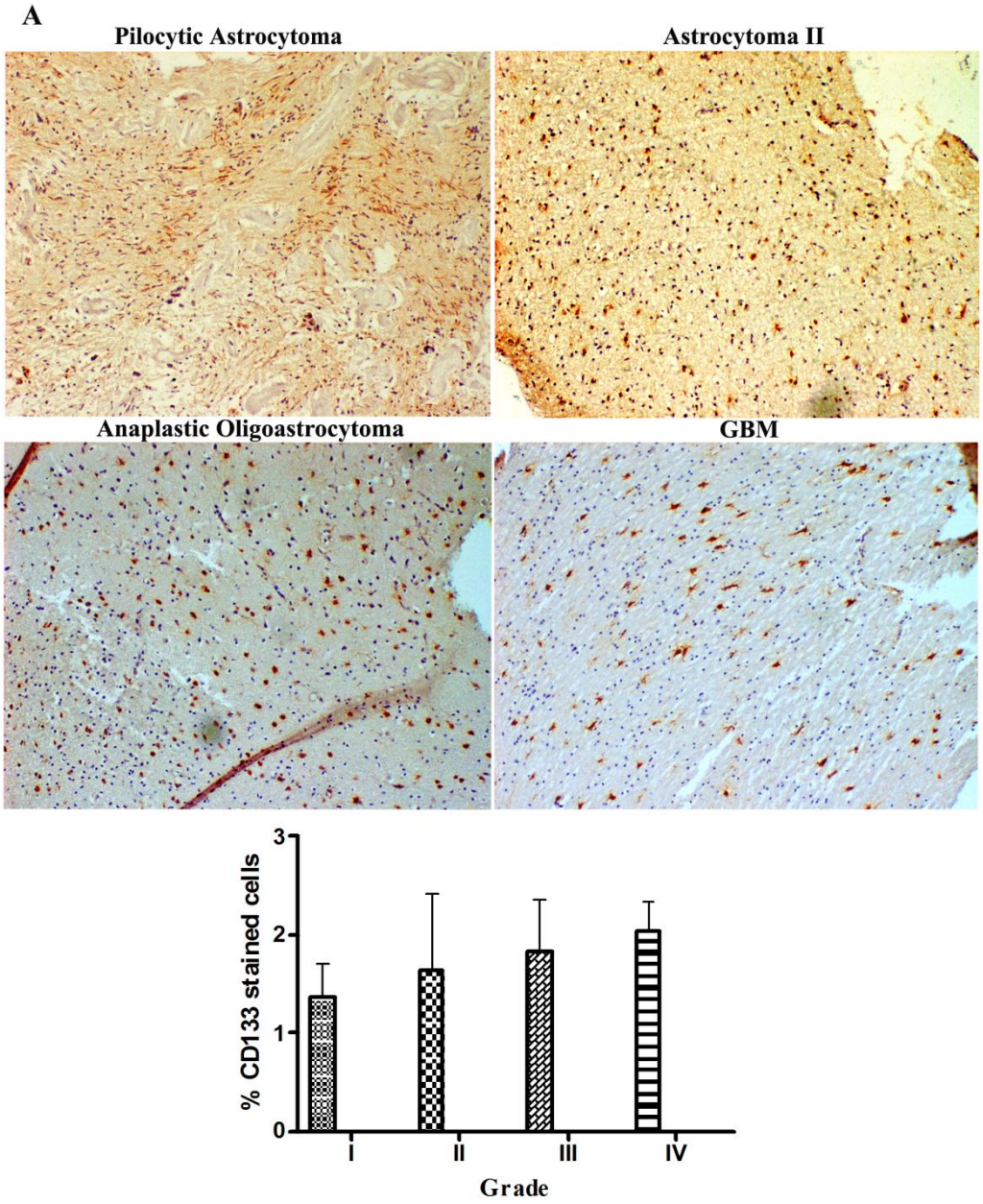
(A) Pilocytic astrocytoma, (B) Astrocytoma II, (C) Anaplastic astrocytoma, (D) GBM and (E) flow cytometric determination of the percentage of CD133 positive cells.

Gliomaspheres were dissociated using TrypLE and processed for flow cytometry as described under methods. Flowcytometry was used to determine the proportion of CD133 positive cells from different grades of glioma.

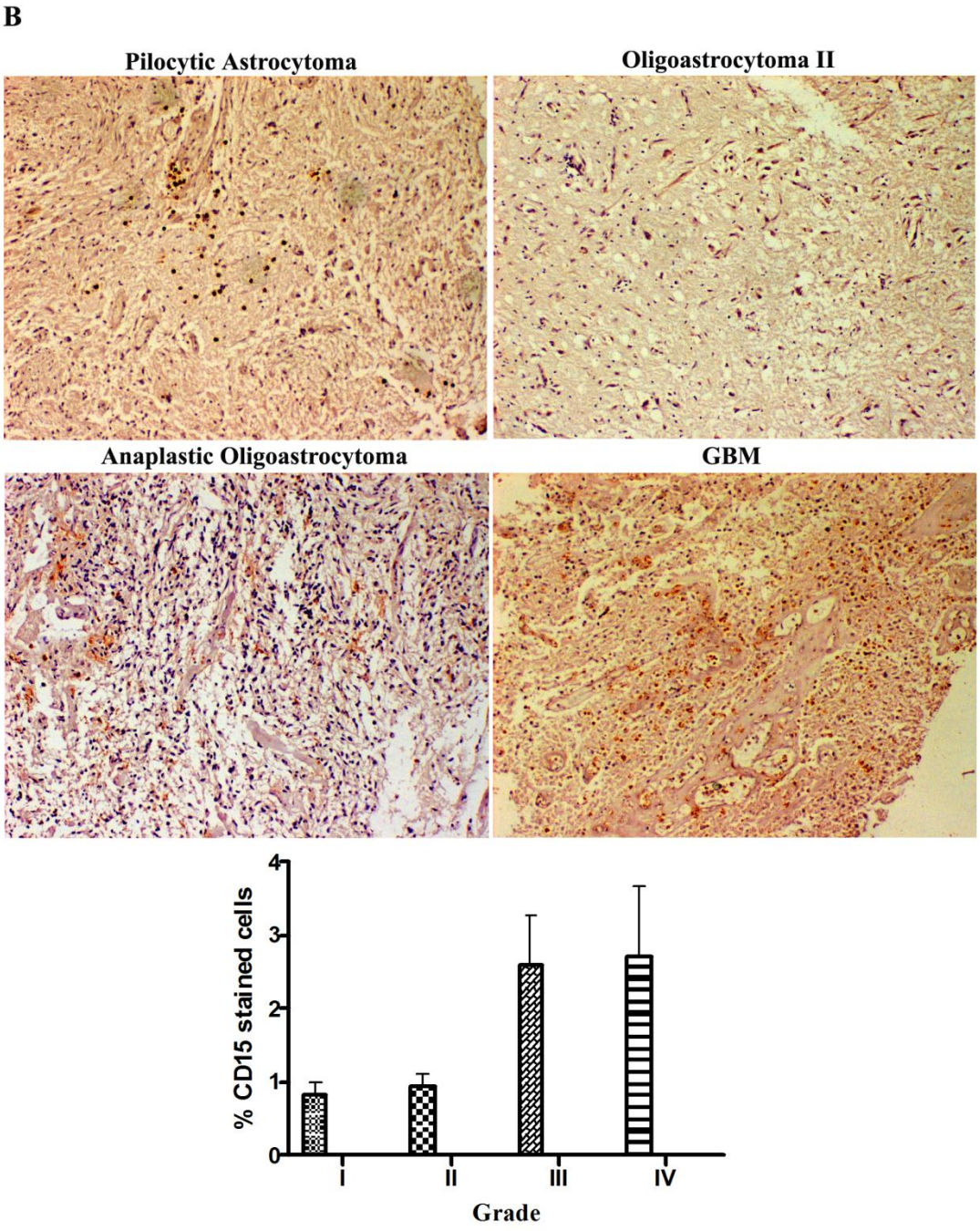
IV.3.6. Immunohistopathologic analysis of human glioma whole tissue sections for neural stem cell and lineage markers showed variation with clinical staging

Paraffin embedded sections of all the four grades of glioma were immunostained for CD133, CD15, nestin, GFAP and β-tubulin III. Expression of antigens was assessed in a panel of 48 gliomas of different pathological types. The expression levels of antigens in human glioma tissues showed considerable variability with different clinical grading ranging from complete lack/very low immunoreactivity to high intensity staining. The markers characteristic of NSCs, CD133 (Figure 22A) and CD15 (Figure 22B) were detected mainly in higher malignant grades of glioma, whereas nestin (Figure 22C) was present diffusely throughout the tumors in all grades of glioma. The expression of stem cell markers CD133 and CD15 was either not detectable or expressed only in very small fraction of cells. The expression of nestin is more intense in high grade gliomas compared to low grade. GFAP (Figure 22D) and β-tubulin III (Figure 22E), the markers characteristic of glial and neuronal differentiation respectively, was detectable in all tumors and present diffusely throughout the section. The results demonstrate that the GFAP and β-tubulin III protein expression was present in all grades of glioma. 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.

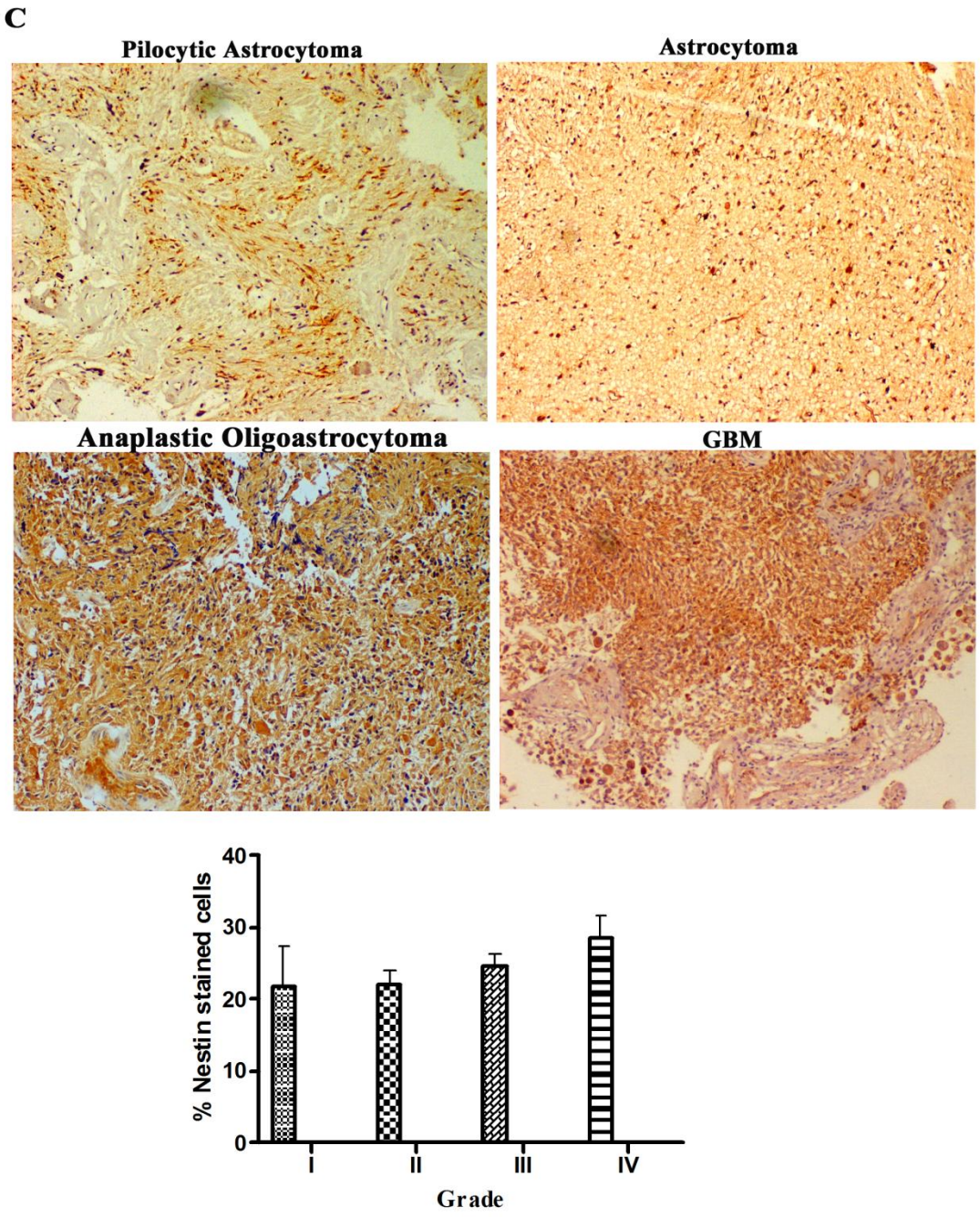
Figure 22. Histopathologic features of four grades of glioma tumors showing expression of CD133, CD15, nestin, GFAP and β-tubulin III (100X). The representative images are from (A) CD133 (B) CD15 (C) nestin (D) GFAP and (E) β-tubulin III.



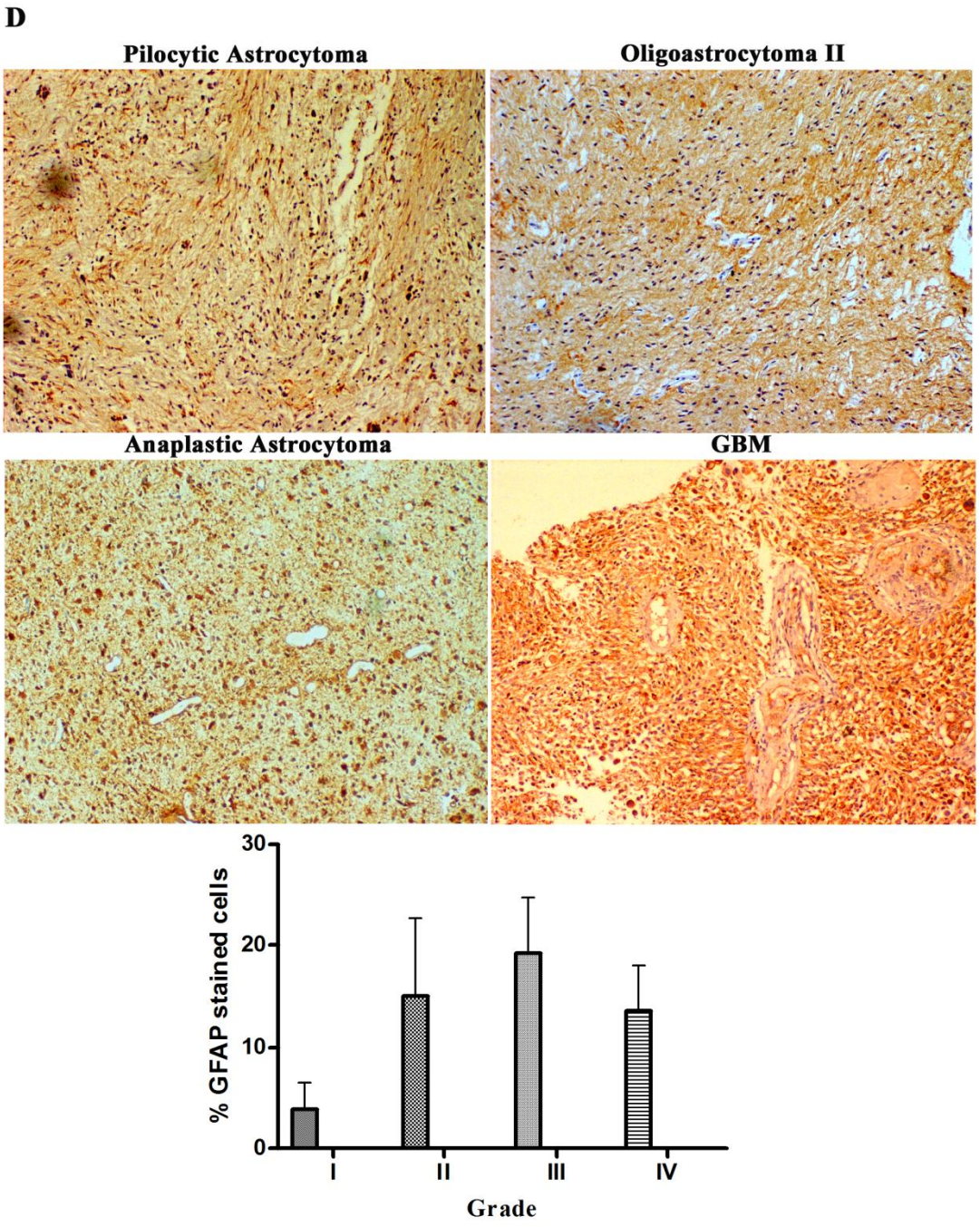
Representative images of IHC staining demonstrating the expression of CD133 marker (NSC marker) in different grades of glioma (Pilocytic astroctoma-grade I, Astrocytoma II-grade II, Anaplastic oligoastrocytoma-grade III, GBM-grade IV). The tissue sections were formalin fixed and stained by human specific antibody against CD133 and were also labelled with hematoxylin (blue) to identify nuclei.



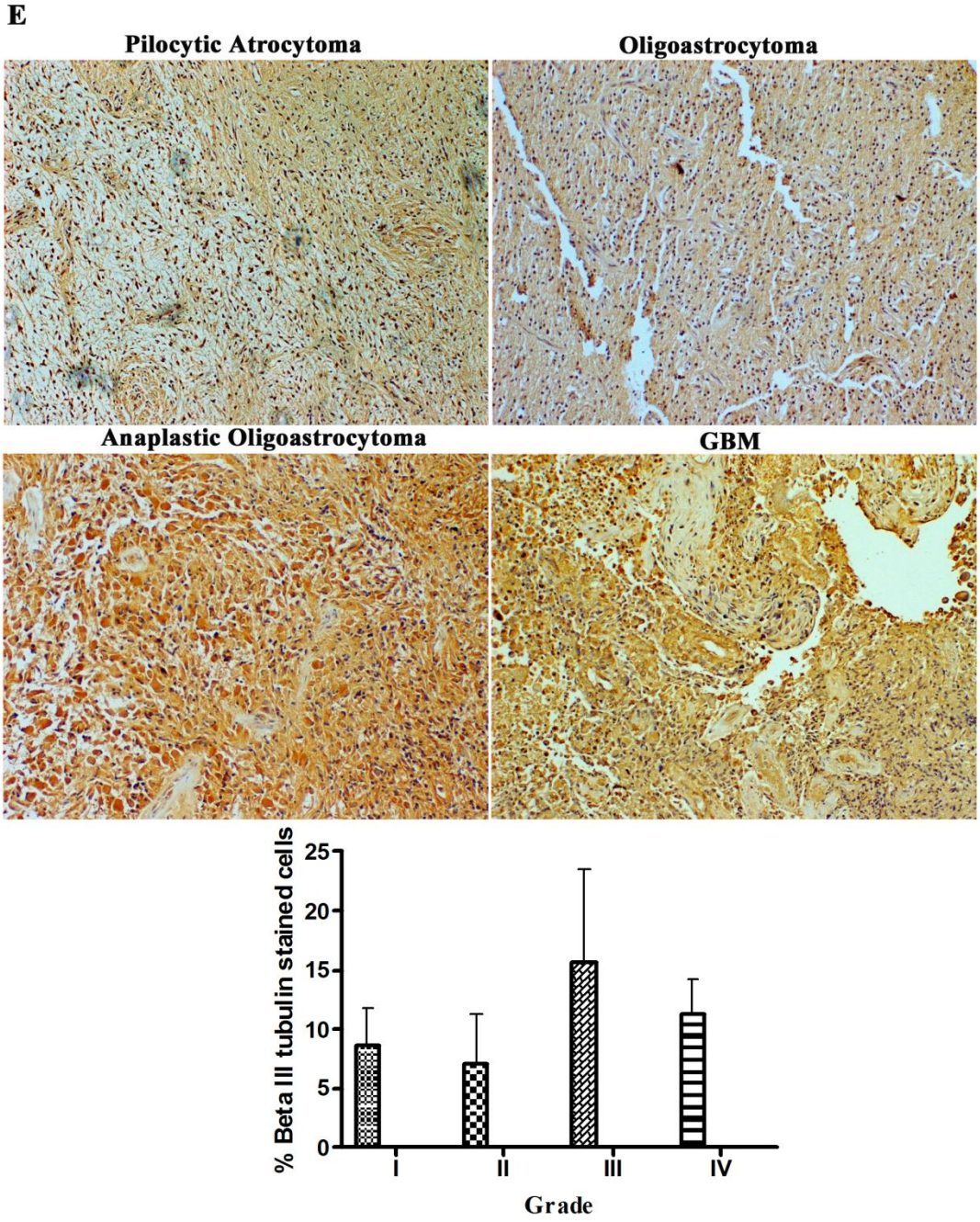
Representative images of IHC staining demonstrating the expression of CD15 marker (NSC marker) in different grades of glioma (Pilocytic astroctoma-grade I, Oligoastrocytoma II-grade II, Anaplastic oligoastrocytoma-grade III, GBM-grade IV). The tissue sections were formalin fixed and stained by human specific antibody against CD15 and were also labelled with hematoxylin (blue) to identify nuclei.



Representative images of IHC staining demonstrating the expression of nestin marker (NSC marker) in different grades of glioma (Pilocytic astroctoma-grade I, Astrocytoma II-grade II, Anaplastic oligoastrocytoma-grade III, GBM-grade IV).The tissue sections were formalin fixed and stained by human specific antibody against nestin and were also labelled with hematoxylin (blue) to identify nuclei.



Representative images of IHC staining demonstrating the expression of GFAP marker (glial lineagemarker) in different grades of glioma (Pilocytic astroctoma-grade I, Oligoastrocytoma II-grade II, Anaplastic astrocytoma-grade III, GBM-grade IV). The tissue sections were formalin fixed and stained by human specific antibody against GFAP and were also labelled with hematoxylin (blue) to identify nuclei.

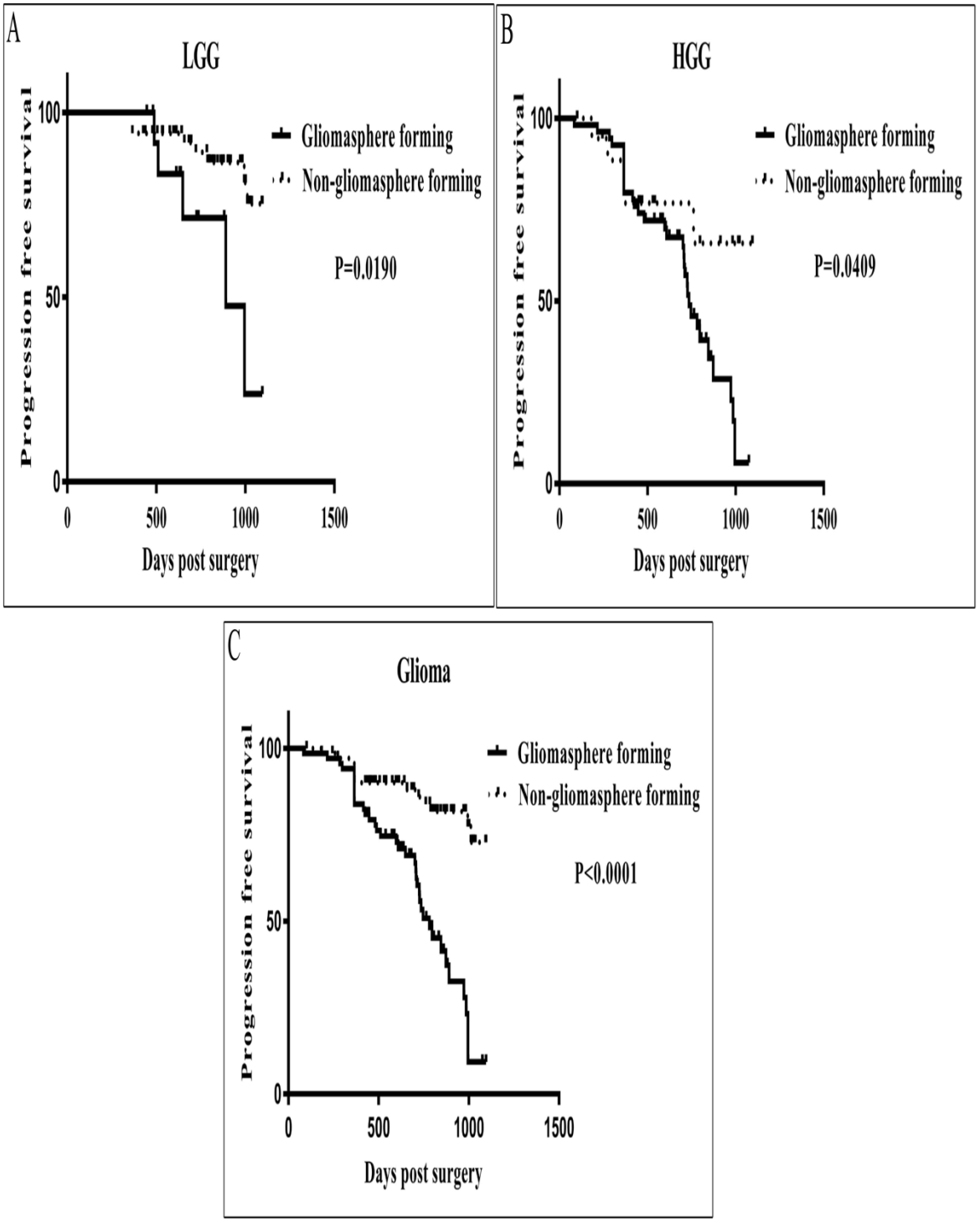


Representative images of IHC staining demonstrating the expression of Beta III tubulin marker (neuronal lineage marker) in different grades of glioma (Pilocytic astroctoma-grade I, Oligoastrocytoma II-grade II, Anaplastic oligoastrocytoma-grade III, GBM-grade IV). The tissue sections were formalin fixed and stained by human specific antibody against Beta III tubulin and were also labelled with hematoxylin (blue) to identify nuclei.

IV.3.7. Gliomasphere Formation is Associated with PFS

We then analyzed whether there is a relation between gliomasphere formation and PFS in glioma patients. The Kaplan–Meier survival analysis using log rank tests indicated that patients whose tumor showed ability to form gliomaspheres in culture were associated with shorter PFS in both LGGs ($P = 0.0190$, Figure 23A) and HGGs ($P = 0.0409$, Figure 23B). The median PFS (3 years, PFS) of gliomasphere forming and nonsphere forming patients were 890 and greater than 1,095 days, respectively, for LGG ($n = 67$) patients, whereas 735 and greater than 1,095 days respectively for HGG ($n = 73$) group. Similarly, the median PFS (3 years, PFS) duration is 781 days for gliomasphere forming patients and greater than 1,095 days for non-sphere forming patients ($P < 0.0001$) in the full cohort of glioma (Figure 23C). After 3 years, in LGG, the gliomasphere forming group had 24% survival, whereas the non-sphere forming group had 75% survival. In the sub-population of HGG, the neurosphere forming group had 6% 3-year PFS whereas the nonsphere forming group had 66% survival. After 3 years, in the full population of glioma, the neurosphere forming group had 9% 3-year PFS whereas the non-sphere forming group had 73% survival.

Figure 23. Evaluation of the relationship between gliomasphere formation and PFS of LGG and HGG patients using Kaplan–Meier method. Kaplan–Meier estimates of PFS in (A) LGG, (B) HGG, and (C) full cohort of glioma.



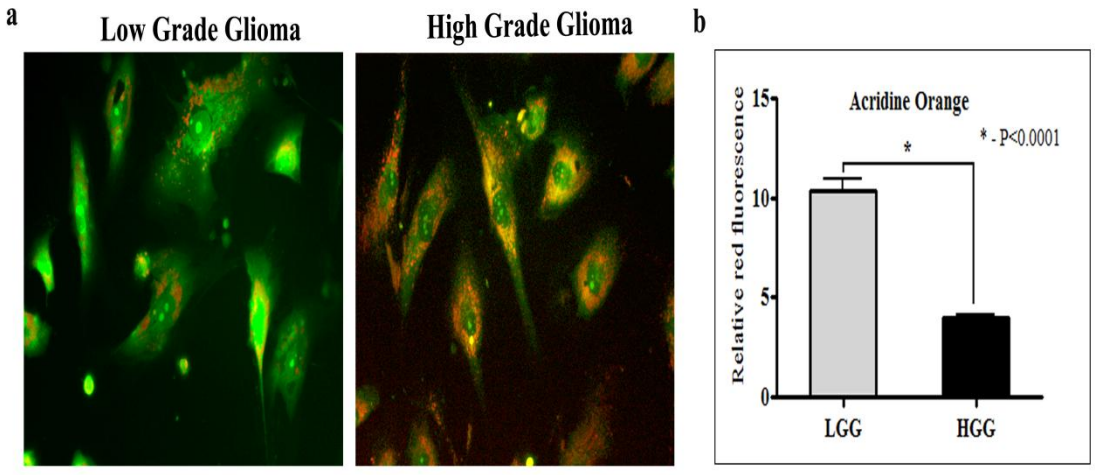
Kaplan–Meier estimates of PFS in (A) LGG, (B) HGG, and (C) full cohort of glioma. Gliomasphere formation was related to shorter PFS in LGG and HGG.

IV.4. Monitoring autophagic status among different grades of glioma

IV.4.1. Glioma cells showed an accumulation of acidic vesicular organelles in LGG and HGG

To determine whether autophagy is non-defective, autophagic flux analysis after treatment with Bafilomycin A1 was done on primary cultured glioma cells. Autophagy is characterized by development of acidic vesicular organelles (AVOs), which is detected in primary cultured glioma cells using the lysosomo-tropic agent acridine orange. Supravital staining of glioma cells using acridine orange showed accumulation of AVOs (fluoresce bright red) in LGG and HGG and also observed an enhanced staining of HGG over LGG (Figure 24). This data indicates that glioma cells have autophagolysosome formation and the enhanced acridine orange staining of HGG over LGG confirms that autophagosome formation is higher in HGG compared to LGG.

Figure 24. Acridine orange staining in glioma cell cultures



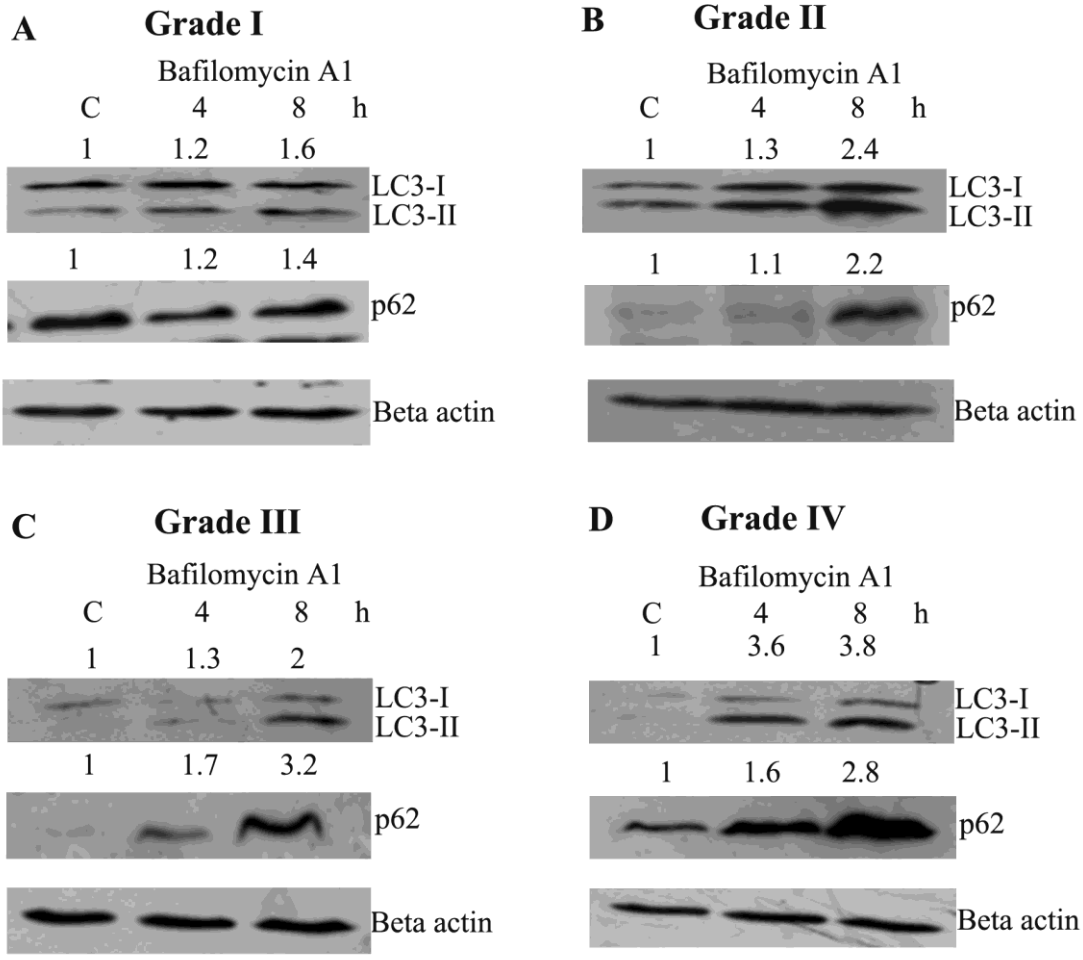
Glioma cells from LGG and HGG were stained with acridine orange, the bright red fluorescent spots indicating increased autophagosomes was observed under fluorescence microscope and photographed. Degree of red acridine orange staining was quantified in low and high-grade glioma derived cells using ImageJ software.

IV.4.2. Autophagy is non-defective in glioma cells isolated from human tissue

To further confirm the autophagy status, primary cultured glioma cells were immunobloted for LC3-II and p62 markers (grade I (2), grade II (3), grade III (3) and grade IV (2)). LC3-II is the autophagosome membrane protein, whereas p62/sequestosome (SQSTM1) is a protein that tags the cargo for degradation via autophagy, and is found to be aggregated in many pathogenic scenarios where autophagy is defective. The increase or decrease in autophagy cannot always be correlated with autophagy since, the decrease in LC3-II and p62 might be due to the over activation of autophagy leading to enhanced degradation whereas its increase may be due to the suppression of degradation. 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. Bafilomycin A1 is the widely used autophagic inhibitor that blocks final stage of autophagy (autophagosome-lysosome fusion) and forestalls degradation of contents thereby accumulating LC3 and other autophagy substrates.

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 (Figure 25a–d). The rate of accumulation of LC3-II and p62 proteins was low in LGG than in HGG (Figure 25a, d), 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.

Figure 25. LC3-II and p62 expression showing induction of autophagy in different grades of glioma derived cells after BafilomycinA1 treatment



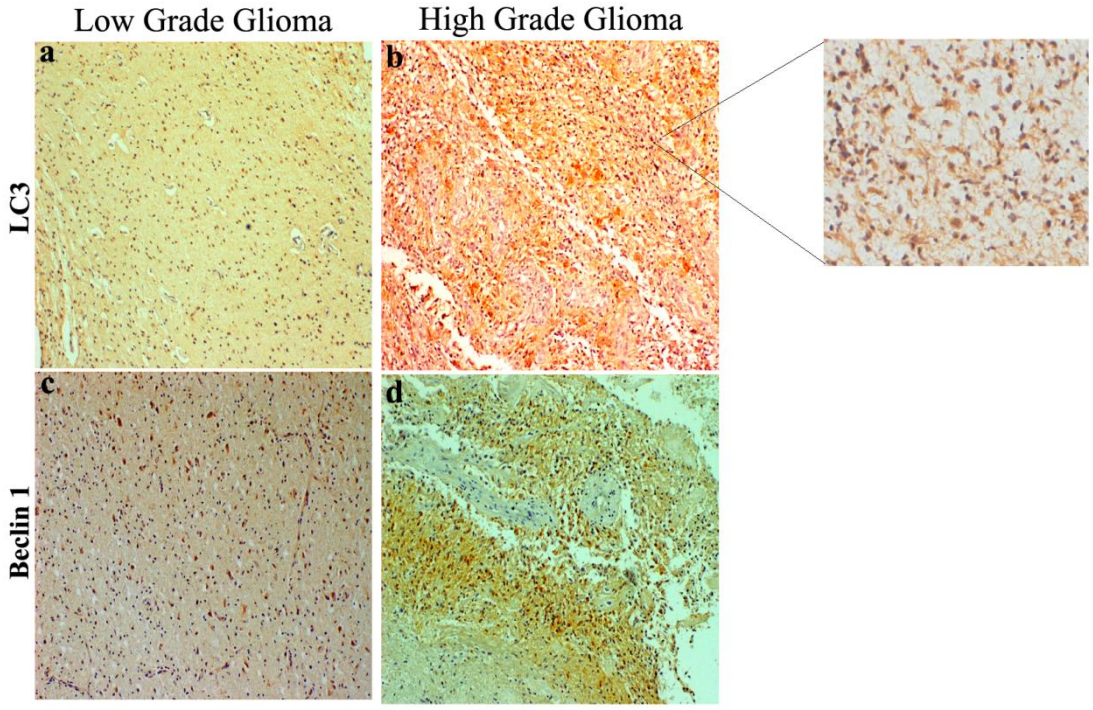
(*A) Pilocytic astrocytoma (B) Astrocytoma II (C) Anaplastic astrocytoma and (D) GBM. Primary cultured glioma cells treated with Bafilomycin A1 for 4 h were analyzed for the accumulation of autophagy markers LC3 and p62 by Western blotting. Bafilomycin A1 treatment increases the accumulation of LC3 and p62 in all the four grades of glioma cells. It was normalised to β-actin.*

IV.4.3. Immunohistochemical analysis of autophagy markers detected autophagy in all the four grades of glioma

To determine whether autophagy is prevalent in different grades of glioma tissues by using traditional approach, paraffin embedded sections from all the four grades of glioma were immunohistochemically stained for autophagy markers LC3-II and beclin 1. There was a diffuse staining pattern for LC3 and beclin 1 in human glioma

tumor tissues. In some sections, LC3-II formation was detected in a punctate pattern. The markers characteristic of autophagy was detected in tissue sections from all the four grades of glioma (Figure 26).

Figure 26. Characterization of autophagy in glioma tissue sections using antibodies against LC3 and beclin1



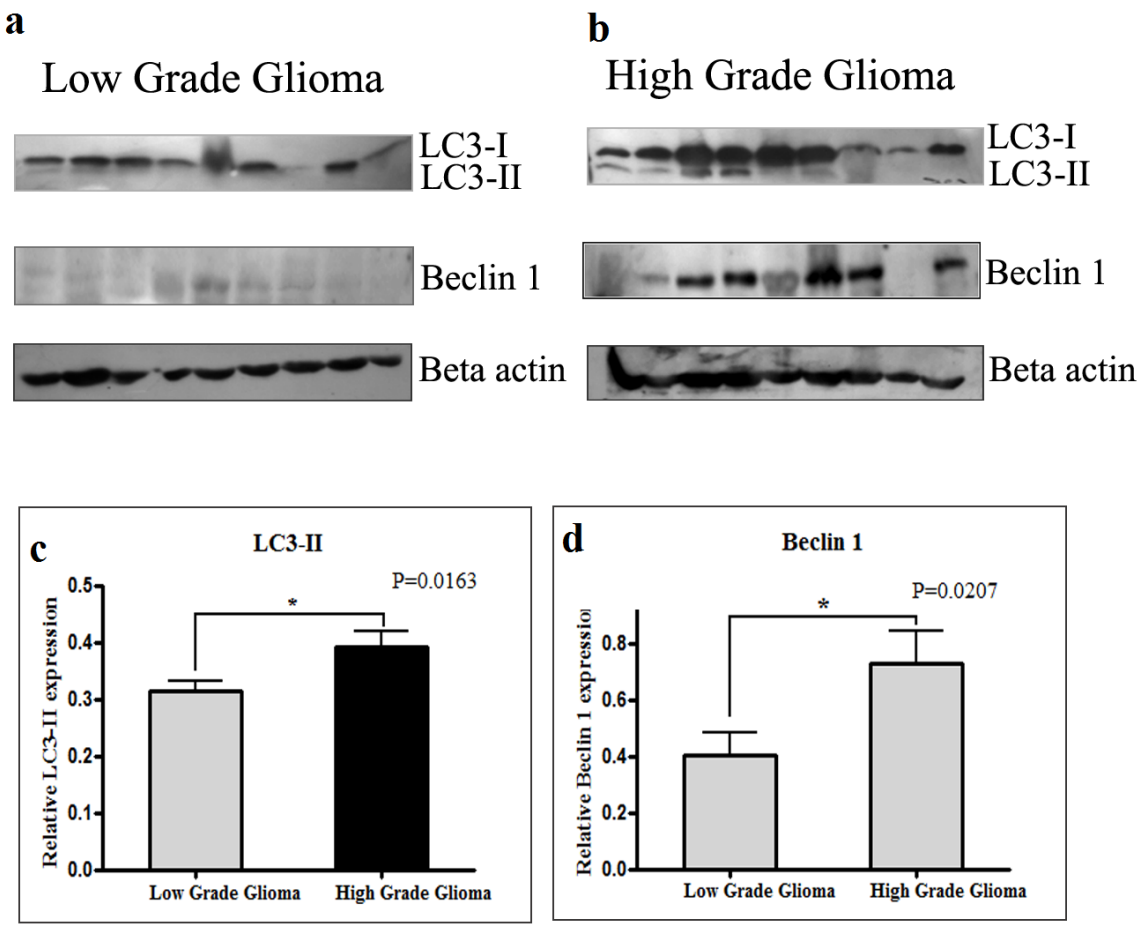
Human glioma tissue sections were fixed with buffered formalin and expression of autophagic markers was examined using antibodies against LC3 and beclin 1 and detected by HRP conjugated secondary antibody and DAB as chromogen. The sections were counterstained by hematoxylin (10X).

IV.4.4. Detection of autophagy markers in different grades of human glioma tissue by western blotting

Western blot analysis showed that the expression levels of LC3-II and beclin 1 proteins were significantly higher in high grade than in low grade-gliomas. LC3 has two isoforms: LC3-I is cytosolic and LC3-II is present on the autophagosomal membrane. Therefore an increase in LC3-II is an indication of autophagy. LC3-I expression was observed in all grades of glioma whereas LC3 II and beclin 1 induction, an indication of autophagy was absent in LGG but present in HGG. The

lack of expression of autophagy proteins in LGG may be because of the low detectable level of autophagy (Figure 27). 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 Figure 27a-d.

Figure 27. Detection of autophagic proteins in glioma tissues by western blotting

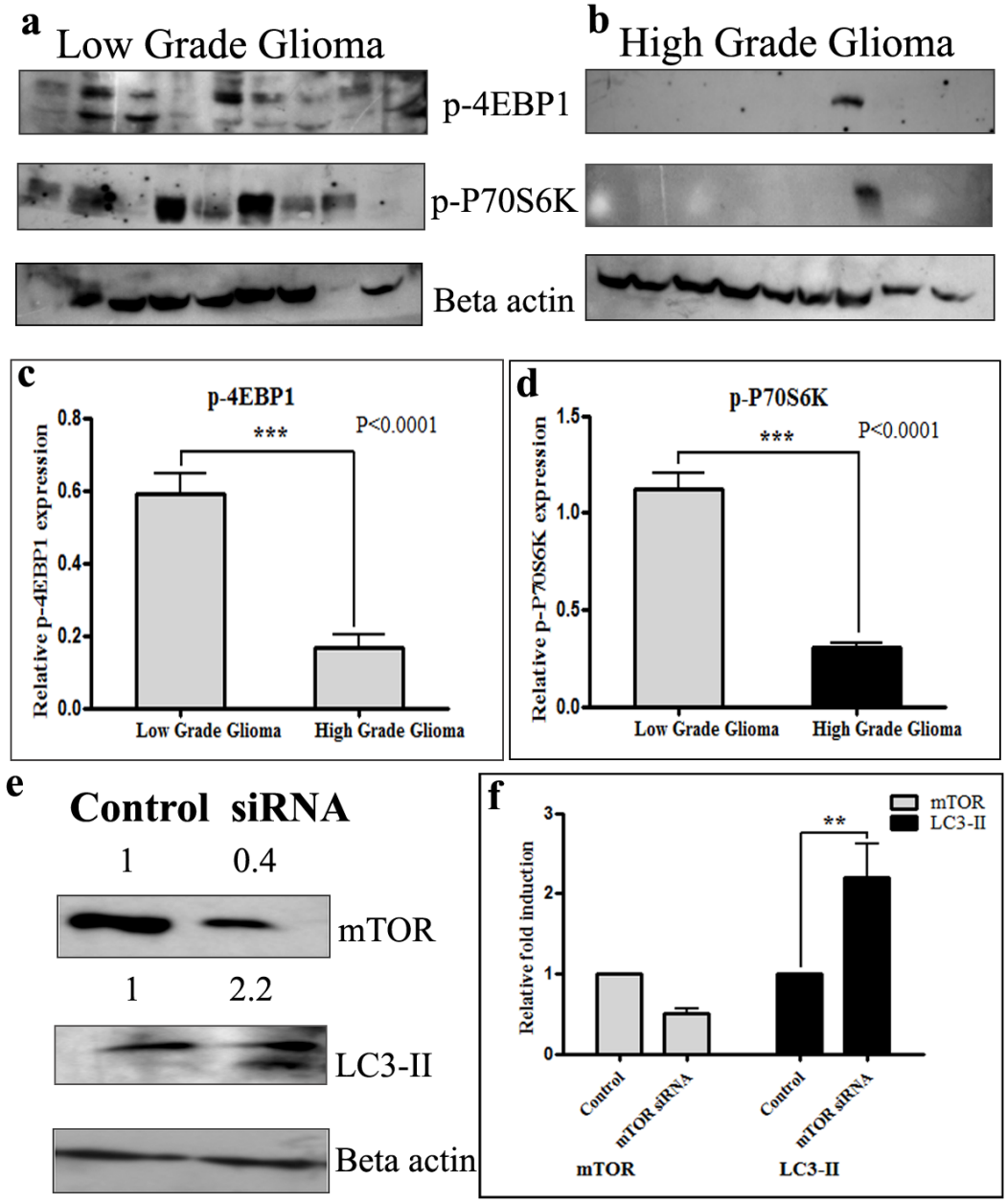


Representative blots of autophagic proteins in low and high grade gliomas (e) Astrocytoma II (f) GBM. 100 µg of protein was used for Western blotting assay with autophagic markers. β-actin antibody was used as loading control. g, h Relative expression of autophagy proteins (LC3-II and Beclin 1) was compared between the grades (LGG: n = 30 and HGG: n = 25 patients). Data represented as mean ± SEM

IV.4.5. mTOR status correlates with autophagy in different grades of glioma tissue

mTOR is a kinase protein and its activation has been found to negatively regulate 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 analysed (Figure 28a–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 (Figure 28e–f), that verify the role of mTOR activity on autopahgic rate in glioma tissues.

Figure 28. mTOR expression in relation to autophagy in gliomas.

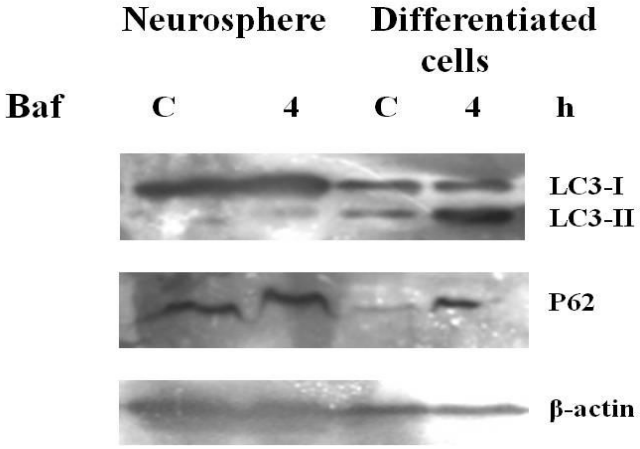


a–d Western blotting of mTOR substrates p-4EBP1 and p-P70S6kinase expression. Representative blots of phosphorylation status of mTOR substrates in low and high grade gliomas (a) Astrocytoma II (b) GBM. c,d Quantitative analysis of the p-4EBP1 and p-P70S6kinase levels compared between the grades. e, f 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)

IV.4.6. Differentiated cells exhibit increased autophagic activity than undifferentiated gliomaspheres

To compare the autophagic status of differentiated glioma cells and undifferentiated gliomaspheres, autophagic flux analysis using Bafilomycin A1 treatment for 4 h was performed and expression of LC3-II and p62 was assayed by western blotting. The growing body of literature indicates that the glioma growth, propagation and oncogenic potential are maintained by a small sub-population of cells in a tumor called GSCs. A recent study has demonstrated that GSCs has impaired autophagy and the activation of autophagy can induce its differentiation thereby sensitizing tumors to therapy. Our result shows that compared with gliomaspheres grown in SFM, the autophagy protein expression was higher in differentiated glioma cells grown in SCM (Figure 29).

Figure 29. Autophagy status of gliomaspheres and differentiated glioma cells

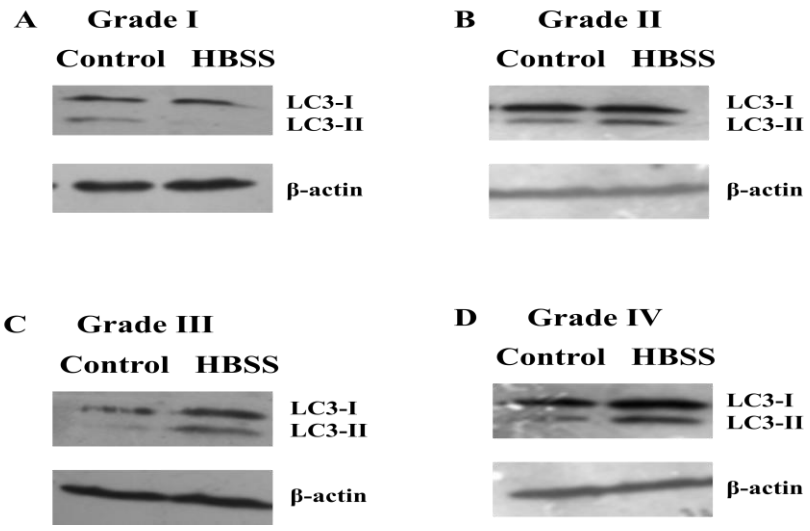


Gliomaspheres grown in SFM and differentiated glioma cells in 10% DMEM/F-12 were treated with Bafilomycin A1 for 4 h and analyzed for the expression of autophagy markers LC3-II and p62. Differentiated glioma cells show marked increase in autophagy activity compared to gliomaspheres.

IV.4.7. Primary cultured Glioma cells induce autophagy under starvation conditions

To analyse the activation of autophagy during starvation, expression status of autophagic molecular marker LC3-II; which gets incorporated into autophagosome membranes was assayed by immunoblotting. The primary cultured glioma cells treated with HBSS for 4 h has showed increased expression of LC3-II when compared to control cells cultured in complete medium (10% DMEM/F-12) (Figure 30). This demonstrates autophagy induction during starvation conditions in primary cultured glioma cells.

Figure 30. Autophagy is induced under starvation conditions



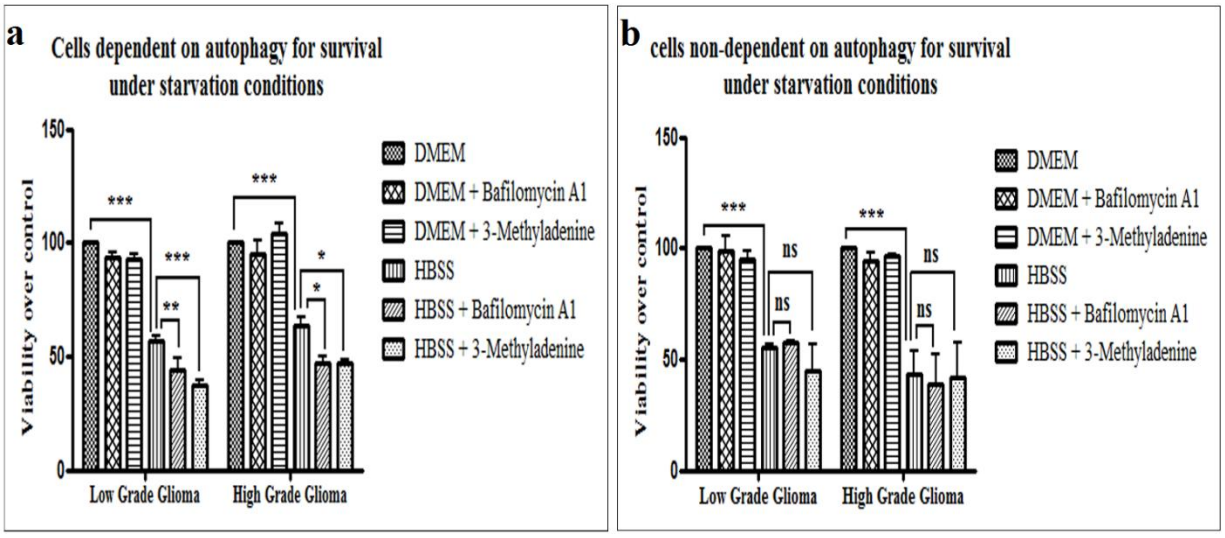
(A) Pilocytic astrocytoma (B) Astrocytoma II (C) Anaplastic astrocytoma and (D) GBM. In the all four grades of glioma, LC3-II expression was more intense in cells grown in starvation condition (HBSS) when compared to control cells grown in 10% DMEM/F-12 for 4 h. Normalised to β-actin.

IV.4.8. The role of autophagy during starvation on primary cultured Glioma cells

The effect of starvation on primary cultured glioma cells whether survival or death was analyzed by cell viability assay. When glioma cells were subjected to starvation

in HBSS (glucose present, aminoacids and other growth factors deprived) for 12 h, cell exhibited decreased viability of 30-50%. (Figure 31a, b). 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 (Figure 31a) 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 (Figure 31b). These results suggest that the extent at which the glioma cells depends on autophagy for overcoming the starvation condition may vary among the tissues.

Figure 31. The viability of primary cultured glioma cells in starvation medium and upon inhibition of autophagy

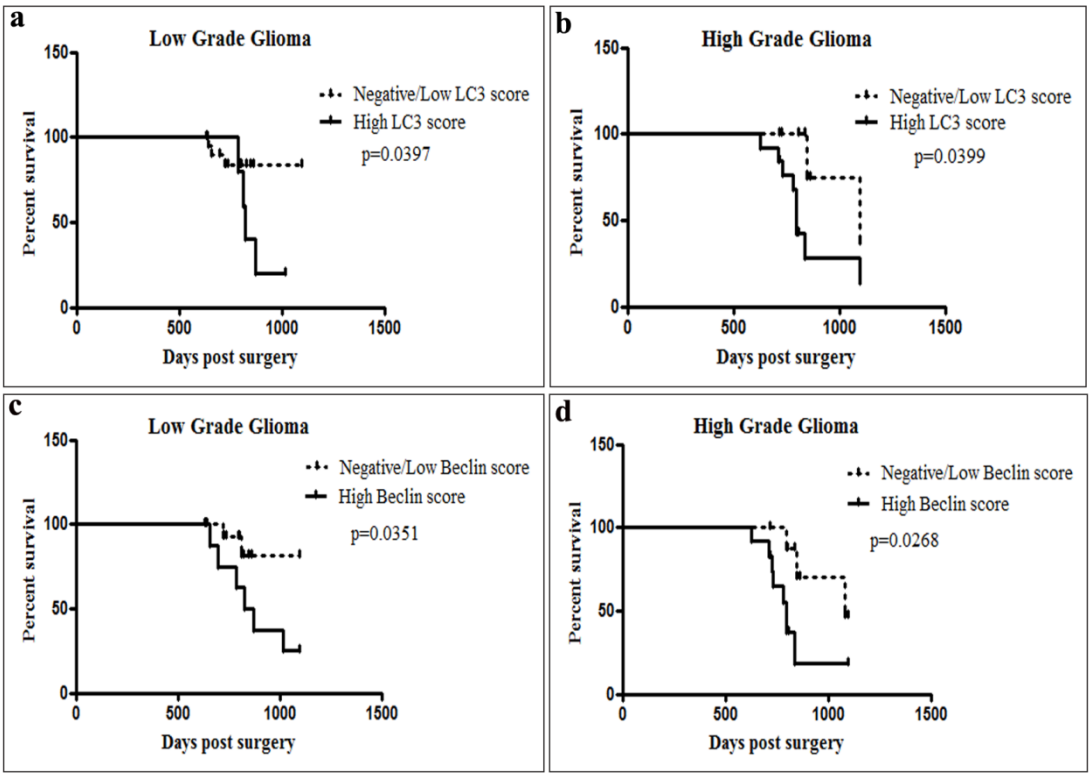


*(a) Cells dependent on autophagy for survival and (b) Cells non-dependent on autophagy for survival under starvation conditions. Cell viability was determined by MTT assay after a period of 12 h starvation in HBSS and also co-treatment with autophagy inhibitors, bafilomycin A1 and 3-MA. Error bars are 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)*

IV.4.9. 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 were associated with shorter progression free survival in LGG (LC3: 19%; $p = 0.0397$, Figure 32a and beclin 1: 27%; $p = 0.0351$, Figure 32c) and HGG (65%; $p = 0.0399$, Figure 32b and beclin 1: 62%; $p = 0.0268$, Figure 32d). This suggests that high LC3-II and beclin 1 expression is associated with increased malignancy in glioma.

Figure 32. 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 and beclin 1 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 and beclin 1 expression was related to shorter progression free survival in low and high-grade glioma.

V.1. Gliomasphere formation ability of different grades of glioma

Gliomas are the most common primary brain tumors with poor patient outcome and short survival. These tumors have been shown to follow a cancer stem cell (CSC) model of tumorigenesis, where a glioma stem cell (GSC) population is responsible for the generation, maintenance, propagation and the recurrences of the tumour that occur after current treatments (Galli et al., 2004; Hambardzumyan et al., 2008; Vescovi et al., 2006). These populations would demonstrate an ability to grow as gliomaspheres, increased self-renewal and multipotent differentiation *in vitro* and also have the ability to serially recapitulate the original tumour *in vivo* (Singh et al., 2003). To date, different studies have indicated the presence of GSCs in glioma thus, it is critical for glioma therapy that treatments must target and eliminate GSCs. The clinical relevance of GSCs has not been fully elucidated and therefore, the study of GSCs is extremely pertinent for the development of novel therapies. If this is the case the need to isolate and study GSCs from different grades of glioma is very important from both scientific and therapeutic stand point. Gliomasphere formation is a specifying feature of the presence of GSCs *in culture* and this neurosphere/sphere forming assay is the widely used technique for isolation and characterization of GSCs as well as providing quantitative information about the stem cell frequency (Reynolds et al., 1992).

This work presents the results on the isolation of GSCs from different grades of glioma and patients from all of the four grades both low (I and II) and high (III and IV) grade were included in this study. For this study, we have used the neurosphere assay technique a phenotypic selection of cells grown as gliomaspheres in a stem cell

promoting medium to isolate and functionally characterize the human GSCs from different grades of glioma. Many of the studies in glioma are either focused on high grade gliomas mainly GBM (grade IV, highly malignant glioma) or brain tumors generally; on the other hand, very few studies are available for low grade gliomas thereby making these tumors incompletely explored. However, except grade I glioma, all other grades of glioma (II, III and IV) are highly malignant with increased potential for invasion and the patient succumbs to death within some years or months after diagnosis. Even though grade II comes under low grade glioma category but about 70% of them progress to high grade within 5-10 years (Pouratian and Schiff, 2010; Soffietti et al., 2010). Despite the frequency and severity of this disease, very little research exists that compares the relationship between gliomasphere forming ability among different grades of glioma. None of the studies have extensively studied the relationship between gliomasphere formation and clinical aggressiveness of the tumor. In this regard, a comparative analysis using *in vitro* cultures of both low and high grade gliomas would represent a way to get insight into these crucial aspects. The study was performed on human glioma tissue and hence created an in vitro model that closely mimed the properties of patient tumor.

V.1.1. Assessing the relationship among gliomasphere formation in different grades of primary cultured human glioma cells

The studies in glioma has reported that the ability to form gliomaspheres in culture shows the clinical and biological nature of the tumor thereby pointing the use of neurosphere assay as a clinically relevant effort to predict tumor prognosis and progression. Gliomas are well studied and treated on the basis of grade as the most significant prognostic factor (Qaddoumi et al., 2009). But the major caveat

associated with entirely depending on grade as the prognostic factor for treating a highly heterogeneous tumor like glioma is that even in the same grade itself there will be tumors with different risk factors (high risk as well as low risk to progression or death) and behaviour to therapies. It was well established in earlier studies which have shown the presence of low and high risk groups within the same grade of glioma (Laks et al., 2009). In previous studies, it was reported that neurosphere formation is independent of grade and is a predictor of clinical outcome in glioma (Laks et al., 2009; Panosyan et al., 2010). The same group also have shown among the GBM tumors a sub-population with substantial hazard to death and the study suggested that neurosphere formation indicates the prognosis/patient outcome of high grade gliomas (HGGs), but not that of low grade gliomas (LGGs) (Panosyan et al., 2010). But that study has used a small sample size to assess possible association between the neurosphere formation and clinical outcome of tumor. Because of the small sample size used in the previous study and high heterogeneity in glioma, we have now investigated the relationship between gliomasphere formation and grade in glioma.

The fundamental step in our study is the isolation and expansion of GSCs from different grades of glioma. First and foremost question in our study was whether, GSCs be isolated and grown from all the four grades of glioma, especially low grade as there were no extensive studies that focussed on CSC population in LGGs. Initially under *in vitro* conditions, we assessed whether all the four grades of glioma have sphere formation ability. Our results demonstrated that all the four grades of glioma had the capacity for gliomasphere formation. Since gliomasphere formation

in culture is an underlying feature of GSCs, this supports the idea that stem cell population exists in all grades of glioma studied.

Next, we analyzed the gliomasphere formation rate among different grades of glioma. There were previous reports which showed that renewable neurosphere is predictive of tumor progression and is independent of grade (Laks et al., 2009). The reports from different studies also suggest the use of neurosphere culture system as a tool in brain tumor research. Those studies have assessed the sphere formation in correlation to progression/survival and also suggested that neurosphere formation predicts the prognosis of high grade gliomas, but not of the low grade (Panosyan et al., 2010). In this study, the gliomasphere formation capacity of both high and low grade glioma were analyzed.

Our study has shown that grade II gliomas have the highest primary sphere forming ability. Even the HGGs (III and IV) comes behind grade II in the primary sphere formation capacity at the same time grade I glioma which hardly advance to other grades has a very low primary sphere formation ability. This can be explained in connection with the previously reported data (Laks et al., 2009; Panosyan et al., 2010) which showed that neurosphere formation can be predictive of tumor progression, as grade II gliomas have higher capacity for tumor progression into grade III and IV gliomas. According to reports, even though low grade gliomas are slow growing, 70% of grade II glioma progress to high grade glioma within 5-10 years (Pouratian and Schiff, 2010). An alternative explanation to the observations made in this report surrounding primary sphere formation ability of grade II gliomas, is that perhaps progenitor cell population might be higher in grade II gliomas, which

has increased capacity for proliferation compared to stem cell populations. Thus the higher levels of primary sphere formation in grade II gliomas compared to grade III and IV may be a factor of enhanced proliferation capacity of progenitor cells. In concurrence with previous reports, we have found that there is a direct relationship between primary gliomasphere formation capacity and tumor progression, as the LGG grade II with increased potential to relapse/progress has shown higher primary sphere forming capacity. To the best of our knowledge, this is the first study that compares extensively the potential association between gliomasphere formation and grade of glioma, especially LGGs.

To truly assess whether all the four grades of glioma possess a GSC population, it is important to study the renewal gliomasphere formation ability. The earlier reports suggest that self-renewal or secondary sphere formation capacity is more indicative of the stem cell compartments within the tumor as the progenitor populations get eliminated during their propagation in neural stem cell culture conditions (Nolte et al., 2013). Thus we have analyzed the ability to form multipassage gliomaspheres among different grades of glioma as the former reports indicate the relationship between secondary sphere formation (self-renewal) and worst clinical outcome. Our study suggests a direct relationship between self-renewal capacity and grade of the tumor which correlates with the clinical severity, as HGG (III and IV) showed increased secondary gliomasphere formation compared to LGG. In our study, the grade IV glioma which is the most malignant of all brain tumors has shown the highest self-renewal capacity compared to all the other grades studied. A study by Panosyan et al., has shown in pediatric brain tumors that multipassaged sphere

formation capacity is a reflection of worst clinical outcome (Panosyan et al., 2010). In agreement with these reports, the higher secondary sphere formation rate for HGGs compared to LGGs can be explained as the ability to form multipassaged spheres correlated with clinical severity of the tumor.

The association of other variables, such as age and gender with the gliomasphere formation were also included in our study. Previous studies reported that survival/prognosis was inversely associated with age (Laithier et al., 2003; Qaddoumi et al., 2009; Sanders et al., 2007). In our study, the spearmans correlation analyses of gliomasphere with age and gender did not show any significant difference in the sphere formation rate.

Next, we analyzed whether there is any correlation between gliomasphere formation and PFS in LGG and HGG. In concurrence with previous reports, we have found that there is a direct relationship between the capacity for gliomasphere formation and grade of the tumor, as the HGGs have the highest sphere forming ability compared to LGGs ($P<0.0001$) (Laks et al., 2009; Panosyan et al., 2010) . However, when evaluating the PFS, gliomasphere formation retained its predictive value by showing a shorter survival in both LGGs and HGGs. Among the non- sphere forming group there was an increased survival advantage for LGGs compared to HGGs suggesting a strong association between PFS and gliomasphere formation along with the grade. To the best of our knowledge, this is the first study that compares extensively the potential association between gliomasphere formation and grade of glioma, especially in LGGs.

In summary, all grades of glioma possess a stem cell population as indicated by the gliomasphere formation. Our study also suggests that gliomasphere formation may serve as an indicator of stem cell population within the tumor thereby predicting the clinical aggressiveness/outcome of the tumor.

V.1.2. Expression of stem cell markers in glioma and the differentiation potential of GSCs in gliomaspheres

Gliomaspheres from different grades of glioma were further characterized for the expression of stem cell surface markers such as CD133, CD15 and nestin as well as multilineage differentiation capability. Upon differentiation, NSCs has the capacity to generate both glial and astrocyte cell lineages (Galli et al., 2004). Gliomaspheres obtained from cultures of all the four grades of glioma expressed known stem cell markers and also possess glial and neural lineage differentiation capabilities in response to *in vitro* differentiation cues which was shown by expression of GFAP and β-III tubulin markers respectively. The major obstruction in studying GSCs is the lack of specific markers of these cells. The expression of the stem cell marker may vary from tumor to tumor and also depends on the grade of the tumor and culture conditions such as availability of nutrients. The CD133 is considered as a putative marker for identifying stem cells in glioma (Sato et al., 2010; Singh et al., 2004). There are contradictory reports about whether $CD133^+$ cells or $CD133^-$ cells are tumorigenic (Beier et al., 2007; Wang et al., 2008). Recent studies on GSCs suggest that there may exist more than one marker and also questions the use of CD133 (Wang et al., 2008). Thus CD133 expression is not sufficient to identify GSCs and it is better to depend on a spectrum of stem cell markers rather than relying on a single one for refining the stem cell identification. The studies

investigating the CD133 expression have found to show inconsistent patterns and has found to vary from 1-60% in GBM patients itself (Cheng et al., 2009). On the basis of these reports for our study, we have used multiple stem/progenitor cell markers CD133, CD15 and nestin in an attempt to identify and characterize the presence of GSCs in gliomaspheres and tissue sections. These markers were expressed in the glioma sections studied. Since CD133 is the most accredited marker for GSCs, flow cytometric analysis of CD133 subpopulation of gliomaspheres from different grades of human glioma has been performed and the expression of this marker was found to be higher for HGGs.

But the major problem with the stem cell based studies is that there is no specific marker to discern GSCs from NSCs. GSCs are very similar to NSCs but there is difference in their differentiation potential as there is deregulation of certain developmental pathways that inhibits the normal differentiation of GSCs and contributing to its tumorigenicity (Lee et al., 2008; Penuelas et al., 2009). There are also reports that GSCs do not undergo terminal differentiation after treatment with differentiation inducing serum containing medium (Yuan et al., 2004). This was analyzed in our study, and was found that the gliomaspheres cultured in differentiation promoting conditions were capable of reforming spheres and expressing stem cell marker upon switching to serum free medium with growth factors and supplements. This shows that the gliomaspheres isolated from different grades of glioma have GSCs with an intrinsic potential to resist differentiation and maintain their immature state.

In our study the isolated gliomaspheres were found to express known stem cells markers thereby providing evidence to support that GSCs were isolated from all the

four grades of glioma. Other supporting evidences include the isolated gliomaspheres can self-renew, can differentiate into multiple lineages and display sphere reformation ability showing its intrinsic potential to resist differentiation. The differentiation ability of gliomaspheres to revert the stem cell characteristics after the induction of differentiation is an important feature that helps to differentiate GSCs from NSCs. Taken together, these results suggest that gliomaspheres from all the four grades of human glioma contain a sub-population of GSCs which possess all the defining features of stem cells including self-renewal and multipotent differentiation. Altogether, our results suggests the use of gliomasphere formation as a predictive model to classify each tumor based on its progression potential and clinical severity rather than entirely dependent on grade.

V.1.3. Caveats and limitations

However, the sphere formation assay is prone to experimental variability and subjectivity in sphere identification owing to culture medium composition, cell plating density, duration in culture before plating as well as culture artefacts due to sphere aggregation (Mori et al., 2006). The sphere assay operates on the premise that stem cells are clonal and each sphere is generated from a single stem cell. But, the spheres are highly dynamic structures and are prone to aggregation (Chen et al., 2005). As such, cell plating density is very important and true clonality can be studied only by plating single cell/well. In the case of primary cultured human glioma cells, single cell and low density cultures are found to have decreased sphere forming efficiency or won't form any spheres as plating density has an impact on cell growth due to cell-cell contact and autocrine/paracrine signals and high density

cultures were required for cell ontogenesis (Pastrana et al., 2011). The other major caveat associated with sphere forming assay is that stem cell frequency is a relative measure of stem and progenitor cell frequency. Previous studies suggest that neurosphere culture technique overestimate the stem cell frequency as both stem and progenitor cells have the ability to form neurospheres, but passaging may eliminate the transiently amplifying progenitors (Louis et al., 2008; Reynolds and Rietze, 2005). But recent studies have shown the tumorigenic capacity of *in vitro* neurosphere cultures thereby validating this technique.

Recent studies have seen the realization that gliomaspheres are important models for understanding tumor biology and have an impact on the prognosis of glioma (Laks et al., 2016; Laks et al., 2009). Because of the time taken for performing gliomasphere formation assay, the clinical feasibility of the assay becoming the main diagnostic criteria is limited. Neverthless, it is a better predictor of PFS, but mandates further research. The use of neurosphere culture as an in vitro model system has been supported by the ability of these spheres to preserve all the major mutations associated within the neoplasm and to produce tumors showing the clinical and biological nature as that of parent tumor following xenotransplantation (Laks et al., 2016; Laks et al., 2009; Nolte et al., 2013). It is a fundamental step to isolate GSCs using gliomasphere as in vitro model for tumors and that serve as a tool for elucidating the genes and molecular pathways that account for the proliferation and tumorigenic potential of glioma.

V.2. Autophagic status among different grades of glioma

Autophagy is a process in which different cellular organelles are packaged within specific vesicles called autophagosomes whose contents get digested within

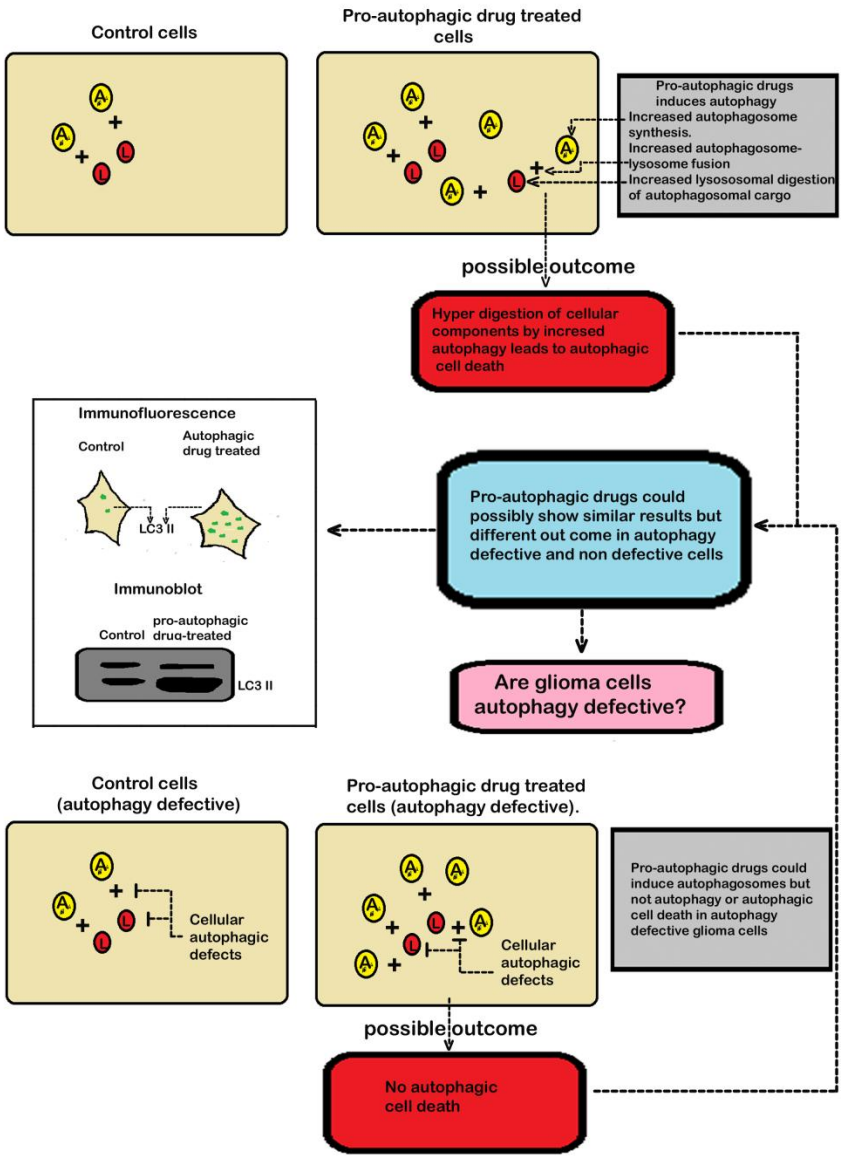
lysosomes. Autophagy, like apoptosis, can be utilized as a cell death mechanism since autophagy is capable of systematic dismantiling of cells by hyperdigesting cellular components. The therapeutic insensitivity of glioma, mainly due to its intrinsic resistance to apoptosis (type I programmed cell death), are attempted to overcome by activating autophagy using pro-autophagic drugs (Lefranc et al., 2005; Lefranc and Kiss, 2006). However, the utilization of pro-autophagic drugs has not yielded expected benefits in terms of tumor control or patient prognosis. The probable reasons for such failure have not yet been thoroughly investigated.

Autophagy alterations/malfunctioning is often associated with the etiology of important human diseases, such as neurodegenerative disease (Alzheimer's disease, Frontotemporal dementia, Parkinson's disease or Huntington's disease) and cancer (Lee, 2012). This raises the question "Is autophagy defective in glioma cells?" and may be the reason for pro-autophagic drugs not showing significant therapeutic benefit. Autopahgy, as an intracellular clearance pathway, plays an important role in removing the waste materials and preventing the accumulation of unwanted components inside cells (Ventruti and Cuervo, 2007). Brain is a highly specialized and dynamic organ which consists of slow/non-dividing cells mainly post-mitotic neurons that are more sensitive to accumulation of toxic components than rapidly dividing ones. Thus, quality control of autophagy is essential for the survival and normal functioning of brain. In addition, brain is a highly energy dependent organ and autophagy plays an important role to provide energy through breakdown of complex macromolecules during starvation. Thus, a constitutive level of basal autophagy is essential for the proper functioning of brain in terms of maintaining cellular homeostasis, constitutive removal of abnormal proteins and also providing

an alternate metabolic pathway by recycling and degrading components under nutrient deficient or stress conditions thereby preventing neurodegeneration and other diseases (Vellai et al., 2007). Recent series of studies have supported the concept that autophagy is very essential for the maintenance of normal neuron function and the deficiency or mutation of autophagy genes has been associated with many cancers and several pathological conditions in brain (Lee et al., 2013a). In this context, whether any defect in autophagy exists in glioma is not as such convincingly analyzed. However, given the complexities of glioma and no significant improvement after treatment with pro-autophagic drugs, it is important to assess autophagy.

Most of the traditional methods analyze pro-autophagic response of cells aganist the drugs by analyzing an increase in the expression of LC3-II, an autophagosome marker protein, either using immunoblotting or immunohistochemsitry. Increase of autophagosomes, in a strict sense, could not be considered as an increase in autophagy since completion of autophagy requires the fusion of autophagosomes with lysosome and the digestion of autophagosomal cargo within lysosomes (Asanuma et al., 2003). There is a theoretical possibility that the cells that are defective in autophagosome-lysosomal fusion machineries or lysosomal defects could exhibit an increase in autophagosomes/LC3-II during pro-autophagic drug treatment, but could not induce proper autophagy or autophagic cell death. However, the traditional approach of assaying autophagy by analyzing the autophagosomal increase in cells could not detect such an autophagic defect, if exists. The Figure 33 depicts the possible outcomes after pro-autophagic drug treatment in glioma cells.

Figure 33. Possible outcomes after pro-autophagic drug treatment in glioma cells

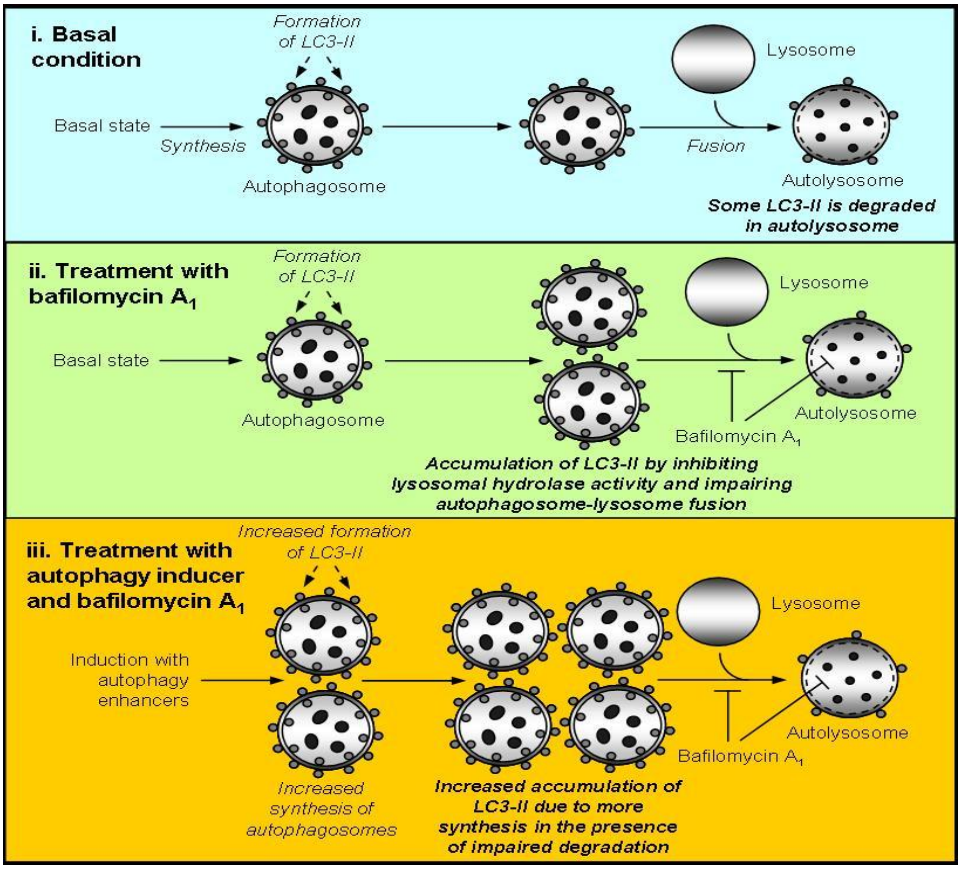


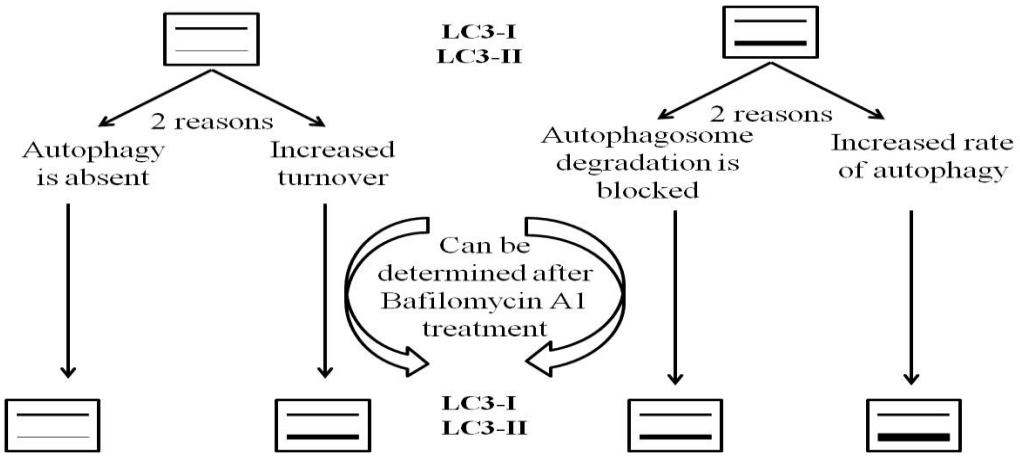
Pro-autophagic drugs can induce autophagy leading to increased synthesis of autophagosomes and eventually cell death, whereas in autophagy defective conditions therewill be an increase in autophagosomes but no cell death. Thus pro-autophagic drugs can show similar results, but different consequences in autophagy defective and non-defective cells.

The doubts on such limitations of this traditional approach could be overcome by performing an autophagic flux analysis using inhibitors that block autophagy at the

specific stages that will give an idea of the real autophagic activity (Tanida et al., 2005). For that purpose, Bafilomycin A1 which blocks the final step of autophagic degradation has been used to study the autophagy flux. Accumulation of autophagosomes/LC3-II after Bafilomycin treatment confirms that autophagy is occurring without any defect (Kawai et al., 2007; Klionsky et al., 2008b). None of the studies hitherto has checked the basal autophagic status of glioma cells. In this scenario, analyzing the basal status of autophagy by performing a flux assay in different grades of primary cultured glioma cells was done. The Figure 34 depicts the expression status of autophagosomes/LC3 II during increased or blocked autophagy.

Figure 34. Image showing the expression of LC3-II levels under conditions of blockage and induction of autophagy





LC3-II is the main autophagy marker and it gets degraded in the autolysosome, hence the level may fluctuate depends on its turnover rate. Thus autophagy can be exactly determined only after treatment with Bafilomycin A1.

(figure adapted from Sovan Sarkar; Autophagic clearance of aggregate-prone proteins associated with neurodegeneration, Methods in enzymology)

The question whether autophagy is absent or defective in glioma was analyzed in primary cultured cells isolated from different grades of human glioma by performing an autophagic flux analysis after treatment with Bafilomycin A1. Initially, after Bafilomycin A1 treatment acridine orange staining was performed to detect the accumulation of autophagosomal vacuoles associated with autophagy. Even though acridine orange staining provides a primary indication about the increase of autophagosomes in glioma cells it cannot be used alone as fool proof evidence about autophagic increase. To obtain convincing evidence about autophagic activity in glioma cells the endogeneous expression status of autophagic markers LC3 II and p62 was analyzed. LC3-II is a marker protein present in the membrane of autophagosomes whereas p62 is a protein that tags the cargo and directly interacts with LC3-II. During autophagy, autophagosomes fuses with lysosomes forming autophagolysosomes and the components of autophagosomes including LC3 II

present in the autophagosome membrane and p62 with its cargo is degraded in the lysosomes (He and Klionsky, 2009; Mizushima and Yoshimori, 2007). Upon Bafilomycin A1 treatment for a time period, there will be an accumulation of autophagosomal cargo including LC3-II and p62 because of the blocking of final degradation step of autophagy (Kawai et al., 2007; Klionsky et al., 2008b). Under normal conditions, if autophagy is non-defective, a low level of constitutive autophagy is present for clearance of cellular components. In this context, if autophagy is occurring in gliomas, in the presence of Bafilomycin A1 it should accumulate more amounts of autophagosomes/LC3 II and p62 compared to control cells (that was not treated with Bafilomycin A1). Our results showed an increase in the levels of LC3-II and p62 in all grades of glioma after treatment with Bafilomycin A1 when compared with non-treated control thereby suggesting that autophagy is non-defective in all of the four grades of glioma. This is the first study that has analyzed extensively the basal autophagic status of different grades of glioma. To the best of our knowledge, dynamics of p62 degradation in different grades of glioma has not been studied so far.

The expression of LC3-II and p62 in different grades of glioma was also analyzed using traditional approach directly at the tissue level by immunohistochemical analysis and immunoblotting after extraction of proteins from tissue. There was a recent study that suggested the use of LC3-II antibody for immunohistochemical analysis in patient tissues and also in *in vitro* settings to determine autophagy, as there will be a punctuate pattern associated with LC3-II formation in contrary to LC3-I seen in a diffuse pattern (Holt et al., 2011). In our study, the

immunohistochemical analysis of LC3-II and p62 has shown only a diffuse pattern of staining and only some samples has shown a significant punctate pattern associated with autophagy whereas immunoblotting has shown LC3-II expression only in grade IV glioma. The near lack of expression of LC3-II in grade I, II and III may be because of the low detectable level of autophagy. P62 was present irrespective of the grade when immunoblotting was performed. Beclin 1 is a tumor suppressor protein associated with autophagy and studies have suggested that its expression will give an indication of the presence of autophagy in a particular tissue (Miracco et al., 2007). The immunohistochemical data of Beclin 1 has shown a diffuse staining pattern, whereas upon Western blotting at the tissue level this protein was expressed in all grades of glioma except grade I. These results indicate that traditional approach of analyzing autophagy markers *in situ* can only be used when there is an over-expression of protein and it also won't give the overall increase of autophagy at the same time autophagy flux assay would be more appropriate to measure the real autophagy activity.

We also compared the basal autopohagic status between gliomaspheres and differentiated glioma cells by performing flux analysis. A recent study has demonstrated that the glioma stem/progenitor cells which are acclaimed to be responsible for the growth and propagation of glioma have impaired autophagy (Zhuang et al., 2011). Recently there are several studies which suggests GSCs are resistant to TMZ induced autophagy; as the autophagy related proteins were found to be down regulated, that might contribute for the increased resistance and recurrence of tumors following TMZ therapy and induction of autophagy induces the

differentiation of GSCs which sensitizes to therapies (Fu et al., 2009). Our study also demonstrated the resistance of GSCs towards differentiation by the ability of the isolated glioma cells subjected to differentiation cues to reform the gliomaspheres thereby showing an inherent ability to maintain the immature state. In this context, we compared the basal autophagic status of differentiated glioma cells and gliomaspheres by performing flux analysis after treatment with Bafilomycin A1 and probing for LC3-II and p62 expression. LC3-II and p62 proteins were detected in both differentiated glioma culture and gliomaspheres, but their expression was higher for the former compared to latter. Our result concur with the previous findings that gliomaspheres have only a minimal expression of autophagic proteins/decreased autophagy compared to the differentiated cells, thereby indicating that GSCs have low autophagy profile in comparison with differentiated cells. This result suggests the possibility that, the decreased autophagy profile of GSCs compared with differentiated cells might be the probable reason for GSCs getting spared even after multimodality treatment. But conclusive evidence in this regard warrants further studies with more patient samples.

Brain cells are highly vulnerable for starvation effect and the tumor cells from the GBMs have reported to have three times more upregulated glycolysis than in normal brain tissue (Vlashi et al., 2011). Recent reports have shown that short term starvation enhances the sensitivity of glioma cells to chemotherapy and also leads to increased survival (Safdie et al., 2012). Since pro-autophagic drugs are used for the treatment of glioma, we next studied whether the autophgy is getting induced in glioma during starvation. The study has been conducted in both nutrient rich

(DMEM with 10% FBS) and starvation (HBSS) conditions in all grades of glioma. In our study, autophagy was found to be activated during starvation and also suggests that autophagy is inducible in all grades of glioma in response to aminoacid starvation. Autophagy as such cannot be considered as cell death mechanism. Recent reports indicate that autophagy is not only a cell death mechanism but also is induced during adverse conditions such as starvation as a mechanism for producing aminoacids within the cell thereby acting as a nutrient source and rescuing the cells during nutrient limitations (Kang and Avery, 2008). This raises the question what is the exact role of autophagy in glioma: pro-survival or pro-death. To explore the role of autophagy in glioma derived cells, the cells were exposed to three different conditions: starvation (HBSS), starvation along with autophagic inhibitors (both Bafilomycin A1 and 3-methyladenine were separately used with HBSS) and was compared with control grown in 10% FBS containing DMEM, followed by MTT cell viability assay. The study demonstrated the existence of two phenotypes in glioma: one showing a significant decrease in the viability when autophagy was inhibited and other with no change in cell viability even after blocking autrophagy. Our results indicate that autophagy is not just a cell death pathway but also may play a survival role thereby benefitting the tumor growth. But further studies using pro-autophagic drugs and analyzing the role of autophagy after treatment with these drugs are needed to elucidate the exact role.

The phosphorylation status of mTOR substrates were also assessed in different grades of glioma as they are the major regulators of autophagy. mTOR pathway plays an important role in cell growth and survival and also connects autophagy with

the nutrient status of a cell in such a way that mTOR activation results in the negative regulation of autophagy (Fan et al., 2006; Hosokawa et al., 2009). Recent series of reports also suggest that mTOR modulates key signalling pathways that promote uncontrolled proliferation of glioma and thus inhibition of mTOR profoundly sensitizes glioma to therapies (Eshleman et al., 2002; Iwamaru et al., 2007). There were also reports showing the frequency of phosphorylated mTOR and its target proteins p-4E-BP1 and p-p70S6K expression increase with the histological grade in glioma (Annovazzi et al., 2009; Korkolopoulou et al., 2012). Thus the phosphorylation status of mTOR substrates: p70 S6 kinase and 4E-BP1 were analyzed at the tissue level by immunoblotting. The phosphorylation of the mTOR substrates was seen only in low grade gliomas (I and II) whereas it was absent in high grade gliomas. The non-phosphorylation of mTOR substrates reflects a decreased metabolic status of cells and can be explained with previous reports showing high grade tumors were in general less glycolytic than low grade which renders them an ability to live in adverse nutrient deficient conditions (Vlashi et al., 2011). It is in line with what is known from the literature on the relationship between autophagy and mTOR substrate phosphorylation status there exists a inverse correlation, in a way that, glioma grade with expression of phosphorylated mTOR substrates (grade I and II) there was low/no LC3-II expression at the tissue level and vice versa.

The presence of non-defective basal autophagy in low and high-grade glioma has been demonstrated in our study, yet the prognostic relevance of autophagy 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

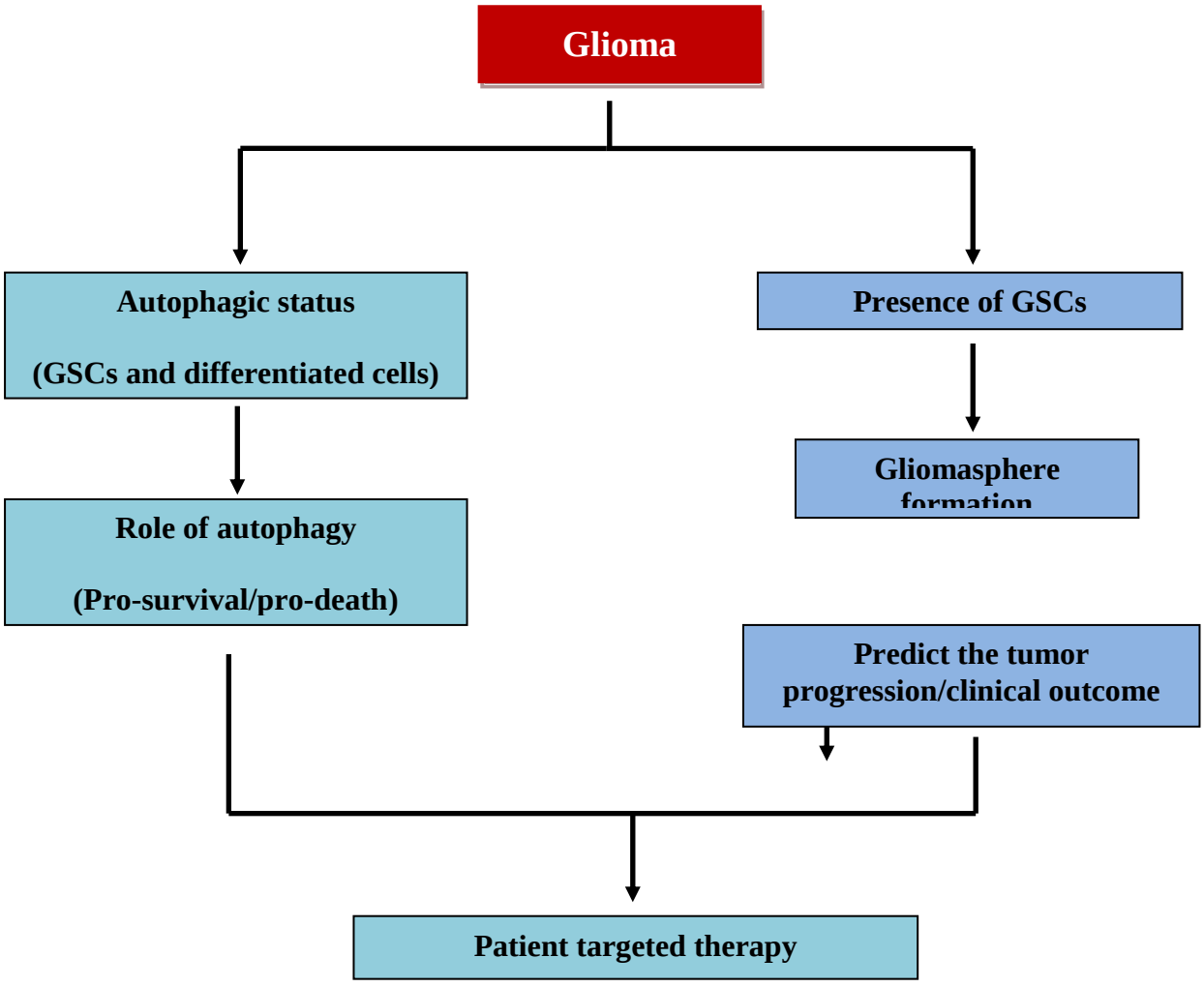
prognosis and treatment outcome in various tumors (Fujii et al., 2008; Hao et al., 2014; Karpathiou et al., 2011; Tang et al., 2013; Wan et al., 2010). High LC3 expression has been associated with metastasis and vasculogenic memory in melanoma (Han et al., 2011). In addition, increased LC3-II and p62 expression were associated with aggressive clinicopathologic features and poor prognosis in oral squamous cell carcinoma (Liu et al., 2014). By contrast, there are also studies that show autophagy suppresses tumor progression and contributes to good prognosis in lung tumors and human hepatocellular carcinomas (Guo et al., 2013; Lee et al., 2013b). Decreased expression or allelic loss of beclin 1 correlates with poor clinical results in ovarian, hepatocellular and breast cancers (Liang et al., 1999). Although there are evidences implicating the prognostic function of autophagy marker expression in HGG and GBM (Hu et al., 2012; Huang et al., 2010; Pirtoli et al., 2009), 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 (Kimmelman, 2011; Knizhnik et al., 2013; Pirtoli et al., 2009). Elevated cytoplasmic expression of beclin 1 (BPCE) score was associated with good prognosis in HGG (Pirtoli et al., 2009). Reduced expression of autophagic proteins (LC3-II and beclin 1) has been shown to be correlated with the progression of astrocytic tumors (Huang et al., 2010). On the contrary, in a study high LC3-II staining was associated with poor prognosis and resistance to radio-and chemotherapy in gliomas (Ge et al., 2009a; Winardi et al., 2014). Several recent studies reports that, autophagy induction has been reported to be associated with tumor cell survival and adaptation to nutrient stress in GBM as well as radio resistance of glioma stem cells (GSCs) (Hu et al., 2012; Lomonaco et al., 2009).

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. Recently, several studies have shown that inhibition of autophagy sensitises tumor cells to chemoradiotherapies (Karagounis et al., 2016; Koukourakis et al., 2015; Sui et al., 2013). Similarly, studies also revealed that inhibition of autophagy sensitized glioma cells to treatment (Ge et al., 2009b; Ito et al., 2005). Recent studies in glioma have shown that autophagy induced in response to radio and chemotherapy (TMZ) can contribute to therapy resistance (Koukourakis et al., 2016; Yan et al., 2016). Our findings regarding high LC3/beclin 1 expression and poor prognosis in gliomas were similar to findings from various recent studies (Ge et al., 2009a; Winardi et al., 2014). This observation indicated that autophagy might promote tumor progression, but owing to small sample size additional studies need to be performed to clarify the relationship.

The insights from this study identifies stem cells and the autophagic status of glioma as a promising area for further understanding the pathophysiology and potential for targeted therapies. Even though the pro-autophagic drugs are used for the treatment of glioma, there was no study that checked the status of autophagy "defective or not" in gliomas, this work is the first one that found out autophagy is non-defective in all grades of glioma. The study also confirmed that autophagy is capable of induction in a starvation condition as recent reports indicate the use of short term starvation to sensitize glioma to chemotherapy. Those abovementioned observations, together with the data presented here, encourage us to speculate that targeting GSCs along with autophagy could be a promising therapeutic strategy to enhance current therapy

in patients as poorly differentiated GSCs are the major fuel for tumor growth. Thus it is worth studying the importance of autophagy in glioma in a large population for further understanding the exact mechanism involved in its regulation and ultimately for designing rationale therapeutic strategies.

Figure 35. Proposed strategy for targeting glioma



The studies suggest that presence of GSCs and loss of function of autophagy plays an important role in treatment resistance of glioma. The induction of GSC terminal differentiation along with autophagy activation may help to sensitize gliomas to therapeutic approaches as poorly differentiated GSCs are the major fuel for tumor growth. Thus Targeting GSCs along with autophagy is a potential therapeutic strategy to enhance current therapy in patients with glioma.

VI. SUMMARY AND CONCLUSIONS

The gliomasphere forming ability and autophagic status of glioma was analyzed in detail. We paid particular attention to gliomasphere formation ability among different grades of glioma because they are not extensively documented, whereas the few articles already present in the literature highlight their marked roles in glioma progression. Based on gliomasphere formation and the expression of known GSC markers, we have shown that all grades of glioma contain a stem cell population *in vitro*. The stem cell markers CD133, CD15 and nestin were expressed in all the four grades of glioma. Differentiation studies have showed that they have a multipotent differentiation capacity to neuronal lineage marker beta III tubulin and glial lineage GFAP. In our study, the generated gliomaspheres were found to express known stem cell markers, thereby providing evidence to support that GSCs were isolated from low and high-grade glioma. Other supporting shreds of evidence include the isolated neurospheres can self-renew, can differentiate into multiple lineages and display sphere reformation ability showing its intrinsic potential to resist differentiation. Taken together, these results suggest that gliomaspheres from both low and high-grades of human glioma contain a subpopulation of GSCs which possess all the defining features of stem cells including self-renewal and multipotent differentiation. Our study also demonstrates a significant association between gliomasphere formation and clinical severity of the tumor as PFS was strongly related to gliomasphere formation and demonstrate for the first time that both LGG and HGG neurosphere forming group showing a significantly shorter PFS with an increased survival advantage in LGG compared to HGG. In conclusion, the present study further highlights the importance of neurosphere culture system for GSC research. It is a cardinal step to isolate GSCs using neurosphere as *in vitro* model for tumors and

may serve as a tool for elucidating the genes and molecular pathways that account for the proliferation and tumorigenic potential of glioma.

The primary sphere formation and secondary sphere generation capacity were extensively studied and was found to provide new insights into the development and behavior of gliomaspheres *in vitro* in both high and low grade glioma. The study suggests that the ability to form renewable gliomaspheres may serve as an indicator of stem cell population within the tumor thereby predicting the clinical aggressiveness/outcome of the tumor. Gliomasphere formation might prove to be an adequate marker that predicts the clinical course, as the biological behaviour of gliomas is not predictable based on the existing prognostic factors. But it warrants further investigations to shed light on the potential value of gliomaspheres to predict the treatment outcome.

The autophagy status in all of the four grades of glioma was analyzed both *in vitro* and also in tissues using autophagy markers. We have used *ex vivo* primary culture studies with glioma cells to understand the autophagic flux in response to Bafilomycin A1 treatment. The present study indicated that autophagy is not impaired and is capable of induction in all the four grades of glioma. To the best of our knowledge, this is the first study that monitors the autophagic flux in different grades of glioma. Since, the primary glioma tissue is used for study; it can be argued that it directly reflects the *in vivo* condition, if not, more close to *in vivo* scenario. However additional studies using increased sample number and assessment of cell viability after treatment with autophagy inhibitors (Bafilomycin A1 and 3-methyladenine) will be required to determine the exact role of autophagy in glioma.

Further experiments with pro-autophagic drugs will be required to describe the response of autophagy in more detail and an understanding of the therapeutic role of autophagy in glioma.

Gliomas are highly heterogeneous tumors because even same grade and pathological subtype are known to have different prognosis and response to treatment. Our study suggests that analyzing the gliomasphere formation rate and role of autophagy will help to determine the clinical otcome of glioma.

VII. REFERENCES

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Publications

Padmakrishnan CJ, Easwer HV, Girish Menon R, Krishna Kumar K, Suresh Nair, Bhavya Bharathan, Neelima Radhakrishnan and Srinivas Gopala. *In vitro neurosphere formation correlates with poor survival in glioma*. IUBMB Life. 2018. Article DOI: 10.1002/iub.1964. **IF=3.236**

Padmakrishnan CJ, Easwer HV, Vinod Vijayakurup, Girish Menon R, Suresh Nair and Srinivas Gopala. *High LC3/Beclin expression correlates with poor survival in glioma: a definitive role for autophagy as evidenced by in vitro autophagic flux*. Pathol Oncol Res. 2017 Oct 11. DOI: 10.1007/s12253-017-0310-7. **IF=1.935**

Vinod V, **Padmakrishnan CJ**, Vijayan B, Gopala S. *'How can I halt thee?' The puzzles involved in autophagic inhibition*. Pharmacol Res. 2014. DOI: 10.1016/j.phrs.2014.03.005. **IF=4.408**

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Conference/Presentations

Oral Presentation -"Isolation and characterization of glioma stem cells from different grades of human glioma tissues" at 4th International conference on stem cells and cancer (ICSCC-2013): Proliferation, Differentiation and Apoptosis, 2013.

Poster Presentation - "Gliomasphere forming ability and autophagic status of cells isolated from human glioma tissue" at International Conference on Advances in Biological Sciences, 2012.

Oral Presentation - "Gliomasphere forming ability and autophagic status of cells isolated from human glioma tissue" at Institute Day Scientific meet, SCTIMST, 2012.

Fellowships

2009 - SCTIMST Institute Fellowship for PhD programme - India (awarded to two candidates).

Awards

Third prize - 4th International conference on stem cells and cancer (ICSCC-2013): Proliferation, Differentiation and Apoptosis, 2013.

Best speaker - Institute Day Scientific Meet, SCTIMST, 2012.

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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.

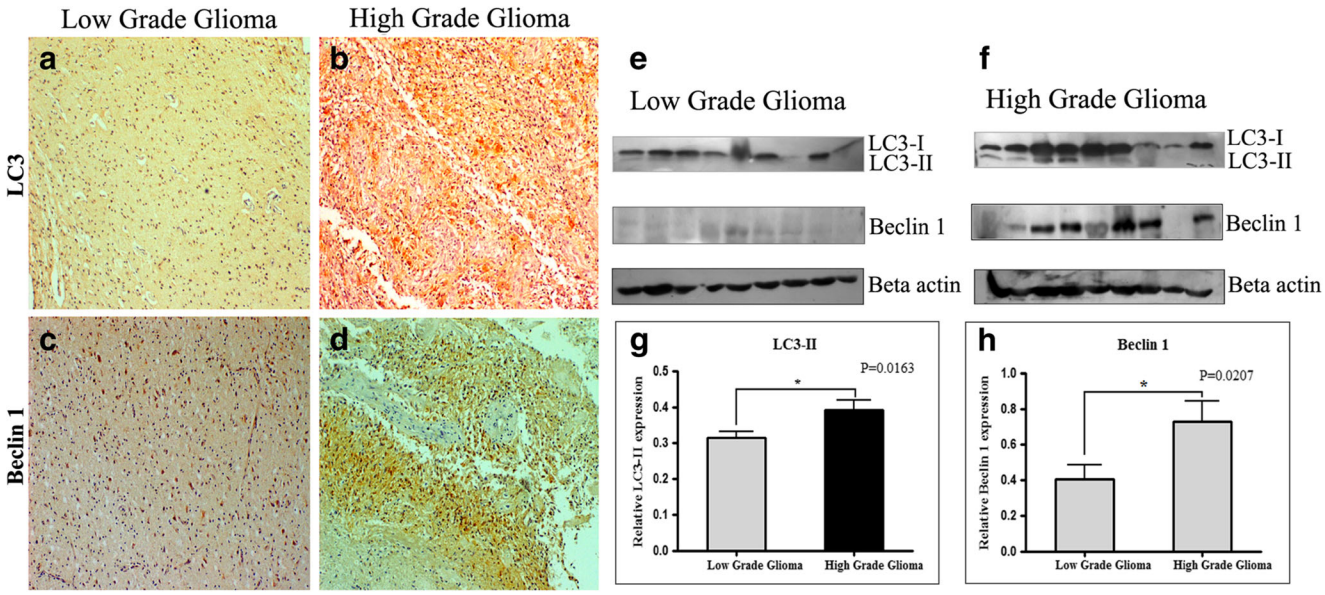

Fig. 1 Evaluation of markers of autophagic process in low and high grade glioma. **a**–**e** Representative immunohistochemistry pictures showing the expression of LC3-II and Beclin 1 (10×) in low and high-grade gliomas. Human glioma tissue sections were fixed with buffered formalin and expression of 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

* $p < 0.05$

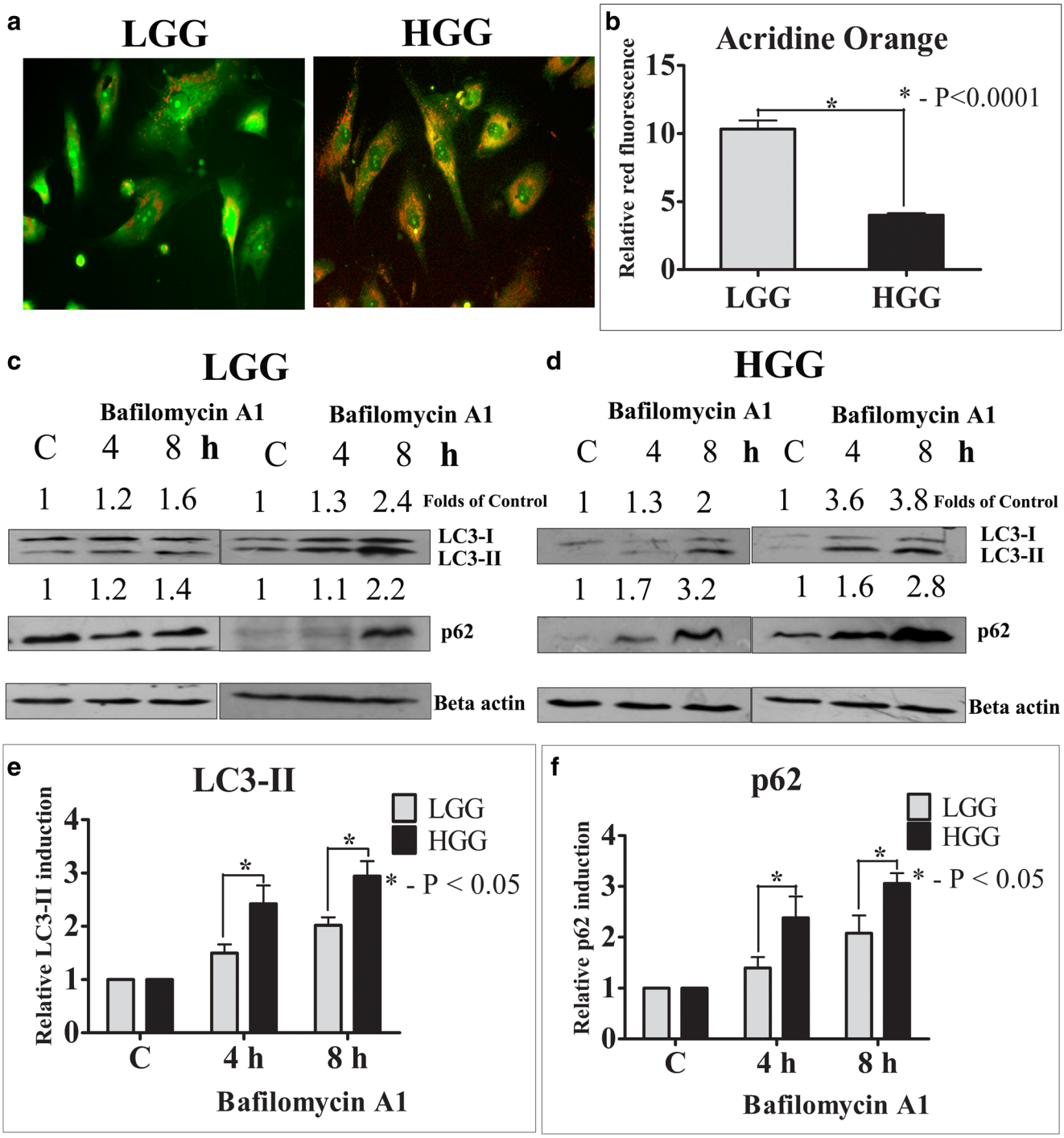

Fig. 2 Determination of autophagic flux in primary cultured glioma cells. **a**, **b** Detection of autophagy in low and high-grade glioma cell cultures before and after bafilomycin A1 treatment by acridine orange staining. **a** Glioma cells from LGG and HGG were stained with acridine orange, the bright 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.

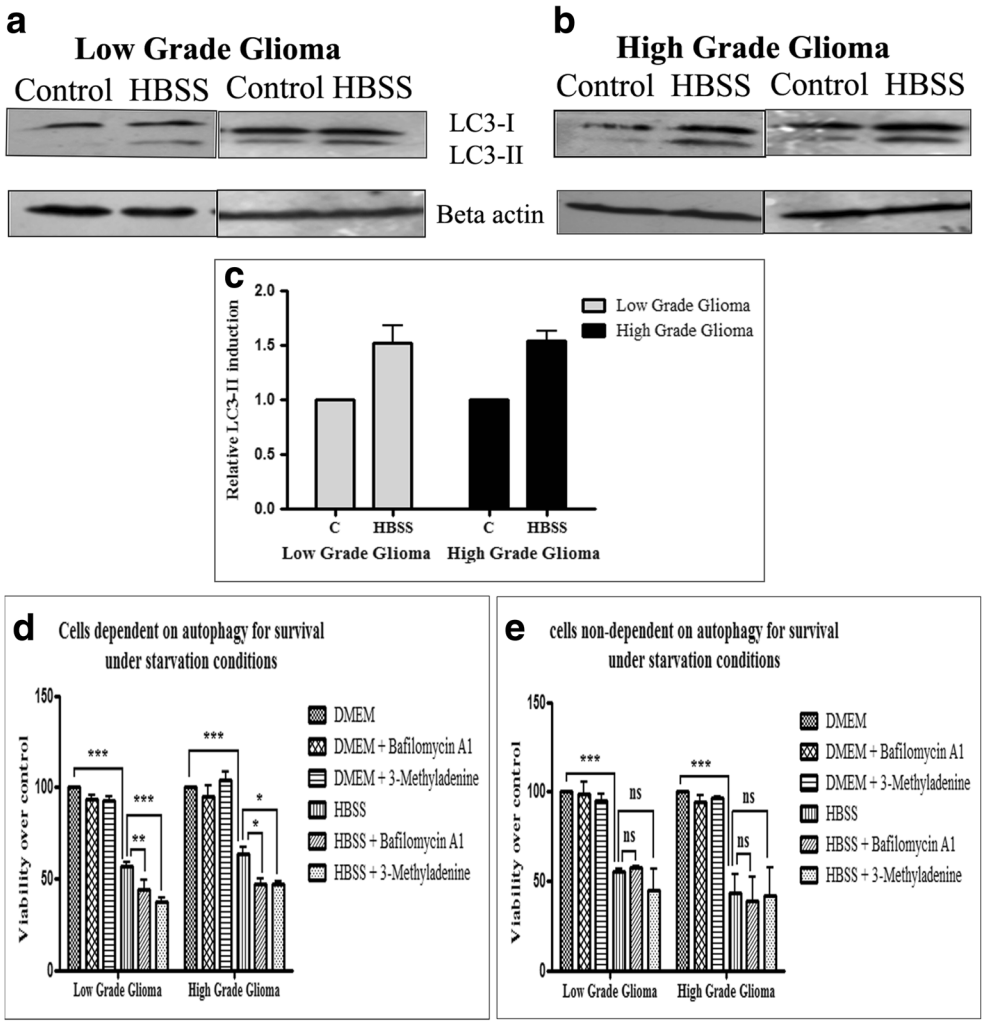

Fig. 3 Autophagy is induced under starvation conditions. **a**–**c** Starvation induces autophagy in both low and high-grades of glioma. **a** LGG **b** HGG. In both low and high-grades of glioma, LC3-II expression was more intense in cells grown in starvation condition (HBSS) when compared to control 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 autophagicy 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

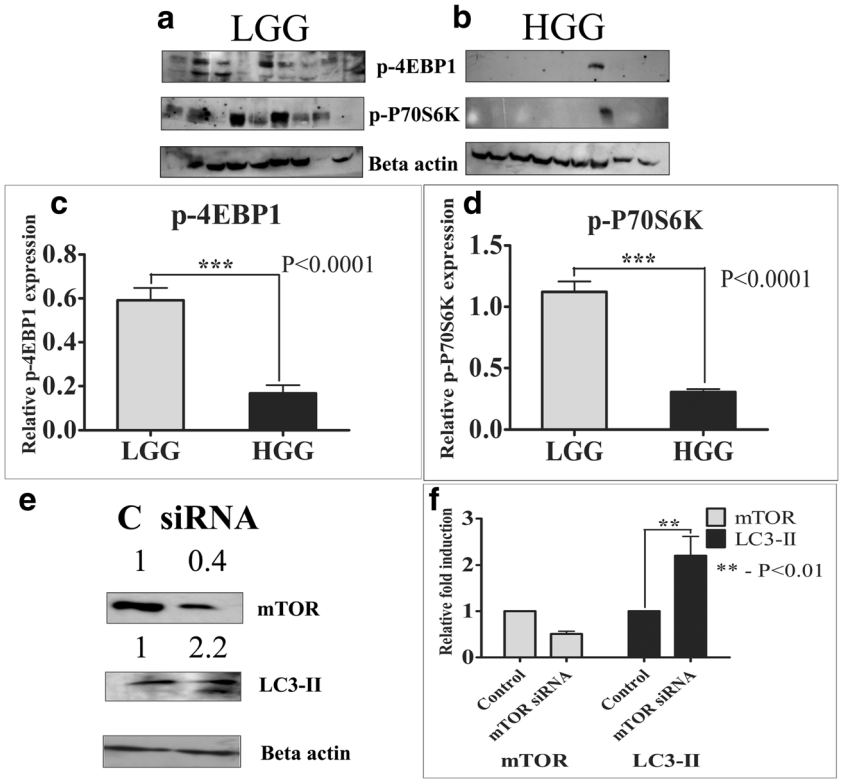

Fig. 4 mTOR expression in relation to autophagy in gliomas. **a**–**d** Western blotting of mTOR substrates p-4EBP1 and p-P70S6kinase expression. Representative blots of phosphorylation status of mTOR substrates in low and high grade gliomas **a** Astrocytoma II **b** GBM. **c**, **d** Quantitative analysis 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)

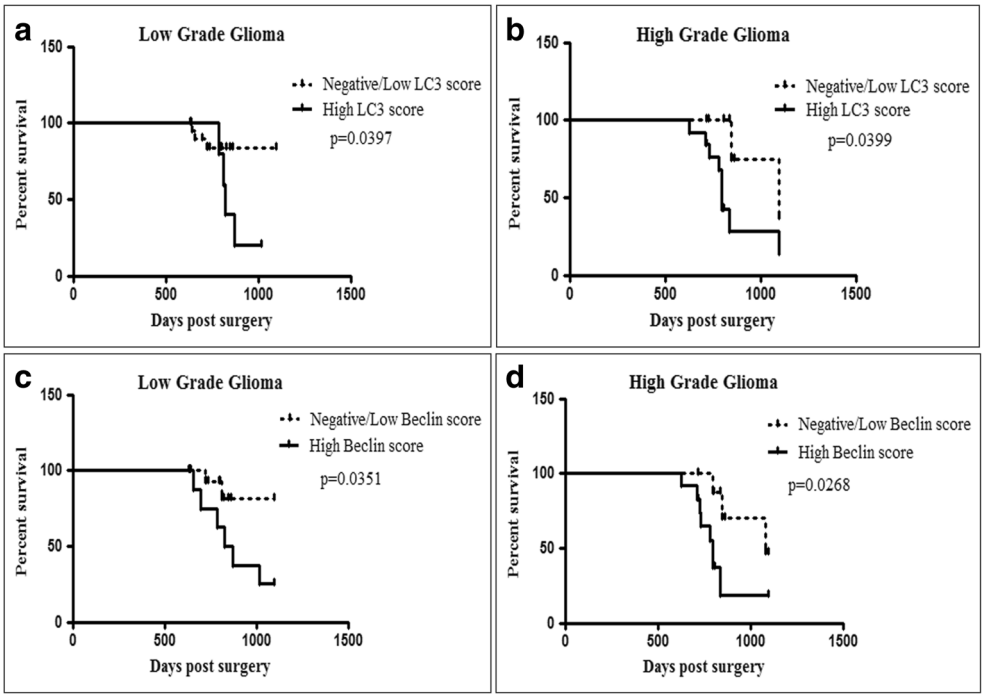

Fig. 5 Evaluation of the relationship between LC3-II and beclin 1 protein expression and progression free survival of low and high grade glioma patients using Kaplan-Meier method. **a**, **b** Plot comparing the high and negative/low LC3 expression with survival. **a** LGG **b** HGG. **c**, **d** Survival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

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Research Communication

In Vitro Neurosphere Formation Correlates with Poor Survival in Glioma

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Abstract

Sphere formation is an indicator of tumor aggressiveness independent of the tumor grade; however, its relation to progression-free survival (PFS) is less known. This study was designed to assess the neurosphere forming ability among low grade glioma (LGG) and high-grade glioma (HGG), its stem cell marker expression, and correlation to PFS. Tumor samples of 140 patients, including (LGG; $n = 67$) and (HGG; $n = 73$) were analyzed. We used sphere forming assay, immunofluorescence, and immunohistochemistry to characterize the tumors. Our study shows that, irrespective of the pathological sub type, both LGG and HGG formed neurospheres in vitro under conventional sphere forming conditions. However, the number of neurospheres formed from tumor tissues were significantly higher in HGG compared to LGG ($P < 0.0001$). Different grades of glioma were further characterized for the expression of stem cell marker proteins and lineage markers. When neurospheres were analyzed, CD133 positive cells were identified in addition to CD15 and nestin positive cells in both LGG and HGG. When these neurospheres were subjected to differentiation, cells positive for GFAP and β-tubulin III were observed. Expression of stem cell markers and β-tubulin III were prominent in HGG compared to LGG, whereas GFAP expression was higher in LGG than in HGG. Kaplan–Meier survival analysis demonstrated that neurosphere forming ability was significantly associated with shorter PFS ($P < 0.05$) in both LGG and HGG. Our results supports earlier studies that neurosphere formation may serve as a definitive indicator of stem cell population within the tumor and thus a better predictor of PFS than the tumor grades alone. © 2018 IUBMB Life, 9999(9999):1–10, 2018

Keywords: glioma; gliomasphere; neurosphere; survival; CD133; CD15

INTRODUCTION

Gliomas are the most common primary brain tumors and patients with glioma have poor prognosis and shorter survival (1, 2). Despite the multimodality therapies, the overall survival of glioma patients has not impr"oved significantly, and to date, there is no definitive cure or treatment for preventing the progression and recurrence of this tumor (3). The major prognosticator of glioma still continues to be the grade of the tumor, but recent studies have shown that even in the same grade there are two subgroups, those that respond to therapies or remain recalcitrant (4). Thus, the search for more molecular or clinicopathological factors that will accurately predict the prognosis of a highly malignant tumor like glioma is continuing.

An important advancement in glioma research is the identification of a subset of cells in the tumor mass with stem cell properties called glioma stem cells (GSCs) (5–7). GSCs were originally characterized and identified by their ability to form loosely attached spherical cluster of cells known as neurospheres (also called gliomaspheres) in defined stem cell promoting medium with the ability to form a tumor in vivo (8). GSCs demonstrate an ability to proliferate indefinitely, differentiate into neuronal and glial lineages, that may underlie the initiation, propagation, recurrence, and therapeutic resistance of the tumor (5). Recently, several reports have shown that GSCs are real cancer reservoirs with an intrinsic resistance to treatments, leading to tumor recurrence, progression, and non-responsiveness to

Abbreviations: *GSC, glioma stem cells; HGG, high grade glioma; LGG, low grade glioma; PFS, progression free survival*

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therapies (5–7, 9–11). The study on GSCs from different grades of glioma would shed light on the biologic basis of tumor aggressiveness and the prognosis of this disease. Recent evidences have suggested that neurosphere formation, an indicator of the presence of GSCs, is a predictor of glioma tumor progression and clinical outcome independent of grade (4, 12).

Neurosphere forming assay is a technique used for isolation and characterization of GSCs from primary brain tumors, providing quantitative information about the stem cells (13). However, proper isolation of stem cells from the bulk tumor and maintenance of culture conditions that are ambient to preserve the genetic and phenotypic characteristics of these cells makes such studies challenging. Another hurdle is the heterogeneity of different stem cell subtypes within the same tumor mass and lack of specific cell surface markers that distinguish GSCs from neural stem cells (NSCs), as both these cells have been found to express the same markers (e.g., CD133, CD15, nestin) (9). The aim of this study was to assess the neurosphere formation ability among low-grade gliomas (LGGs) and high-grade gliomas (HGGs), its stem cell and lineage markers expression, and also whether there exists a correlation between neurosphere formation and progression-free survival (PFS) in glioma.

MATERIALS AND METHODS

Patient Samples and Clinical Information

Human glioma tissues were collected from patients who underwent surgical tumor resection at the Department of Neurosurgery, Sree Chitra Tirunal Institute for Medical Sciences and Technology as approved by the Institutional Ethics Committee (IEC/192). All patients provided written informed consent for participation in the study. Glioma specimens were histopathologically classified according to the world health organization (WHO) classification (2007) by a neuropathologist. The prospective study was conducted during the 2009–2013 period, fresh biopsy samples were taken for protein isolation and for primary culture of glioma cells and a part of the tissue sample was fixed in formaldehyde for immunohistochemical analysis. Data collection was completed in 2015. The neurosphere formation ability was studied in tissue samples obtained from 140 glioma patients, which consisted of 67 LGGs and 73 HGGs. LGGs included grade I and grade II, whereas HGGs consisted of grade III as well as grade IV tumors. Follow-up data of 3 years were available for 112 of the total 140 patients and was obtained from medical records.

Isolation and Culture of Primary Glioma Cells and Spheres

Tumor tissues obtained after surgical resection were washed with Hanks Balanced Salt Solution (HBSS; Sigma Chemical Co., St Louis, MO), minced to small fragments and subjected to enzymatic digestion with 0.2% papain in a laminar hood. The dissociated tumor cells were washed, subsequently resuspended, and cultured in stem cell promoting serum-free medium (SFM) consisting of Dulbecco's Modified Eagle Medium: F-12 medium (DMEM/F-12) supplemented with L-Glutamine (2 mM), B27 supplement (1×), [Invitrogen Carlsbad, CA], basic fibroblast growth factor (bFGF; 20 ng/mL), epidermal growth factor (EGF; 20 ng/mL), [Sigma Chemical Co., St Louis, MO], and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) for enrichment of neurospheres from GSCs. Serum containing medium (SCM) consisting of DMEM/F-12 medium with 10% fetal bovine serum (FBS) was used for the differentiation of glioma cells. For experiments with adherent monolayer culture, the culture supernatant was removed from the flask containing cells at 80% confluence and was trypsinized in Trypsin Phosphate Versene Glucose (TPVG) solution for 2–5 min at 37 °C. Detached cells were suspended in SCM and a known amount of cells was transferred to appropriate culture plates for further experiments.

Sphere Formation Assay

To determine the presence and frequency of a stem cell population within different grades of human glioma, sphere formation assay was performed. For the assay, finely dissociated tumor cells were plated in stem cell promoting SFM at a clonal density of 5×10^3 cells per cm^2 in 25 cm^2 tissue culture flasks (T25). Cultures were kept at 37 °C, 5% CO_2 for 7–27 days in a humidified tissue culture incubator. Neurospheres formed in cultures were monitored using phase-contrast microscope, [IX-51 (Olympus, Melville, NY)]. After 7–27 days the neurospheres were harvested and enzymatically dissociated into single cells using 2 mL TrypLE for 20 min at 37 °C and re-plated in flasks containing SFM and incubated under standard conditions. Secondary neurospheres formed also were subsequently dissociated and plated for the formation of tertiary spheres in SFM. The neurospheres after three passages were considered to have the potential of renewable neurosphere formation (4, 14).

Sphere Size Determination

Sphere diameters from 10 visual fields were measured after 3, 7, 14, 21, and 27 days using an inverted microscope IX-51 (Olympus) along with ProgRes® CapturePro Software (Jenoptik). The average size of the spheres and standard error mean were calculated (15).

Sphere Formation Rate Assay

The sphere formation rate was studied for analyzing the proliferation or self-renewal potential of neurospheres. Neurospheres were harvested and dissociated into single cells using 2 mL TrypLE for 20 min at 37 °C and cells were plated at a density of 5,000 cells/200 μL per well in a 96-well plate. Cultures were kept at 37 °C, 5% CO_2 in a humidified tissue culture incubator. After 7 days, the number of spheres formed was counted by keeping the plate under an inverted microscope IX-51 (Olympus) and was used to estimate the mean number of spheres per 5,000 cells. This assay estimated proliferation rate of sphere derived cells in different grades of glioma (8).

Immunofluorescence Staining

Immunofluorescence of randomly chosen neurospheres (LGGs: $n = 4$ and HGGs: $n = 4$) and differentiated glioma cells (LGGs: $n = 4$ and HGGs: $n = 4$) was used to detect the expression of stem cell and lineage markers. For this, the cultured cells were fixed with ice-cold 4% paraformaldehyde for 15 min at 4 °C and

then were permeabilized, and blocked with Triton X 100 and FBS, respectively. The cells were then incubated with antibodies against CD133 (rabbit polyclonal IgG; 1:50), CD15 (mouse monoclonal IgG; 1:50), nestin (mouse monoclonal IgG; 1:1,000), β-tubulin III (rabbit polyclonal IgG; 1:200), and GFAP (rabbit polyclonal IgG; 1:1,000) overnight at 4 °C. After rinsing in PBS, for detecting primary antibody, cells were incubated for 90 min at room temperature in the dark in DyLight488 conjugated secondary antibody (goat polyclonal IgG; 1:600). The cells were then counterstained with propidium iodide (PI) to reveal the nuclei. For imunostaining of differentiated tumor spheres after differentiation assay, the neurospheres were cultured on SCM for 7 days and immunofluorescence was performed. It was visualized using Zeiss LSM 510 Meta confocal microscope at ×10 magnification under the settings of 543 for PI and 488 for FITC.

Immunohistochemical Staining

The expression levels of stem cell and lineage markers in different grades of glioma tissue were analyzed by immunohistochemical staining in a panel of gliomas of different pathological types. Tissues were fixed in 10% buffered formalin, embedded in paraffin, cut into 3–5 μm sections and mounted on positively charged microscope slides. Briefly, paraffin sections of tumors were then deparaffinized/dewaxed in xylene, hydrated through graded alcohols, treated with heat induced epitope retrieval technique using citrate buffer (pH 6.0) at 95 °C for 5–20 min and allowed to gradually cool in the buffer. The tissue sections were taken out and washed with double distilled water, 3% H_2O_2 for 10 min was used to block intrinsic peroxidise activities. The slides were again washed in distilled water for 15 min and blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS, pH 7.6) for 10 min. The sections were immunohistochemically stained with the following primary antibodies against: CD133 (rabbit polyclonal IgG; 1:50), CD15 (mouse monoclonal IgG; 1:50), nestin (mouse monoclonal IgG; 1:100), β-tubulin III (rabbit polyclonal IgG; 1:250), and GFAP (rabbit polyclonal IgG; 1:5,000) 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 was used as the detection system and the reaction was visualized by using 3,3′ diaminobenzidine-tetrahydrochloride (DAB) as chromogen. The nuclei were counterstained with Mayer's hematoxylin. Slides were mounted using DPX as mounting medium.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5. Two tailed student's *t*-test was used for comparisons of protein expression results between LGG and HGG. Data were expressed as mean ± standard error of the mean (SEM). The survival analysis was evaluated by the Kaplan–Meier method. The level of statistical significance was set at $P < 0.05$. Cumulative incidence function (CIF) for competing risk analysis was done using R and statistical significance assessed using Gray's test.

RESULTS

Gliomas of Different Grades Retain the Ability to Form Neurospheres

In this study, the neurosphere formation ability of LGG and HGG biopsies were analyzed in vitro using primary cells isolated from 140 tissue samples. Within 48–72 h of plating in SFM, phase-bright clones of spherical cellular clusters called neurospheres appeared. Large numbers of spheres, of approximately 3–10 cells in diameter, were observed between 3 and 7 days after plating. Within 14 days, such spheres increased their size to approximately 50–100 cells (Fig. 1A–D). Neurospheres were formed in both LGGs and HGGs (Fig. 1I–L). Under serum containing culture conditions, glioma cells were found to grow as an adherent monolayer (Fig. 1E–H).

Tumor tissue samples that formed neurospheres for a minimum of three passages in culture were categorized as having the potential of renewable neurosphere formation. Numbers of neurosphere forming and non-neurosphere forming tumors were calculated in both LGGs and HGGs. Among the 140 lesions, 67 samples (49%) formed neurosphere in vitro. Twenty-one percent LGGs (20%—grade I and 21%—II) and 74% of HGGs formed neurospheres; in the HGG group, 77% of grade III and 72% of GBM formed primary neurospheres (Fig. 1M). The study also evaluated the relationship between numbers of neurosphere forming and non-neurosphere forming patients and clinicopathological variables, including grade, age, and sex in glioma. The data are summarized in Table 1. The number of HGG samples forming neurospheres was higher compared to the number of LGG samples that formed neurospheres ($P < 0.0001$). When neurosphere formation was examined as a function of age and sex, there was no significant association (Table 1).

Sphere Formation Rate Was Higher in HGG Than LGG

To evaluate the sphere forming capacity in relation to long-term proliferation and self-renewal within the LGG and HGG, a sphere formation rate assay was performed. After 7 days of plating, (which involved dissociation of spheres), culturing and counting the number of neurospheres were performed. The number of neurospheres formed was much higher for HGGs than LGGs ($P = 0.0002$, Fig. 1N) further confirming the presence of enhanced stem cell compartment in HGGs. The diameters of neurospheres, an estimate of growth rate of individual spheres from different samples, was measured on days 3, 7, 14, 21, and 28 after plating. Though there existed no significant correlation ($P = 0.7549$) between the size and the grades, the size of neurospheres was found to increase linearly (Fig. 1O).

Molecular Analysis of Neurospheres and Adherent Monolayer Cultured Samples Showed Significant Differences in Stem Cell and Differentiation Markers

Because the focus of the study was on neurospehere formation in different grades of glioma, only a few samples of the total 140 were utilized for immunocytochemistry. Consistent with the previous studies (5, 16), neurospheres (after three passages) generated from all the four grades of glioma exhibited immunoreactivity for

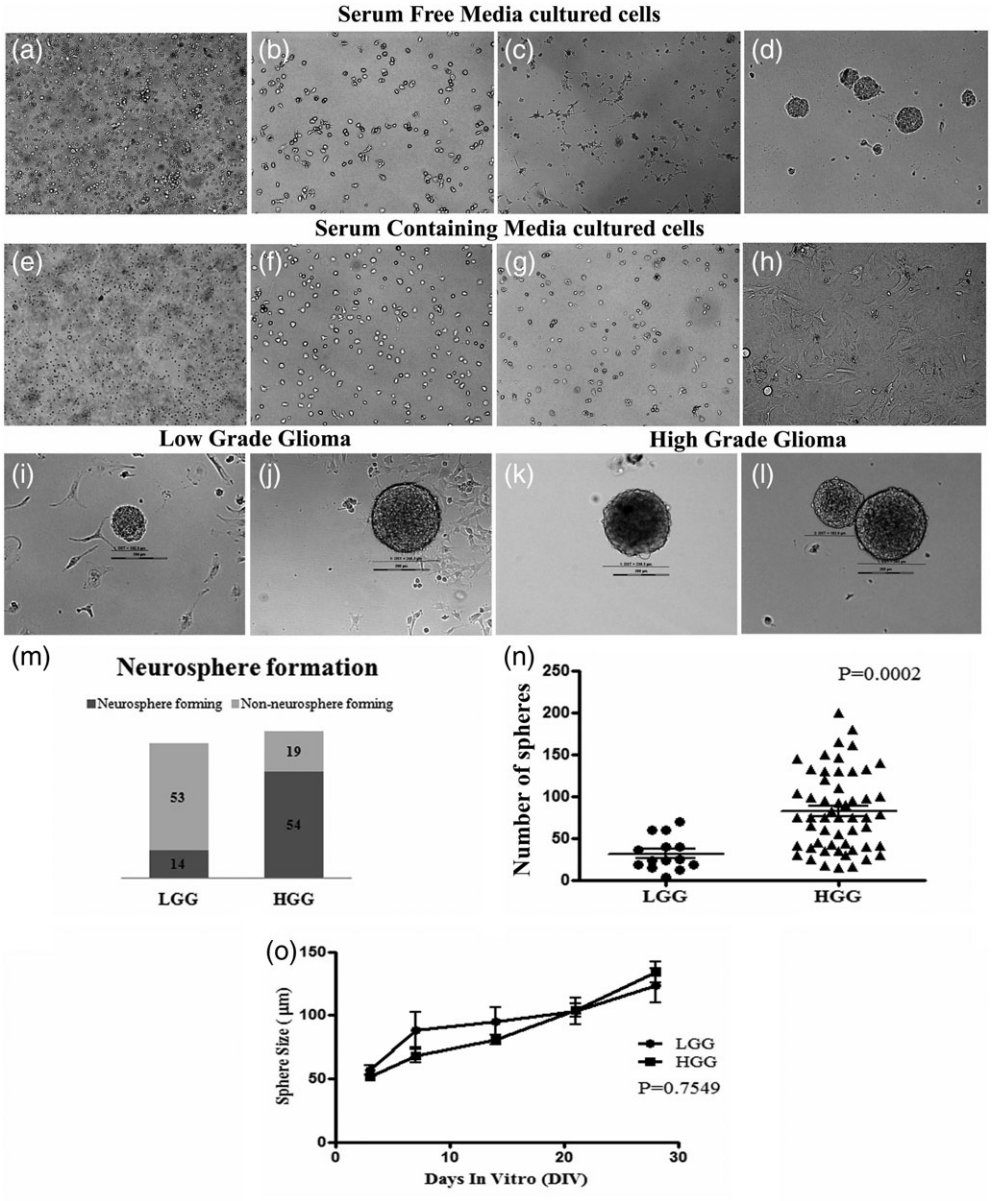

FIG 1

Phase-contrast microphotographs and plots showing examples of cultured glioma cells and neurosphere formation. A–D: Cultured glioma cells isolated as described under "methods" were grown in stem cell promoting SFM consisting of DMEM/F-12 with growth factors and supplements to form neurospheres at different time periods: (A) day 0, (B) day 1, (C) day 2, (D) day 3 (magnification 10×). E–H: Glioma derived cells cultured in DMEM/F-12 medium containing 10% FBS (SCM) for adherent monolayer primary glioma cell cultivation at different time points: (E) day 0, (F) day 1, (G) day 2, and (H) day 3 (10×). I–L: Glioma cells isolated from all the four grades when grown in SFM have the ability to form neurospheres: (I) Pilocytic astrocytoma, grade I, (J) Astrocytoma II, grade II, (K) Anaplastic astrocytoma, grade III, and (L) GBM, grade IV (10×); Scale bar = 200 μm. M: Gliomasphere formation in 140 different grades of glioma patients. First bar stand for LGG, and last bar represents renewable neurosphere formation by HGG. Renewable neurosphere forming tumors are represented in dark bars and non-sphere forming tumors in light bars. The number of tumors is represented in each bar. N: Neurosphere formation rate of different grades of glioma was plotted. The number of neurospheres generated after culturing in SFM for 7 days were compared among LGG and HGG and HGG has the highest neurosphere forming ability. Each dot is representative of one patient sample; bar represents mean of biological replicates. O: Comparison of sphere size among LGG and HGG.

stem cell markers (CD133, CD15, and nestin). $CD133^+$ and $CD15^+$ cells were relatively small populations, 1.88 ± 1.09% (mean ± SEM) and 1.89 ± 0.46%, respectively; $nestin^+$ cells on the other hand, was 32.16 ± 1.99% of cells (Fig. 2A–C). When the same populations were analyzed for lineage markers, only 4.04 ± 0.28% and 0.36 ± 0.02% of the cells were positive for GFAP and β-tubulin III, respectively (Fig. 2D,E). However, cells in non-sphere forming monolayer cultures grown in SCM stained positive for the lineage markers of CNS; GFAP (65.6 ± 5.78%) and β-tubulin III (58.65 ± 1.98%), while had minimal, weak

TABLE 1 *Summary of patient characteristics and gliomasphere formation*

	Gliomasphere forming patients	Non-gliomasphere forming patients	Total number of cases
Low grade glioma	14	53	67
Pilocytic Astrocytoma (I)	2	8	10
Astrocytoma II	6	11	17
Oligoastrocytoma II	2	23	25
Oligodendroglioma II	4	11	15
High grade glioma	**54**	**19**	**73**
Astrocytoma III	5	0	5
Oligoastrocytoma III	9	2	11
Oligodendroglioma III	9	5	14
GBM (IV)	31	12	43
Age group (6 months to 71 years)	42.6 ± 13.7	Mean ± SD (years) 37.1 ± 14.0	39.8 ± 14.0
≤10 years	3	5	8
10–30 years	10	15	25
30–60 years	48	50	98
>60 years	7	2	9
Gender			
Male	46	50	96
Female	22	22	44

expression of NSC and progenitor markers such as CD133 (0.36 ± 0.03%), CD15 (0.81 ± 0.20%), and nestin (25.77 ± 0.82%; Fig. 2F–J). Within the LGGs and HGGs, the expression pattern of stem cell and lineage markers were almost similar, and because of the inability to perform all experiments in 140 samples studied we limited immunocytochemistry experiment to a few samples.

Neurospheres Cultured in SFM Are Multipotent and Show an Intrinsic Potential to Resist Differentiation

We assessed whether tumor-derived neurospheres retained the capacity for multilineage differentiation capacity to generate multiple lineage daughter cells. To test the multipotent ability, the spheres were grown in SCM and thereafter performed fluorescence immunocytochemistry. In SCM, the spheres adhered to the plate and the cells migrated from spheres radially and stained positive for glial and neuronal lineage markers (GFAP for glial and β-tubulin III for neurons) (Fig. 3A,B).

The neurosphere's potential to reintain their immature state, even after growing under differentiating conditions were analyzed. For this, neurospheres were grown in a SCM for 2 weeks. After 2 weeks of differentiation, the cells were then switched to SFM. After 1 week of growth in SFM, the cells were immunostained for stem cell markers and stained positive for CD133, CD15, and nestin (Fig. 3C–E). The result indicates that neurospheres have cells with an intrinsic potential to resist differentiation and maintain their immature state.

Expression of Stem Cell and Lineage Markers Vary with the Grade of the Tumor

Paraffin embedded sections of all the four grades of glioma were immunostained for CD133, CD15, nestin, GFAP, and β-tubulin III. The expression levels of antigens in human glioma tissues showed considerable variability with different clinical grading ranging from a complete lack/very low immunoreactivity to high-intensity staining. The expression of stem cell markers CD133 (Fig. 4A–E) and CD15 (Fig. 4F–J) was either not detectable or expressed only in a very small fraction of cells and were detected mainly in higher grades of glioma (LGGs/HGGs, $P < 0.0001$ for CD133 and CD15). The expression of nestin (Fig. 4K–O) was diffusely present throughout the glioma cells in all tissue sections and was more intense in HGGs compared to LGGs ($P < 0.0001$). GFAP (LGGs/HGGs, $P = 0.0373$, Fig. 4P–T) and β-tubulin III (LGGs/HGGs, $P < 0.0001$, Fig. 4U–Y), the markers characteristic of glial and neuronal differentiation, respectively, was detectable in all tumors and were present diffusely throughout the section. In contrast to the high-level expression of β-tubulin III and other stem cell markers in HGG specimen's analyzed, high intensity of GFAP expression was observed in LGGs compared to HGGs.

Neurosphere Formation is Associated with PFS

We then analyzed whether there is a relation between neurosphere formation and PFS in glioma patients. The Kaplan–Meier survival analysis using log rank tests indicated that patients whose tumor showed ability to form neurospheres in culture were associated with shorter PFS in both LGGs ($P = 0.0190$, Fig. 5A) and HGGs ($P = 0.0409$, Fig. 5B). The median PFS (3 years, PFS) of neurosphere forming and non-sphere forming patients were 890 and greater than 1,095 days, respectively, for LGG ($n = 67$) patients, whereas 735 and greater than 1,095 days respectively for HGG ($n = 73$) group. Similarly, the median PFS (3 years, PFS) duration is 781 days for neurosphere forming patients and greater than 1,095 days for

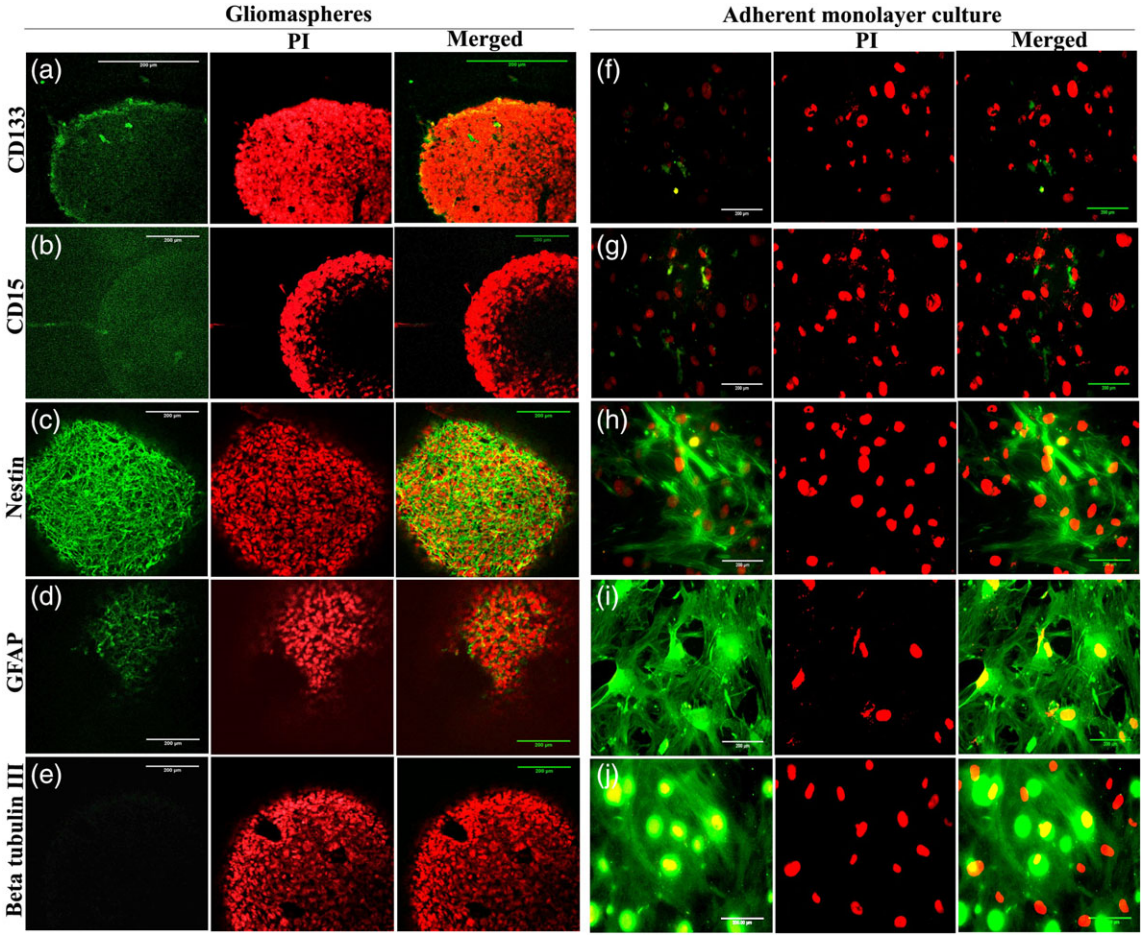

FIG 2

Expression of stem cell and lineage markers in neurospheres and differentiated monolayer cultured glioma cells. Neurospheres were stained by stem cell markers (A) CD133, (B) CD15, and (C) nestin as well as lineage markers for (D) glial cells, GFAP and (E) neuron β-tubulin III. Nuclei were counterstained with propidium iodide (PI). The adherent monolayer culture of differentiated glioma cells grown in DMEM/F-12 containing 10% FBS was incubated with (F) CD133, (G) CD15, (H) nestin, (I) GFAP, and (J) β-tubulin III antibody followed by staining with DyLight 488 conjugated secondary antibody. Nuclei were counterstained using propidium iodide.

non-sphere forming patients ($P < 0.0001$) in the full cohort of glioma (Fig. 5C). After 3 years, in LGG, the neurosphere forming group had 24% survival, whereas the non-sphere forming group had 75% survival. In the sub-population of HGG, the neurosphere forming group had 6% 3-year PFS whereas the non-sphere forming group had 66% survival. After 3 years, in the full population of glioma, the neurosphere forming group had 9% 3-year PFS whereas the non-sphere forming group had 73% survival.

We estimated the cumulative risk of progression/death and no progression in neurosphere forming and non-sphere forming patients. The results of Gray's test equality of CIFs show that in LGG, cumulative incidence curves across neurosphereforming and non-sphere forming groups are not statistically different for progression/death (coded as 1, $P = 0.2253$) and no progression (coded as 2, $P = 0.9325$; Fig. 5D). In HGG, cumulative incidence curves are highly significant for progression/death (coded as 1, $P = 0.0147$) and no progression (coded as 2, $P = 0.0006$) across neurosphereforming and non-sphere forming groups (Fig. 5E). It is important to note neurosphere formation relates to shorter PFS in both LGG and HGG, whereas non-sphere forming group shows a significant difference in PFS with an increased survival advantage in LGG compared to HGG. This suggests that neurosphere formation is associated with increased malignancy in glioma and is a good prognosticator of PFS in association with the grade.

DISCUSSION

Gliomas are well studied and treated on the basis of histopathological criteria and grade as the most significant prognostic factor (17). But the major caveat associated with entirely depending on the grade as a prognostic factor for treating a highly heterogeneous tumor-like glioma is that even in the same grade itself, there will be tumors with different risk factors with variable response to therapies. Earlier studies have shown the presence of low- and high-risk groups within the

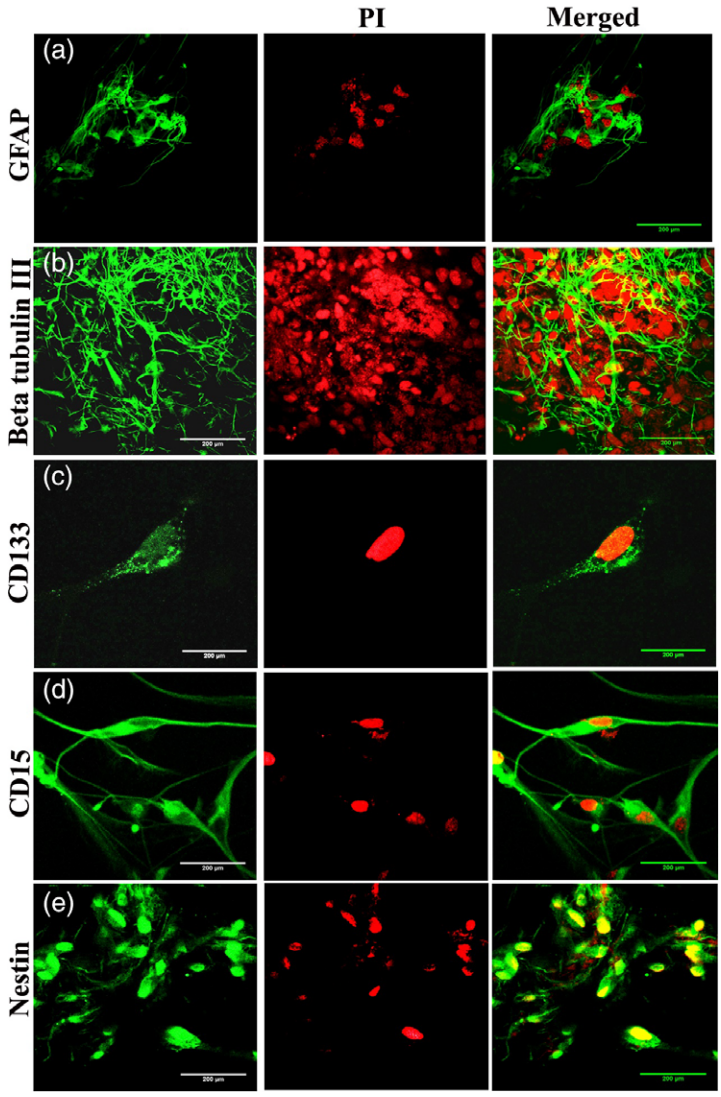

FIG 3

Differentiation potential of neurospheres in SCM and sphere reforming ability of neurospheres after exposing to differentiation cues. Analysis of the multipotency of the neurospheres by immunofluorescence after 7 days of differentiation, many cells expressed markers for (A) neuronal (β-tubulin III) and (B) glial (GFAP) lineage (10×). Neurospheres after growing in 10% FBS containing DMEM/F12 differentiation medium for 7 days was switched to NSC medium to check for its potential to resist differentiation. Neurospheres has depicted an inherent ability to resist differentiation and was shown by the expression of (C–E) stem cell markers even after growing in a differentiation promoting 10% FBS containing DMEM/F12 medium.When the red and green color of the image overlap the pixels appear yellow in some microphotographs.

same grade of glioma (4). According to the previous studies describing the use of the neurosphere culture system as a predictor of clinical outcome in glioma, it was reported that neurosphere formation is independent of the grade (4, 14). This study also suggested that gliomasphere formation suggests the prognosis and clinical outcome of HGGs, but not that of LGGs (14). But that study has used a small sample size to assess the possible association between the neurosphere formation and clinical outcome of the tumor. A recent study on the relationship between gene expressions based on The Cancer Genome Atlas (TCGA) classification and gliomaspheres has shown that gliomasphere system retains the important expression profiles and molecular pathways, thus can be used to study the important genes associated with glioma malignancy (12).

One of the aims of our study was to determine whether GSCs can be isolated and grown from both LGG and HGG, especially low grade, as there were no extensive studies that focused on such population in LGGs (18). According to reports, 70% of grade II glioma progress to HGGs within 5–10 years (18). Our results show that both LGGs and HGGs have sphere formation ability. Since neurosphere formation in culture is an underlying feature of GSCs, and the capacity of sphere generation in both LGGs and HGGs supports the idea that stem cell population exists in all grades of glioma studied. We have also investigated the relationship between neurosphere formation and PFS in LGG and HGG (140 samples). Our results demonstrate that the ability to form multipassaged neurospheres reflects the clinical severity of the tumor as PFS were inversly related to neurosphere formation. This study demonstrates for the first time that both LGG and HGG neurosphere forming group shows shorter PFS with an increased survival advantage in LGGs compared to HGGs.

For the study, we have not sorted the GSCs prior to culturing because of our personal experience and recent reports that demonstrated cell sorting using surface markers negatively affect the viability of cells (19). Even though GSCs can be sorted using cell surface markers, the major difficulty in studying is the lack of specific markers of these cells because of heterogeneity and unavailability of appropriate marker for isolating and distinguishing GSCs from NSCs. Even though CD133 is the most accredited marker for GSCs (20, 21), there are now contradictory reports about whether $CD133^{+}$ cells or $CD133^{-}$ cells are tumorigenic (22, 23). Recent studies suggest that it is better to depend on a spectrum of stem cell markers rather than relying on a single one for refining the stem cell identification (23).

Previous studies also suggest the association of stem cell and lineage marker expression with the clinical severity of tumor (5, 20–27) and there are reports that GSCs do not undergo terminal differentiation after treatment with differentiation-inducing SCM (28–30). The expression of multiple stem and progenitor cell markers by neurospheres generated from LGGs and HGGs and the capability of neurospheres cultured in differentiation promoting conditions to regrow into spheres and to express stem cell markers upon switching to SFM with growth factors and supplements provides evidence to support that GSCs with an intrinsic potential to resist differentiation were present in LGGs and HGGs. Recently, β-tubulin III was found to be of high clinical importance because its expression is related to malignancies and poor prognosis in different cancers (31). It is of considerable interest to note

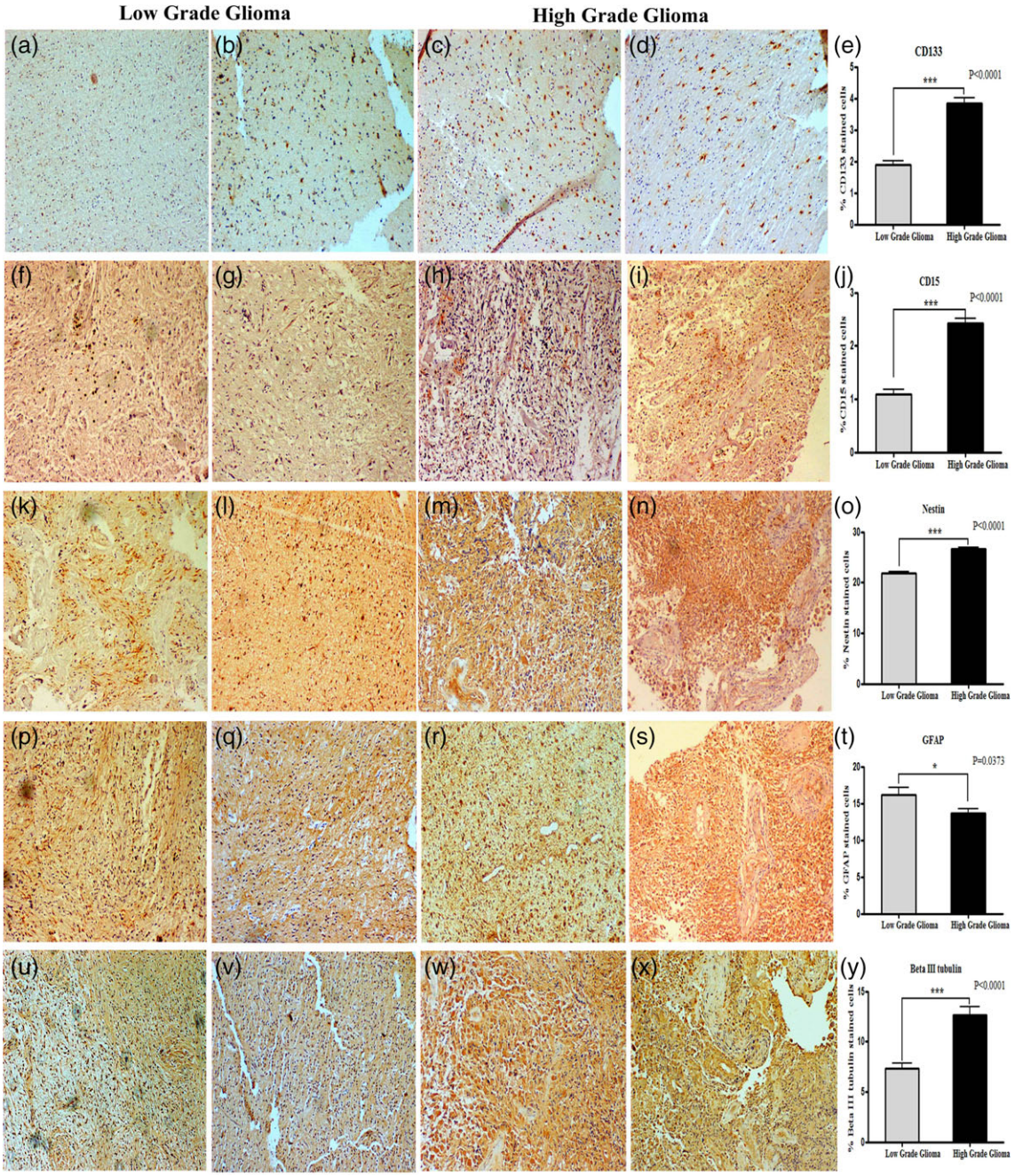

FIG 4 *Histopathologic features of low and high grades of glioma tumors showing expression of CD133, CD15, nestin, GFAP, and β-tubulin III (10×). Representative images of IHC staining demonstrating the expression of (A–E) CD133, (F–J) CD15, (K–O) nestin, (P–T) GFAP, and (U–Y) β-tubulin III in LGG and HGG (Pilocyticastroctoma-grade I, grade II, grade III, GBM-grade IV). The tissue sections were formalin fixed and stained with human specific antibody that was detected by HRP conjugated secondary antibody and DAB as chromogen. The sections were also labeled with hematoxylin (blue) to identify nuclei.*

that relatively high levels of β-tubulin III in HGGs seen in our study is in accordance with recent reports that neuronal markers are aberrantly expressed in human gliomas (32). Further studies are needed to clarify the clinical relevance of β-tubulin III expression in LGGs and HGGs.

Next, we analyzed whether there is any correlation between neurosphere formation and PFS in LGG and HGG. In concurrence with previous reports, we have found that there is a direct relationship between the capacity for neurosphere formation and grade of the tumor, as the HGGs have the highest sphere forming ability compared to LGGs ($P < 0.0001$) (4, 14). However, when evaluating the PFS, neurosphere formation retained its predictive value by showing a shorter survival in both LGGs and HGGs. Among the non-

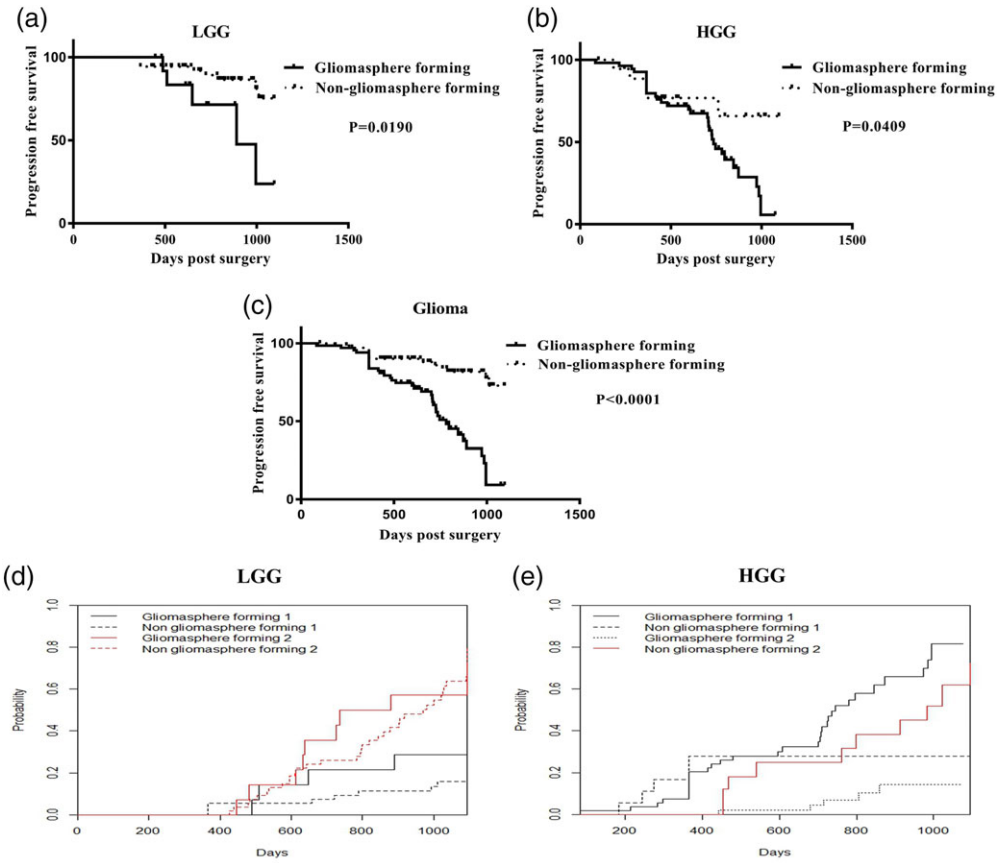

FIG 5 *Evaluation of the relationship between neurosphere formation and PFS of LGG and HGG patients using Kaplan–Meier method and CIF. Kaplan–Meier estimates of PFS in (A) LGG, (B) HGG, and (C) full cohort of glioma.Cumulative incidence curves of progression/death (indicated as 1) and no progression (2) as competing event for neurosphere forming and non-sphere forming patients in (D) LGG and (E) HGG. Neurosphere formation was related to shorter PFS in LGG and HGG.*

sphere forming group there was an increased survival advantage for LGGs compared to HGGs suggesting a strong association between PFS and neurosphere formation along with the grade. Previous studies reported that the survival/prognosis was inversely associated with age (17, 33, 34). In our study, the analyses of neurosphere formation with age and gender did not show any significant difference. To the best of our knowledge, this is the first study that compares extensively the potential association between neurosphere formation and grade of glioma, especially in LGGs.

In our study, the generated neurospheres were found to express known stem cell markers, thereby providing evidence to support that GSCs were isolated from LGG and HGG. Other supporting evidences include the fact that isolated neurospheres can self-renew, may differentiate into multiple lineages under differentiation promoting culture conditions (serum containing media) but display repeated sphere formation ability showing a intrinsic potential to resist differentiation. Taken together, these results suggest that neurospheres from both low and high-grades of human glioma contain a subpopulation of cells, which possess all the defining features of stem cells including self-renewal and multipotent differentiation. Our study also demonstrates a significant association between neurosphere formation and clinical severity of the tumor as PFS was strongly related to neurosphere formation and demonstrated for the first time that both LGG and HGG neurosphere forming group showing a significantly shorter PFS with an increased survival advantage in LGG compared to HGG. In conclusion, the present study further highlights the importance of neurosphere culture system for glioma research.

Limitations and Future Directions

Recent studies have indicated that neurospheres cultured in vitro are important models for understanding tumor biology and have an impact on the prognosis of glioma (4, 12). Because of the time taken for performing neurosphere formation assay, the clinical feasibility of the assay becoming the main diagnostic criteria is limited. Neverthless, it is a better predictor of PFS, but mandates further research. The use of neurosphere culture as an in vitro model system has been supported by the ability of these spheres to preserve all the major mutations associated within the neoplasm and to produce tumors showing the clinical and biological nature as that of parent tumor following xenotransplantation (4, 12, 35). It is a fundamental step to isolate GSCs using neurosphere as in vitro model for tumors and that serve as a tool for elucidating the genes and molecular pathways that account for the proliferation and tumorigenic potential of glioma.

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CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institution and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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