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PROJECT COMPLETION REPORT

1. **Project number:** 5226

2. **Title of the project:** Isolation and characterization of gliomasphere forming cells from Glioblastoma Multiforme: Correlation with prognosis and treatment outcome

3. **Funding Agency Name:** DBT, Govt. of India

4. **Project Reference Number provided by the Funding Agency:** BT/PR11624/Med/30/154/2008

5. **Principal Investigator (Name & Address):** Dr. G. Srinivas, Scientist D, Department of Biochemistry, SCTIMST, Trivandrum, Kerala

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7. **Implementing Institution:** SCTIMST

8. **Collaborating Institutions:** RGCB

9. **Date of Commencement:** 03/08/2010

10. **Duration**: 2 years

11. **Date of completion**: 02/11/2012

12. **Objectives as approved**:

    1. To look for the presence of gliomasphere forming cells within different grades of glioma in vitro.
    2. Assess other GSC like characteristics of different grades of gliomas *in vitro* through self-renewal and differentiation assays.
    3. To evaluate the sphere forming capacity in relation to long-term proliferation and self-renewal within different grades of glioma.
    4. To characterize gliomasphere forming cells with known markers of stemness and lineage and their capacity for multipotency.

5. **Deviation made from original objectives if any, while implementing the project and reasons thereof:** NA

6. **Field/Experimental work giving full details of summary of methods adopted, data collected supported by necessary tables, charts, diagrams and photographs**:

Methods

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 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).

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 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.

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.

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).

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)

Characterization of glioma derived cells *in culture*

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.

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) to reveal the nuclei. For immunostaining of differentiated tumor spheres after differentiation assay, the tumorspheres were cultured on SCM for seven days and immunofluorescence was performed.

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).

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).

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.

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.

7. Detailed analysis of Results

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 1 lists the patient characteristics.

Table 1. 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

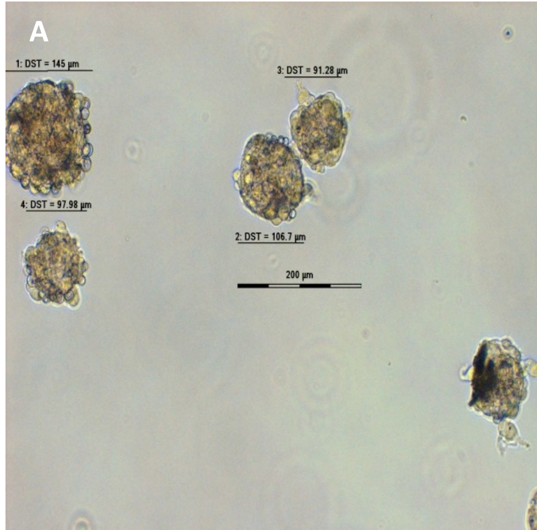
Abbreviations: M – male, F – female, Y – years

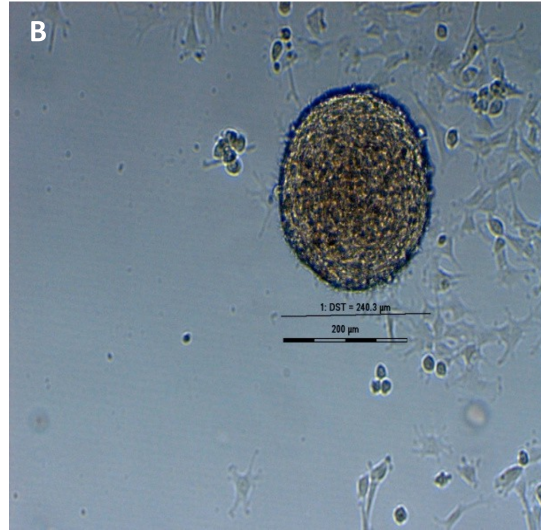
Gliomas of different grades has the ability to form neurospheres

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 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 1).

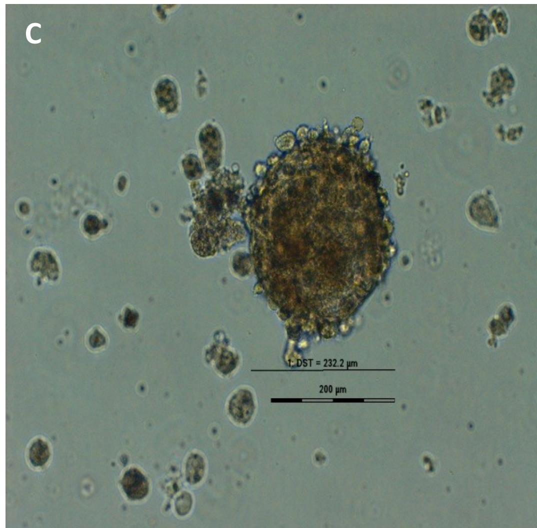
Figure 1. Phase-contrast microphotographs showing examples of gliomasphere forming ability among different grades of glioma.

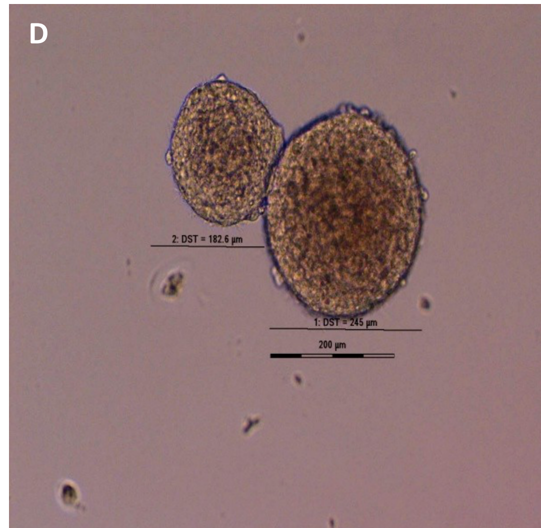
Grade I **Grade II**





Grade III **Grade IV**





(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 FGF have the ability to form gliomaspheres.

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

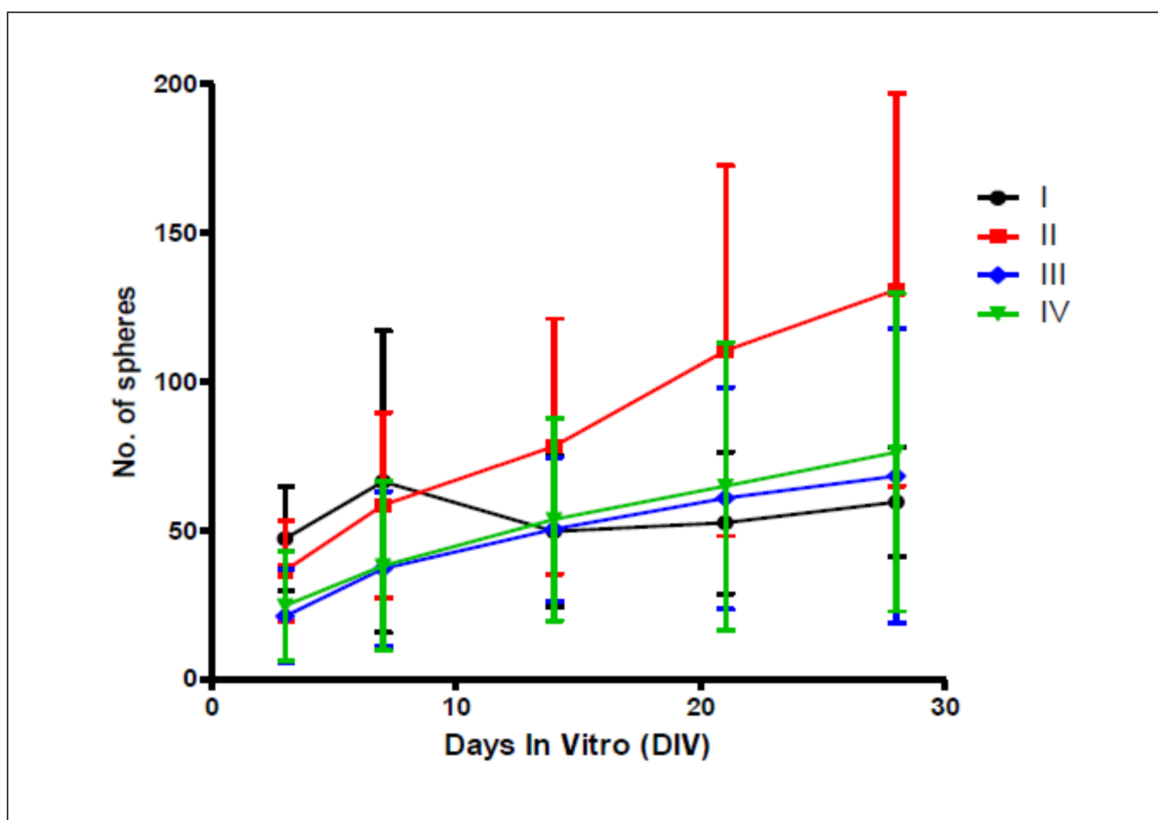
Primary sphere formation analysis was performed on to quantify the frequency of stem cell population within the tumor. The generation of neurospheres from different grades of glioma provides an indication of the clonogenicity of tumors. Tumor samples from 140 patients with glioma, including a subpopulation 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 2). 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 2).

Table 2. 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 2. 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. 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 3). 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 3. 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 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).*

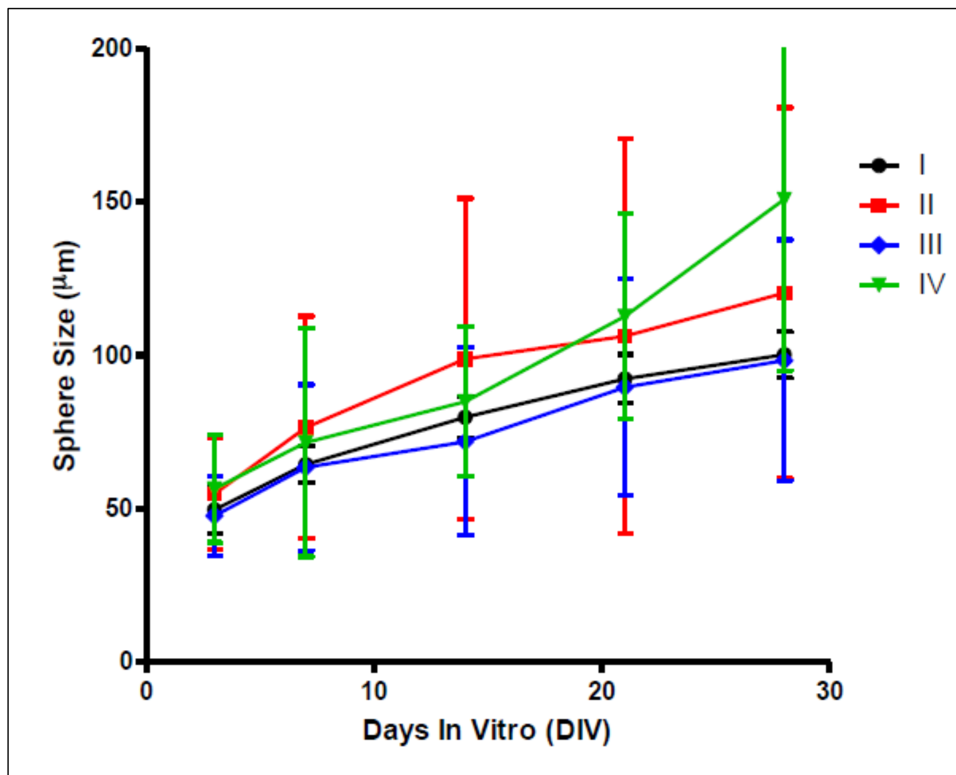
Gliomasphere growth rate shows no significant difference between grades of glioma

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 after plating was found to increase linearily (Table 4). Even though the size of gliomaspheres was increasing, but when compared between grades there exists no significant correlation.

Table 4. 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 3. Graph showing the growth rate of different grades of glioma

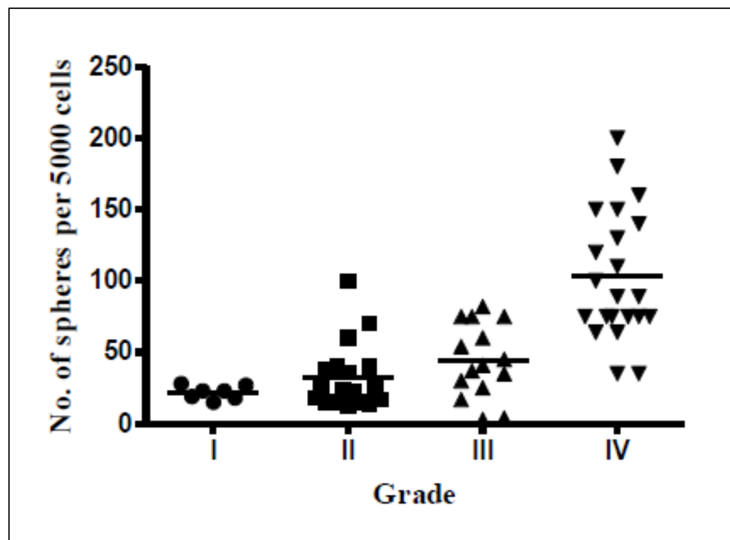


Association among sphere size and grades of the glioma were also examined using spearman's rho correlation analysis. There was no significant correlation between the grade of glial tumors and sphere size (Figure 3). Furthermore, when we examined gliomasphere formation as a function of age and sex, there was no significant correlation.

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

Self-renewal is one of the defining feature of NSCs, thus we tested whether individual cells derived from gliomaspheres had the ability to form new spheres. To evaluate the sphere forming capacity in relation to long-term proliferation and self-renewal within different grades of glioma, sub-sphere formation experiments were performed after seven days of plating, which involved dissociation of primary gliomaspheres and culturing at a density of 5000 cells/well. The sub sphere formation assay allows determining the number of stem cells in a sphere by assessing the number of secondary spheres generated after plating of cells from dissociation of primary spheres. All the four grades of primary gliomaspheres have shown the capacity to form secondary tumor spheres, suggesting that gliomaspheres contain stem like cells with the ability to self-renew (Figure 4). However, the capacity for secondary sphere formation was much higher for grade IV glioma than the other grades of (I, II and III) glioma (Table 5). 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 increased ability to form secondary or higher spheres is likely due to their enhanced stem cell compartment in the high grade gliomas.

Figure 4. Secondary sphere formation of glioma



Secondary sphere formation of different grades of glioma was plotted and grade IV has the highest gliomasphere forming ability. Each dot is representative of one patient sample; bar represents mean of biological replicates.

Table 5. 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	7
Grade II	30.2 ± 2.4	18
Grade III	59.9 ± 10	15
Grade IV	122.7 ± 11.1	22

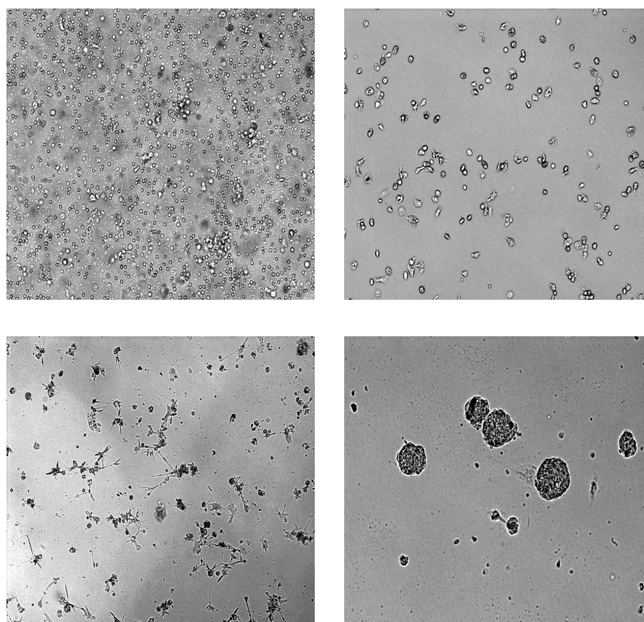
Characterization of gliomaspheres and differentiated glioma cells isolated from different grades of glioma

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 (1), 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.

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 5). 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 6).

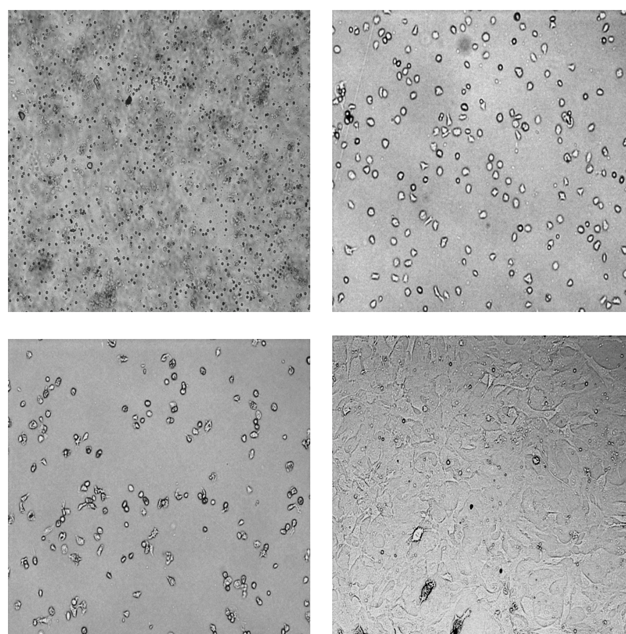
Figure 5. Phase-contrast microphotographs showing glioma derived cells cultured under neural stem cell conditions at different time periods.



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

10.



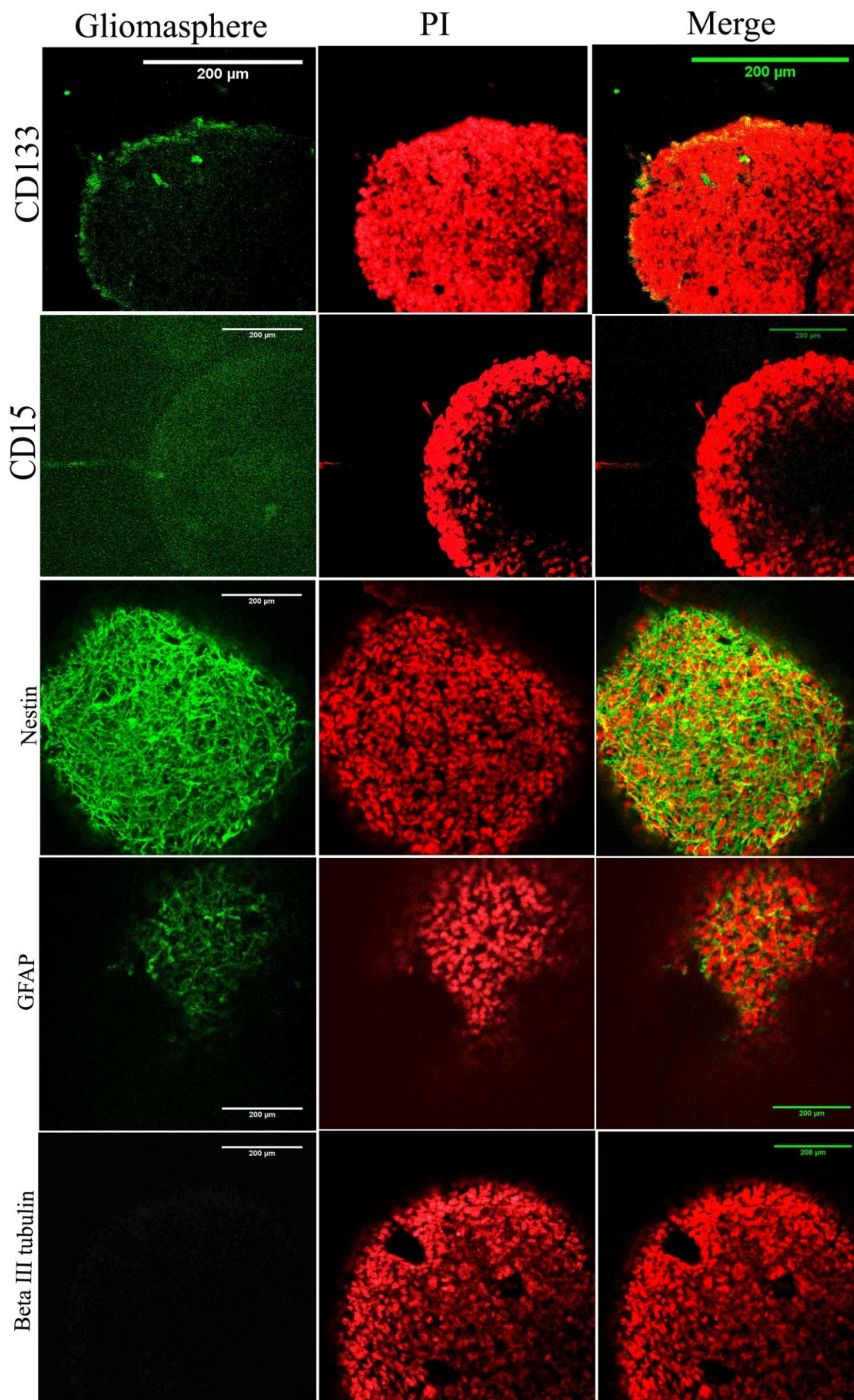
(A) Day 0 (B) Day 1 (C) Day 2 and (D) Day 3 at confluence (100X).

Tumor cells isolated as described in 'methods' were grown in DMEM/F-12 medium containing 10% FBS for adherent monolayer primary glioma cell cultivation.

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 & 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 7). It was visualized using Zeiss LSM 510 meta confocal microscope at 10x magnification under the settings of 543 for PI and 488 for FITC.

Figure 7. 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).

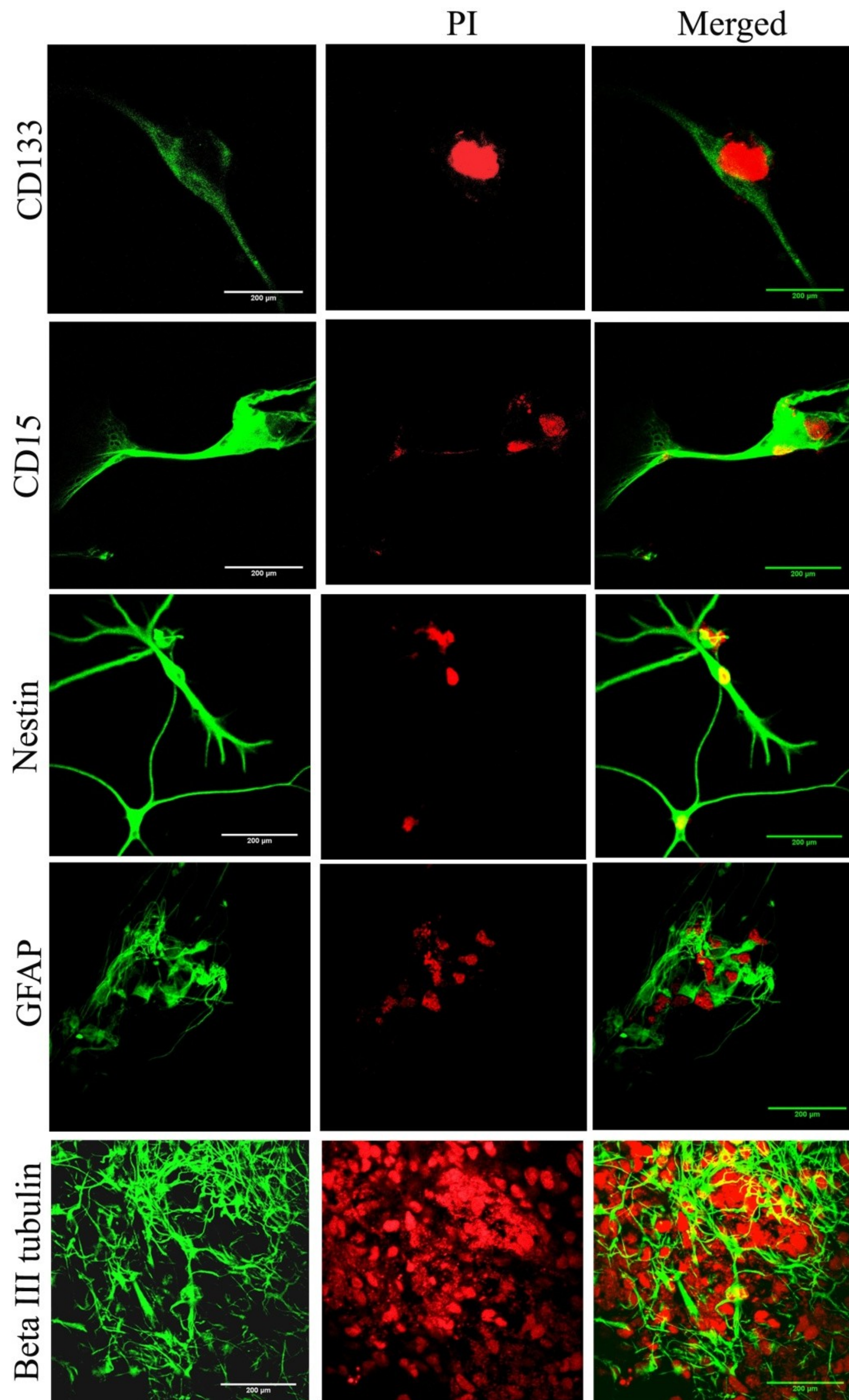
Gliomaspheres are multipotent

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 8). 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.

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

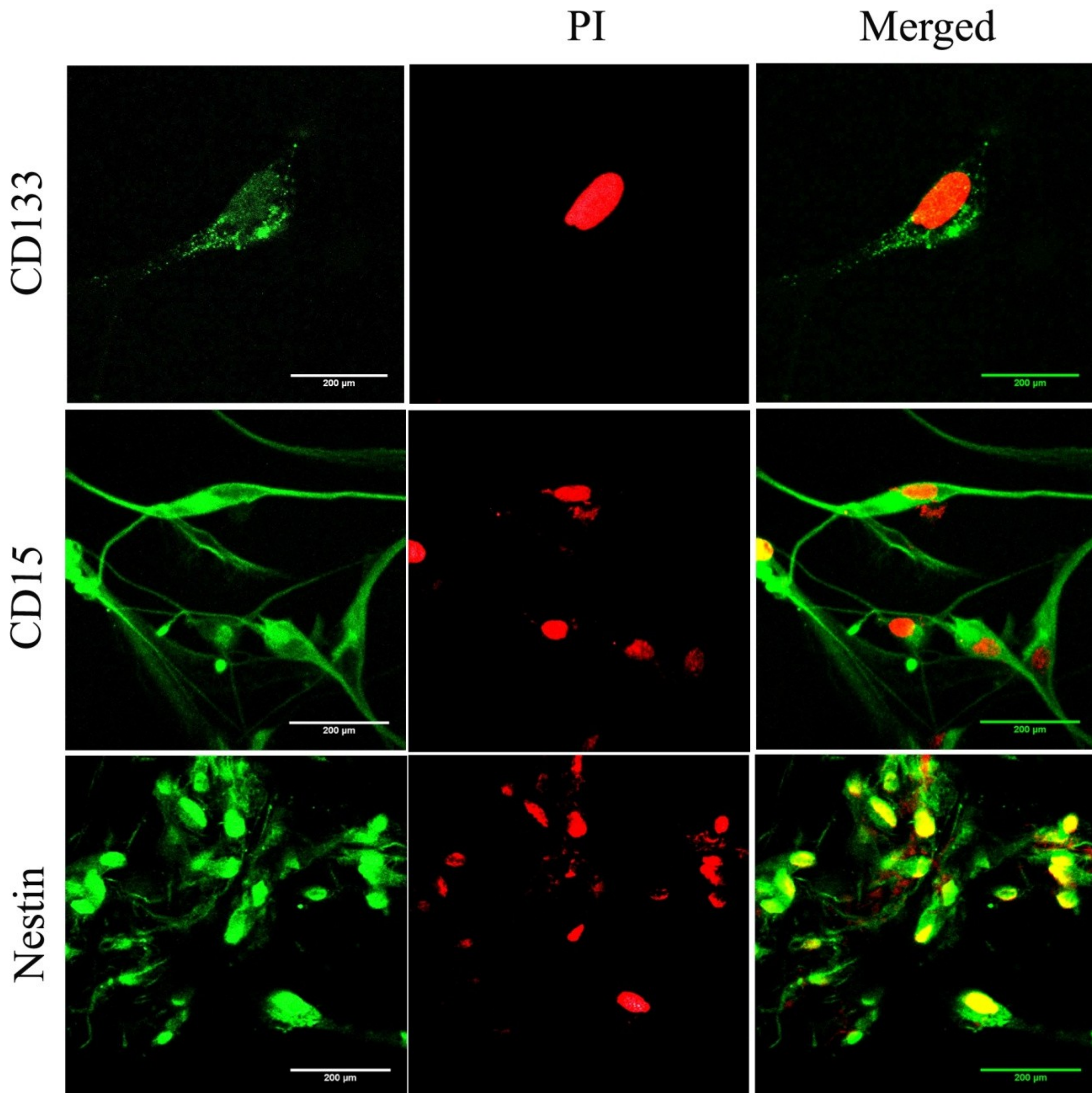
The gliomasphere's (1 grade I, 3 grade II, 3 grade III and 1 grade IV) potential to maintain their immature state even after growing under differentiating conditions for two weeks was analyzed. For the study, gliomaspheres were grown in a serum containing differentiation promoting DMEM/F12 medium for 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 as well as lineage markers. These cells stained positive for stem cell markers (Figure 9) showing that gliomaspheres from different grades of glioma have GSCs with an intrinsic potential to resist differentiation and maintain their immature state.

Figure 8. Expression of stem cell and lineage markers in differentiated gliomaspheres



Gliomaspheres 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.

Figure 9. Sphere reforming ability of gliomaspheres after exposing to differentiation cues



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.

CD133 expression in human glioma cultures

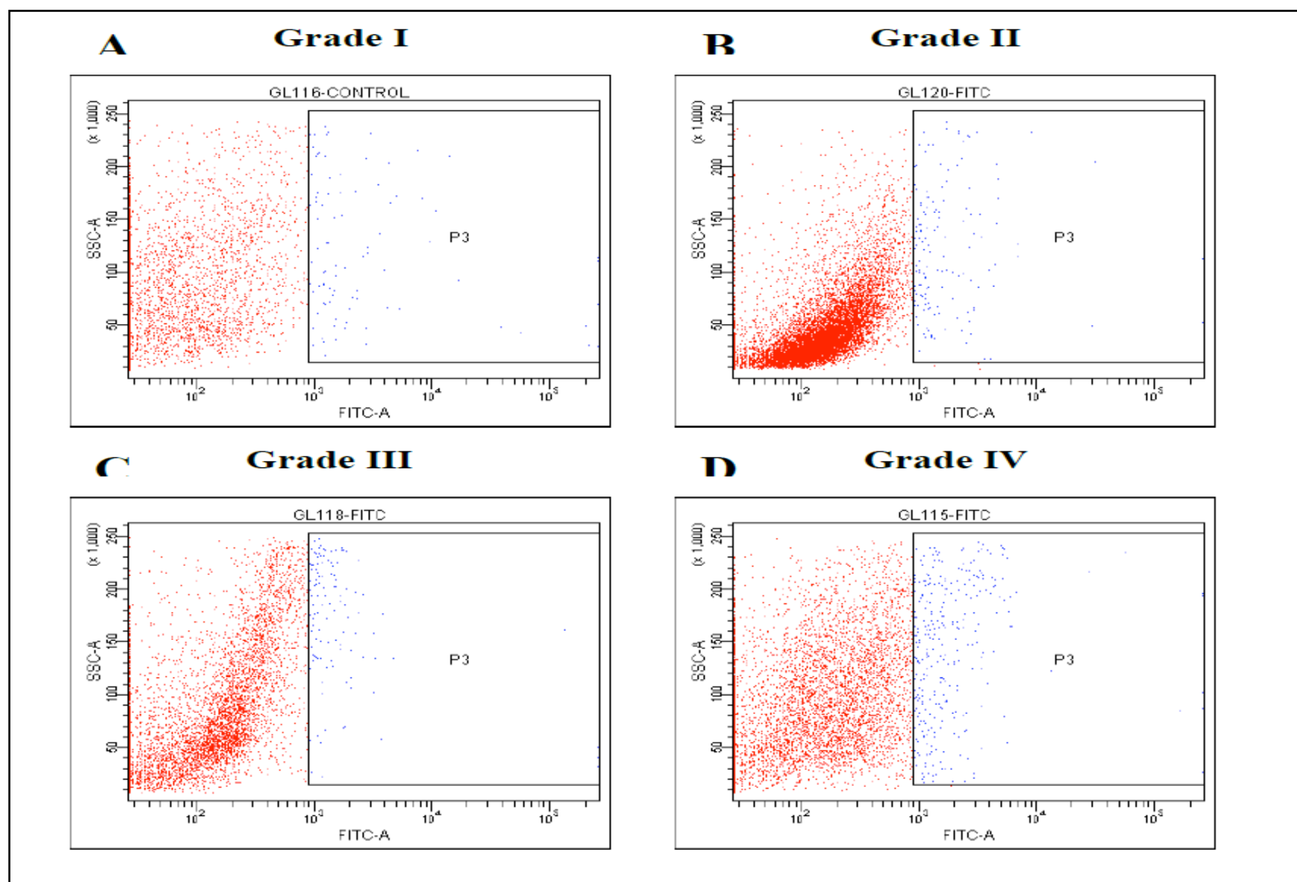
Since, CD133 is a putative marker that has been frequently used for the identification of and characterization of NSC/GSCs. Gliomaspheres from all the four grades of glioma were analyzed for their expression of CD133 marker using flowcytometry. The CD133 expression in different grades of human glioma ranged from 0.1 to 20% (Figure 10). 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 6). 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 6. 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%
11. Astrocytoma II (n = 3)	0.3 - 0.9%
12. Anaplastic Astrocytoma (n = 3)	1 – 5.4%
13. GBM (n = 4)	0.7 – 20%

Figure 10. 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.

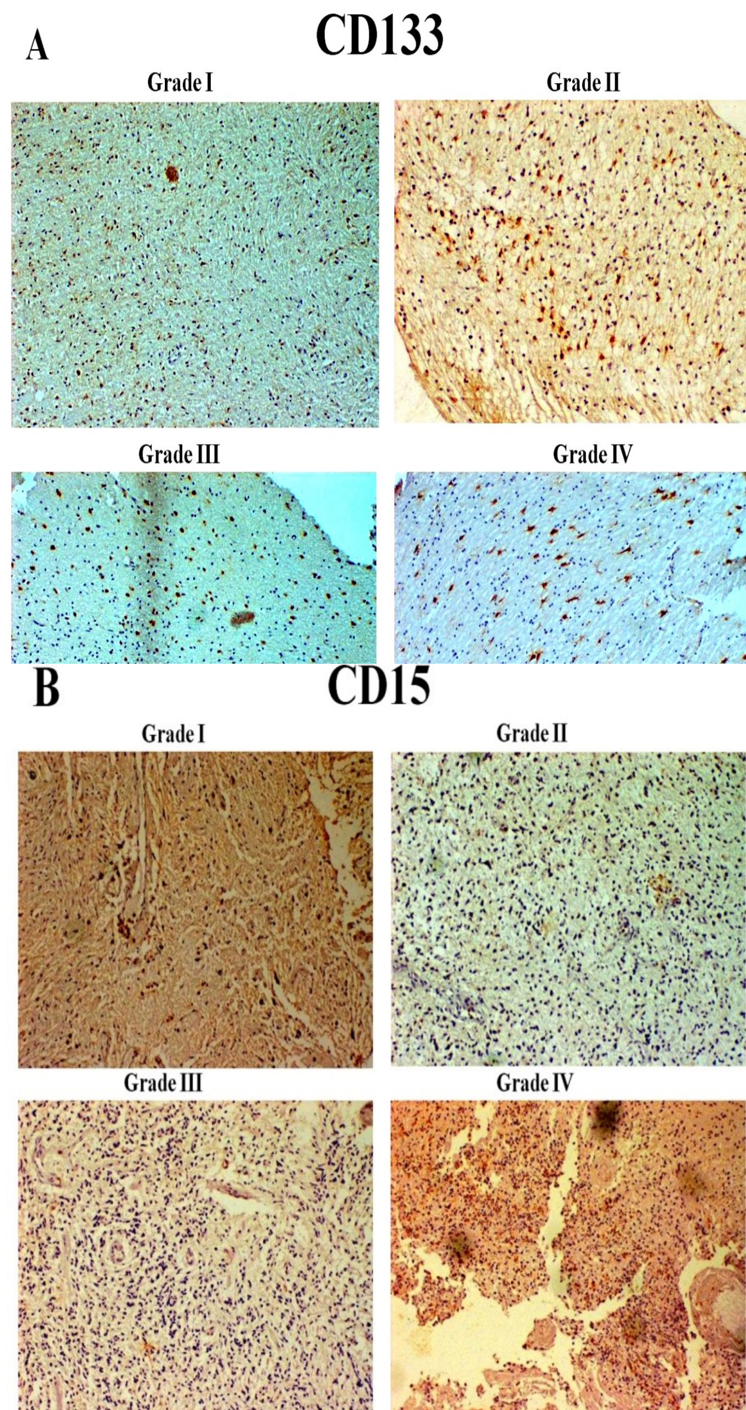
Gliomaspheres were dissociated using TrypLE and processed for flow cytometry as described under methods. Flowcytometry was used to determine the proportion of CD133 positive cells from different grades of glioma.

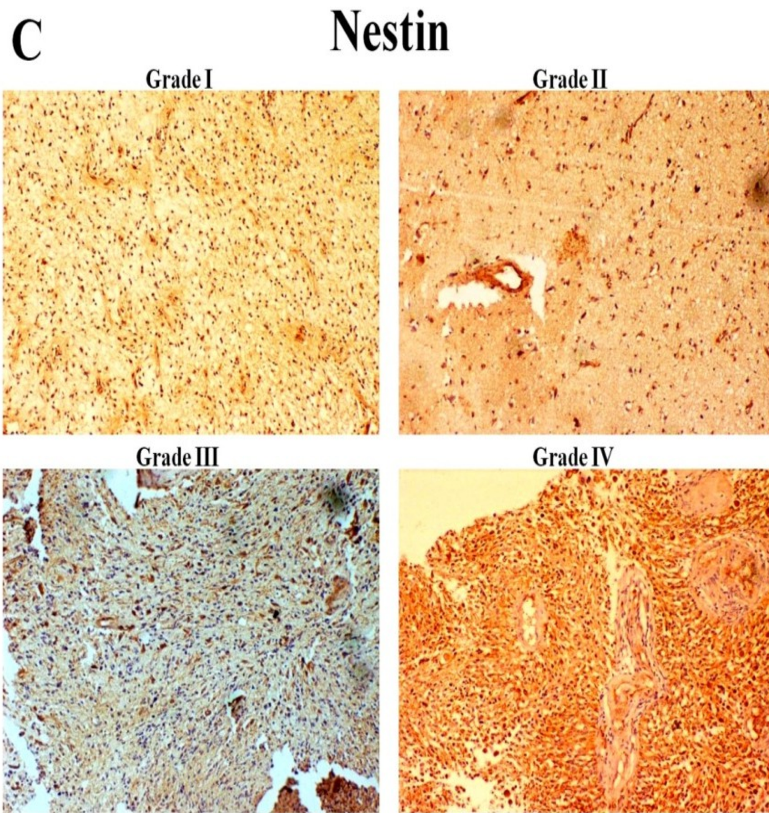
Immunohistopathologic analysis of human glioma whole tissue sections for neural stem cell and lineage markers showed variation with clinical staging

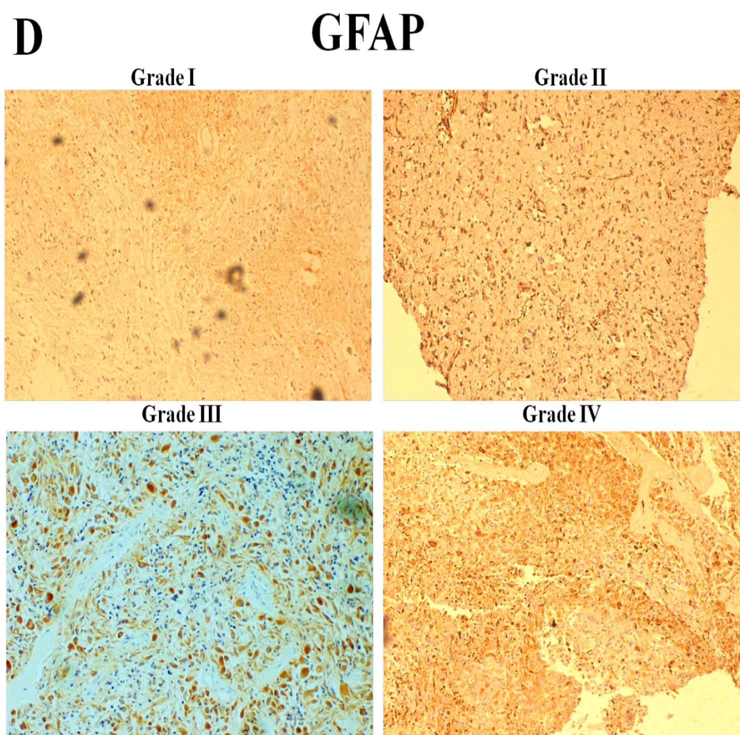
Paraffin embedded sections of all the four grades of glioma were immunostained for CD133, CD15, nestin, GFAP and β-tubulin III. Expression of antigens was assessed in a panel of 48 gliomas of different pathological types. The expression levels of antigens in human glioma tissues showed considerable variability with different clinical grading ranging from complete lack/very low immunoreactivity to high intensity staining. The markers characteristic of NSCs, CD133 (Figure 11A) and CD15 (Figure 11B) were detected mainly in higher malignant grades of glioma, whereas nestin (Figure 11C) 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

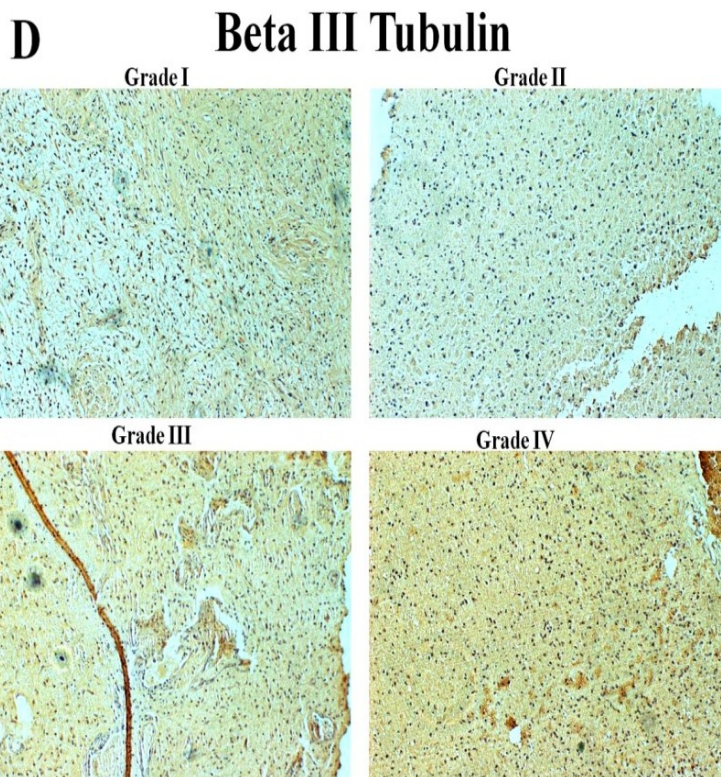
fraction of cells. The expression of nestin is more intense in high grade gliomas compared to low grade. GFAP (Figure 11D) and β-tubulin III (Figure 11E), the markers characteristic of glial and neuronal differentiation respectively, was detectable in all tumors and present diffusely throughout the section. The results demonstrate that the GFAP and β-tubulin III protein expression pattern decreases with increase in grades of glioma.

Figure 11. Histopathologic features of four grades of glioma tumors showing expression of CD133, CD15, nestin, GFAP and β-tubulin III (100X).




C
Nestin
Grade I
Grade II
Grade III
Grade IV


D
GFAP
Grade I
Grade II
Grade III
Grade IV



The representative images are from pilocytic astrocytoma (I), astrocytoma (II), anaplastic astrocytoma (III) and GBM (IV) (A) CD133 (B) CD15 (C) nestin (D) GFAP and (E) β-tubulin III.

The tissue sections were formalin fixed and stained by human specific antibody against NSC and lineage marker. The sections were also labelled with hematoxylin (blue) to identify nuclei.

15. Science and Technology benefits accrued:

a. List of Research publications with complete details:

1. 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***, 71: 244-253, 2019.
2. Padmakrishnan CJ, Easwer HV, Vinod Vijayakurup, Girish Menon R, Suresh Nair, Srinivas Gopala. High LC3/Beclin expression correlates with poor survival in glioma: a definitive role for autophagy as evidenced by *in vitro* autophagic flux. ***Pathology Oncology Research***, 25: 137-148, 2019.

b. Manpower trained on the project:

i.	**Research Scientists or Research Fellows**	**:**	1
ii.	**No. of PhD's produced**	**:**	1
iii.	**Other Technical Personnel trained**	**:**	1

c. **Patents taken, if any** : Nil

d. **Products developed, if any** : Nil

16. Procurement of/Usage of Equipment:

a. Details of Equipment:

Sl.No.	Name of equipment	Make/Model	Cost (Rs.)	Date of Installation	Utilisation	Remarks regarding maintenance breakdown
1	MACS	Biorad	3 lakhs		40 %	Working
2	CO_2 incubator	Eppendorf	3 lakhs		90 %	Working
3	Biosafety cabinet	Micro-Filt (India)	2 lakhs		90 %	Working
4	Trans Blot Apparatus	Biorad	1 Lakh		90%	Working

b. Suggestions for disposal of equipment(S): All equipments are in working condition

(Name and Signature of PI)