

Response of Human Glioblastoma Cells to Hyperthermia: Cellular Apoptosis and Molecular Events

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Research Article

Keywords: Glioblastoma multiforme, Cancer stem cell, Astrocyte, Hyperthermia, Apoptosis

Posted Date: October 26th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-983908/v1>

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Version of Record: A version of this preprint was published at Tissue and Cell on February 1st, 2022. See the published version at <https://doi.org/10.1016/j.tice.2022.101751>.

Abstract

Glioblastoma multiforme (GBM) also categorized as a grade IV astrocytoma, is an aggressive brain tumor which invades the surrounding brain tissue. Hyperthermia is known to be effective for chemo-radiotherapy to sensitize cancer cells to radiation as a treatment option for patients with GBM. The current study was performed in order to assess and compare the properties of the astrocyte and cancer stem cells isolated from glioblastoma exposed to hyperthermia. Astrocytes and cancer stem cells were isolated from human glioblastoma tissue. Glioblastoma tissues were digested and cultured in culture medium supplemented with B27, basic fibroblast growth factor and epidermal growth factor. The morphology and specific markers were evaluated in astrocyte and cancer stem cell of human glioblastoma through immunocytochemistry and quantitative real-time RT-PCR. The multipotentiality of cancer stem cells was presented using differentiation potential into neurons, oligodendrocytes, and astrocytes. For hyperthermia, cells were exposed to temperatures at 42-46°C for 1h using a water bath. Cell survival rate by MTT assay and apoptosis using quantitative real-time RT-PCR and western blot were evaluated. Results demonstrated that there were two morphology types in cell culture including epithelioid morphology and fibroblastic morphology. Astrocytes were confirmed via expression of the Glial fibrillary acidic protein (GFAP) protein; whereas, cancer stem cells (CSCs) were round and floating in the culture medium. Immunocytochemical staining indicated that nestin, CD133 and SRY-box 2 (SOX2) antigens were positively expressed in primary neurospheres. Results indicated that cancer stem cells of glioblastoma are multipotent and are able to differentiate into neurons, oligodendrocytes, and astrocytes. The current study obtained evidence via apoptosis evaluation that CSCs are resistant to hyperthermia when compared to astrocytes isolated from glioblastoma. Furthermore, hyperthermia was demonstrated to decrease cell resistance, which may be effective for chemo-radiotherapy to sensitize cancer cells to radiation. Taken together, CSCs of glioblastoma could be used as a powerful tool for evaluating the tumorigenesis process in the brain and developing novel therapies for treatment of GBM.

Introduction

Glioblastoma multiforme is an aggressive and lethal brain tumor among the various human cancers because this tumor has a recurrence potential [1]. Despite current therapeutic approaches including radiotherapy and chemotherapy after surgical resection, glioblastoma mortality rate remains high owing to poor prognosis of this tumor and high rate of recurrence [2, 3]. To increase treatment efficacy of glioblastoma and attempt to inhibit disease recurrence, it is essential to evaluate tumor recurrence at the cellular and molecular level. Research evidence shows cancer stem-like cells (CSCs) or tumor initiating cells as a subpopulation of cells within glioblastoma are capable of proliferation, possess self-renew capacity, differentiation. CSCs are also reported to be observed in the formation of new tumors in mice [4, 5].

First reports demonstrating the presence of CSCs in brain tumors was observed by Singh and colleagues [6]. Then, Yuan et al. have identified expression of CD133 antigen (prominin-1) in these cells [7]. CSCs support the development and progressive growth of tumors and it has been proposed that the origin of

tumor malignancy is CSCs [6, 8]. Evidence in research suggests that not only is CSCs likely to be responsible for high treatment resistance of glioblastoma and lead to tumor recurrence; but ultimately also leads to an increase in mortality due to DNA repair potential to be increased in CSCs [9]. Studies have also suggested that CSCs depend on the microenvironmental conditions of the brain for growth and differentiation [10, 11]. Another important factor that may play a principle role in the formation of glioblastoma is the cell of origin. At first, astrocytes were believed to be the cell of origin of glioblastoma owing to the expression of glial fibrillary acidic protein (GFAP), a glial marker [5]. Glioblastoma tumors are therefore composed of heterogeneous cells that include CSCs and astrocytes that, together perhaps, lead to an abundance of problems that makes these patients particularly resistant to treatment [12]. It is perhaps these three regions that can pose a threat to treatment including periphery, intermediate and core in the glioblastoma and is proposed to at least partially contribute to the persistent resistance of glioblastoma to the current treatments available. Each area of a glioblastoma has specific properties, conditions and function.

Persana and colleagues reported the high proliferation of the core or necrotic region of tumor [13] as well as high hypoxic conditions, observation of CSCs, expression of antigens of CD133 as well as nestin and resistance to chemotherapy. The intermediate area, similar to the core area, however, differentiated cells and CSCs are shown according to expression of mixed lineage antigens. The periphery of the tumor was observed to demonstrate low proliferation, high vascularization, the presence of differentiated cells such as astrocytes and to be sensitivity to chemotherapy [13]

Therapeutic options and novel methodology for study have been ongoing and due to characterized infiltration of GBM tumors into healthy tissue current therapies have limited benefit and effective therapies are lacking. Therapies that more readily reduce recurrence are critical making further investigations critical particularly in GBM. Different studies have introduced various treatment procedures that would be non-invasive including hyperthermia [14–17]. Different methods can be used to hyperthermia such as microwaves [18], radiofrequency [19], lasers [20], ultrasound [21], magnetic fluid hyperthermia [22], hot water bath [23]. Hyperthermia as a form of “Anti-cancer” treatment is not new. Research over decades had demonstrated its use in human patients; yet it still had not become a part of the standard of care treatment early in its discovery. Challenges always exist and perhaps kept it out of the main stream early on. Hyperthermia leads to the increase of the body temperature, which in turn stops cell proliferation and apoptosis induction in cancer cells [24]. Hyperthermia seems to selectively damage tumor cells and also, sensitize cancer cells to radiotherapy and chemotherapy [25, 26]. In the present study, we addressed that CSCs isolated from glioblastoma can differentiate to other cells and also, we indicated that cancer stem cells of human glioblastoma are more resistant to hyperthermia than astrocytes isolated from glioblastoma.

Materials And Methods

The human glioblastoma tissues were prepared from patients with glioblastoma under neurosurgical operation in the Shohada Tajrish Hospital. Informed consent was obtained from patients for

their participation in this study. Glioblastoma multiforme was confirmed using pathological assessment. Study method was approved by the Ethical Committee of the Shahid Beheshti University of Medical Sciences.

Isolation and Culture of human glioblastoma stem cells

Stem cell isolation was achieved according to a previously published procedure with minor modifications [27]. The tissues were obtained from the glioblastoma core area. The sample of glioblastoma was placed in cold calcium and magnesium free Hank's balanced salt solution (HBSS) with 1% penicillin and streptomycin, and washed 3 times with phosphate buffered saline (PBS). Then, the tissue was cut into small pieces, and was trypsinized in 3-5 ml of 0.05% trypsin-EDTA for 10-15 minutes at a 37°C water bath. Enzymatic digestion was inhibited using trypsin inhibitor (80 µg/ml; Sigma) in HBSS solution, following centrifugation performed (110 g, 5 min, room temperature). The tissue pieces were resuspended in 1ml of sterile NSC basal medium and were triturated using a flamed polished Pasteur pipette (approximately 3-7 times). The cell suspension was filtered with a 40µm strainer. The cell suspension was centrifuged at 110g for 5 min. The cells were resuspended and cultured in complete neural stem cell (NSC) medium including 20 ng/ml, epidermal growth factor (EGF), 20 ng/ml, basic fibroblast growth factor (bFGF), 1% penicillin–streptomycin (Sigma). The cells were incubated at 5% CO₂, 37°C. The cell culture medium was changed every 2–3 days.

Isolation and Culture of Human glioblastoma astrocyte

The primary astrocyte was isolated from the peritumoral brain zone according to a previously explained method by Hashemi et al. [28,29]. Glioblastoma tissue was digested with 0.05% trypsin–EDTA for 10 min at a 37°C water bath. Following centrifugation at 180g for 5 min, cells re-suspended in DMEM/F12 medium containing 1% (100 U/ml) antibiotic/antimycotic and 2% FBS. The cells incubated at 5% CO₂, 37°C. The volume of FBS was gradually increased from 2 to 10% in culture medium for 2 weeks. Specific marker of astrocyte (GFAP) was assessed using immunocytochemistry.

Isolation and Culture of Adult Rat SVZ-neurosphere

Subventricular zones (SVZ) were obtained aseptically from 2 adult male Wistar rat (200–250g body weight) brains. The rats were provided from the Animal House of Shahid Beheshti University of Medical Sciences (SBUM). The rats were anesthetized and decapitated. Subventricular zones of lateral ventricles were isolated from the rat brain. This work was performed under the approval of the Ethics Committee of Shahid Beheshti University of Medical Sciences. SVZs were isolated from lateral ventricles and were digested with 0.02% trypsin-EDTA for 5 min at 37°C.

The tissues were triturated using Pasteur pipette and filtration 0.02% trypsin was done by a 70-micrometer cell strainer to produce a single cell suspension. The cells were cultured in a neural stem cell (NSC) medium with 20 ng/ml EGF, 20 ng/ml bFGF and 1% penicillin–streptomycin (Sigma) for 2 weeks. One half of the culture medium was changed with fresh medium twice per week. Cells derived from SVZ were

applied as a positive control for NSC and proliferation markers in the assessments of real-time RT-PCR and immunocytochemistry.

Differentiation of human GBM-SCs into neuron, astrocyte and oligodendrocyte in vitro

Glioblastoma and SVZ neurospheres were plated onto poly-D-lysine and laminin coated 48 well-plates at $5-20 \times 10^3$ cells per well in the neurobasal media supplemented with 10% FBS for 21 days. The neurospheres were incubated at 37°C with 5% CO₂ for differentiation analysis through immunocytochemistry assessment. Half of the medium was changed every 3 days.

Quantitative real-time RT-PCR

Total RNA was extracted from the cells using the TriPure Isolation Reagent (Roche, Germany) according to the manufacturer's instructions. First-strand cDNA synthesis was performed by adding 1 µg of each RNA sample to reverse transcriptase (Takara, Japan) with Random Hexamer, Oligo DT and RNase inhibitor and reaction was regulated using a thermal cycler for 5 min at 85°C and 15 min at 37 °C. RT-PCR was done using SYBR Premix Ex Taq II (Tli Plus) (Takara, Japan) according to the manufacturer's instructions through the qRT-PCR detection system (Applied Biosystem, one step, RT-RCP Germany). HPRT1 applied as internal control and adult rat SVZ-NSCs were selected as a reference sample for glioblastoma neurospheres. The primer sequences were as follows: nestin, 5'-GTAGCTCCCAGAGAGGGGAA-3' and 5'-CTCTAGAGGGCCAGGGACTT -3'; SOX2, 5'-AACCAGAAAAACAGCCCGGA-3' and 5'-ACCAGAAAAACAGCCCGGA-3'; CD133, 5'-GGAATCCTTTCCATTACGGCG-3' and 5'-CGAGGGATGCGGAAGAATGT-3'; BAX, 5'-CAA ACT GGT GCT CAA GGC -3' and 5'-CAC AAA GAT GGT CAC GGT C-3' and Bcl-2, 5'-GTA CTT AAA AAA TAC AAC ATC ACAG-3' and 5'-CTT GAT TCT GGT GTT TCC C-3', and housekeeping Hypoxanthine Phosphoribosyltransferase1 (HPRT1), 5'-CCT GGC GTC GTG ATT AGT GA-3' and 5'-AAG ACGTTC AGT CC TGT CCA T-3'. All qRT-PCR were achieved in triplicate. Gene expression levels were evaluated using the $\Delta\Delta C_t$ method.

Immunocytochemistry (ICC)

Cells were fixed 4% paraformaldehyde in 0.2M phosphate-buffered saline (PBS) for 20 min, were permeabilized in 0.3% Triton X-100 in PBS for 5 min and blocked with 10% normal goat serum for 10 min at room temperature, respectively. Then, cells were incubated with the appropriate antibody overnight at 4°C. The primary antibodies were rabbit anti-nestin (1:250; Abcam), rabbit anti-CD133 (1:500; Abcam), rabbit anti-GFAP (1:300, Abcam), rabbit anti-MAP2 (1:300, Abcam), rabbit anti-OLIG2 (1:300, Abcam). Following washing with PBS, the cells were incubated with secondary FITC-conjugated goat anti rabbit antibody (1:1000; Abcam) and FITC-conjugated goat anti mouse antibody (1:1000; Abcam) for 2 h at room temperature. Nuclei were stained with DAPI (blue). Finally, immunopositive cells were observed using inverted fluorescence microscopy.

Hyperthermia

Hyperthermia was induced 1 time using a water bath of 42-46°C was used for 1h. After hyperthermia, cultures were maintained for 24h in an incubator at 37°C with a 5% CO₂ and saturated humidity. The control group received a temperature of 37°C. SVZ-NSCs were considered as positive control.

MTT

After 24h, cells survival rate was examined using tetrazolium-based colorimetric assay (MTT assay, 0.5 mg/ml). Absorbance was read by an ELISA plate reader with a test wave-length 570 nm and a reference wavelength at 690 nm, and subtracts the 690 nm background absorbance from the 570 nm.

Western blot

Cells were heat treated to 1 time at temperature of 44°C for 1h. After 24h incubation, cells were homogenized with RIPA lysis buffer. 30 µg protein was loaded in 12% SDS–polyacrylamide gel electrophoresis (PAGE). Electroblotting was performed using wet blotting system and blocking stage of membrane in 5% non-fat milk in TBST for an hour. Membrane containing protein was incubated with rabbit polyclonal β-actin primary antibody (1:200; Santa Cruz Biotechnology Inc., USA), rabbit polyclonal Bax primary antibody (1:200; Santa Cruz Biotechnology Inc., USA) and rabbit polyclonal Bcl-2 primary antibody (1:200; Santa Cruz Biotechnology Inc., USA) overnight at 4°C. After washing with TBS-T, the membrane incubated with HRP-conjugated goat anti-rabbit IgG (1:10,000; Santa Cruz Biotechnology Inc., USA) for 1h in room temperature in the dark. Finally, protein was exposed using chemi-luminescence kit (Amersham Biosciences, Orsay, France) on an X-ray film after a 10s exposure.

Statistical analysis

All data were shown as mean ± standard error of the mean. Results were provided from three independent tests. The statistical analysis of data was performed using SPSS 20 statistical software through two-way analysis of variance (ANOVA) followed by Tukey's test. P value less than 0.05 was considered statistically significant.

Results

Morphology and identification of neurospheres derived from human glioblastoma multiforme

We isolated CSCs from human glioblastoma core zone (Fig. 1, A). Human glioblastoma specimens were cultured in serum free medium associated with the growth factors such as EGF and bFGF. The single cells were floated in the culture medium and started to divide and primary neurospheres were generated between 7-8 days. The tumor stem-like cells or neurospheres proliferated in the form of clusters. The morphology of the tumor stem-like cells was similar to SVZ-NSCs (as an external control positive). Neurospheres grew to form free floating or loosely attached growing spheres in the condition of serum free medium (Fig. 1, B). Immunocytochemistry was used to evaluate nestin and CD133 antigens. Immunocytochemistry test indicated that glioblastoma neurospheres were positively reacted with specific antibodies of nestin and CD133 markers (Fig. 1, C). In addition, the glioblastoma CSCs were assessed

using real-time RT-PCR analysis for CSCs markers expression including nestin, SOX2 and CD133. Expression of the nestin, SOX2 and CD133 markers in the glioblastoma CSCs were shown by RT-PCR analysis. The expression of nestin, SOX2, CD133 markers was similar to SVZ-NSCs, however, their expression levels were significantly increased in the glioblastoma neurospheres (Fig. 1, D). Taken together, according to morphology and expression of NSC specific markers suggested that glioblastoma-isolated neurospheres may be cancer stem cell.

Morphology and identification astrocytes isolated from human glioblastoma multiforme

Astrocytes were isolated from the human peritumoral brain zone. We observed two morphology types in the cell culture medium. 1) The epithelioid form was observed to be large, abundance cytoplasm and interconnecting processes (Fig. 2, a). 2) The fibroblastic form was slender, elongated, and spindle-shaped (Fig. 2, b). The expression of GFAP antigen in the cells was positive in the immunocytochemistry test, confirming the presence of the astrocyte in the culture medium (Fig. 2, B).

Assessment of the multipotentiality of neurospheres derived from human glioblastoma multiforme

We evaluated whether neurospheres derived from glioblastoma have differentiation potential into astrocyte, oligodendrocyte and neuron. The floating neurospheres were cultured in a neurobasal medium containing 10% FBS without growth factors (FGF and EGF) for 21 days. It seems differentiation process of neurospheres was performed after removal of growth factors. The neurospheres differentiation was assessed via immunocytochemistry test for specific markers expression of astrocyte, oligodendrocyte and neuron. Immunocytochemistry staining indicated that GFAP, Olig2 and MAP2 markers were expressed in astrocyte, oligodendrocyte and neuron, respectively. Results confirmed that the glioblastoma has tumor stem-like cells that are able to differentiate (under specific conditions) to astrocyte, oligodendrocyte and neuron (Fig. 3).

Survival rate of cells after induced hyperthermia

To examine the effects of hyperthermia with different temperatures in glioblastoma cells, we assessed hyperthermia with temperatures 42-46°C for 1 h. The condition of cells was observed before and after hyperthermia. After 24h hyperthermia, the results shown that 42-46°C for 1 h could stop cell proliferation and decrease cell viability in a temperature dependent manner in glioblastoma cells. Cell viability was significantly decreased at temperature 42°C, 44°C and 46°C compared with the control at temperature 37°C. Astrocytes isolated from glioblastoma and SVZ-NSCs were more sensitive to hyperthermia than glioblastoma CSCs and a decrease in cell viability was significantly observed when compared to glioblastoma CSCs (fig. 4).

Levels of mRNA and protein expression of apoptotic markers in CSCs and astrocyte of glioblastoma

Cellular apoptosis was assessed using mRNA and protein expression levels of Bax and Bcl-2. In this study, we observed an increase in mRNA and protein expression of Bax and decrease in mRNA and protein expression of Bcl-2 after hyperthermia. The expression of Bax in astrocyte and SVZ-NSCs was

significantly increased compared to glioblastoma CSCs at temperature 44°C, suggesting astrocyte isolated from glioblastoma (as a differentiated cell) is more sensitive to high temperature than glioblastoma CSCs (as an undifferentiated cell) to hyperthermia (Fig. 5, A, B).

Discussion

In this study, we have assessed a population of cancer stem cells in the core region of glioblastoma. Antigens expression of cell surface including CD133, nestin and SOX2 were identified in these cancer stem cells [30, 31]. We have observed cancer stem cells derived from glioblastoma as clusters similar to neurospheres. They have potential to self-renew and proliferate and could be differentiated to neuron, astrocyte and oligodendrocyte [32]. Our study indicated that properties of the neurosphere isolated from glioblastoma seem to have the same to characterization of the neural stem cell derived from the subventricular zone. Simultaneous assessment of normal neural stem cells to glioblastoma cancer stem cells can find a link between brain neurogenesis and tumorigenesis [33]. Additionally, research has shown the increased expression of the markers CD133 and nestin to be associated with advanced disease [34]. Studies have shown that glioblastoma is phenotypically and also functionally heterogeneous and can be formed from both undifferentiated and proliferating cell such as cancer stem cell and differentiated and mature cells including astrocyte [35].

The data reports that glioblastoma originates from cancer stem cells in the tumor core that expresses CD133 and nestin markers. Astrocytes in the peripheral part of tumor seem to differentiate from cancer stem cells of the presence in the part of tumor core, indicating the cancer stem cells are able to differentiate in the intra-tumoral environment [36]. However, differentiation of cancer stem cells to astrocyte leads to over-expression of the astrocytic marker GFAP that is expressed in astrocytoma [34, 37, 38]. It was postulated that cancer stem cells possess tumorigenesis potential, therapy resistance and disease recurrence in patients with glioblastoma [9]. Cancer stem cells derived from glioblastoma can form brain tumors when transplanted into nude mice [7]. The mechanisms of cancer stem cells resistance to therapy seem to be combination of slow cell cycle activity, resistance to DNA damage and oxidative stress, inhibiting cell death, hypoxia and multidrug resistance [39–42]. Cancer stem cells may be in a quiescent status and lead to treatment resistance compared to dividing cells [43]. In the parallel these reports, we observed that neurospheres isolated from glioblastoma are more resistant than astrocytes derived from the glioblastoma.

Thus, different minimally invasive therapies are being evaluated which includes hyperthermia (HT). Hyperthermia was introduced as a therapeutic approach for brain tumors several years ago. Hyperthermia is defined as a temperature increase of body tissue in a given time period, which is used to inhibit growth and induce apoptosis of glioblastoma cells [44]. Various studies have been reported that hyperthermia could be effective for therapy of liver cancer [16], gastric cancer [45] and breast cancer [46]. In addition, hyperthermia can be effective in inhibiting proliferation and apoptosis induction in human glioma cells [17]. Hyperthermia can sensitize cells to chemo-radiotherapy through change of protein structure, inhibition of DNA repair and stop of cell cycle [47].

Therefore, we induced hyperthermia in vitro as much as 4246°C for 1 hour and the status of glioblastoma cells observed before and after hyperthermia. We found that hyperthermia could stop cell growth and inducing apoptosis in a temperature dependent manner in glioblastoma cells. Wang et al. have shown that hyperthermia could decrease the growth, angiogenesis rate and hardness degree of tumor at condition of 44°C for 1 hour, in subcutaneous tumor models of glioma. Numerous basic and clinical studies have presented that 4245°C are considered as suitable temperatures for hyperthermia through microwave or water bath [48, 22]. Temperature level is a challenge for hyperthermia of clinical studies, as 41.8°C is used for wholebody temperature in humans, high temperatures such as 4245°C strikes a problem and advanced equipment is required [26]. However, results of several studies of hyperthermia don't support the inducing of anti-tumor effects, but one research demonstrated that hyperthermia at 3941°C for 90 min could in fact induce cellular and humoral antitumor reactions [49]. According to current clinical status, hyperthermia would damage normal brain tissue at higher temperatures (45°C) [50]. Hyperthermia could be introduced as an effective therapy in the future when nanotechnologydependent hyperthermia and treatment approaches were developed for malignant cancers [51].

Conclusion

Cancer stem cells of glioblastoma could be introduced as an important tool for the assessment of the tumorigenic process in the central nervous system, and will be critical in developing therapies such as hyperthermia technology for treating malignant tumors.

Declarations

Acknowledgements

We thanksfunctional neurosurgery research center, Shahid Beheshti University of medical sciences for financial support.

Author Contributions

Study design and performing tests: M. Hashemi, surgical procedure: S. oraee-yazdani, Analysis and interpretation of data: M. Hashemi, Drafting of the manuscript: M. Hashemi, editing of the manuscript: J. Lenzer, A. Abbasiyam, S. oraee-yazdani. All authors contributed to the article and confirmed the submitted version.

Data Availability

All data generated or analysed during this study are included in this published article. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval

All procedures performed in study according to the Ethical Commission of the Shahid Beheshti University of Medical Sciences. Ethical code of study is IR.SBMU.RETECH.REC.1399.015.

Consent to Participate

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

Consent to Publish

Not applicable.

Conflict of interest

The authors declare that they have no conflict of interest.

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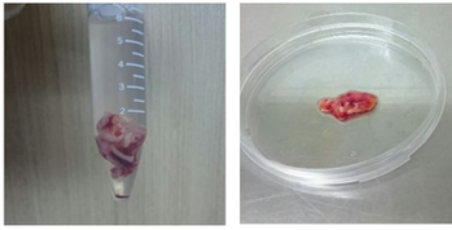
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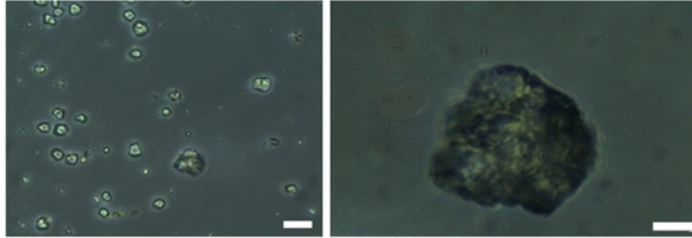
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Figures

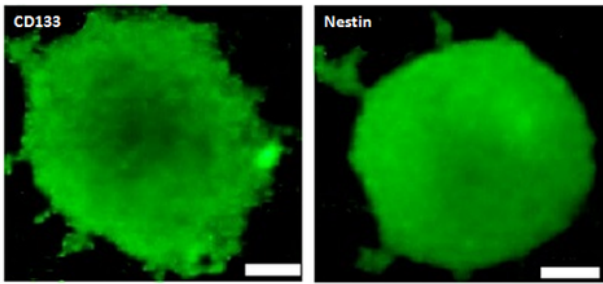
A



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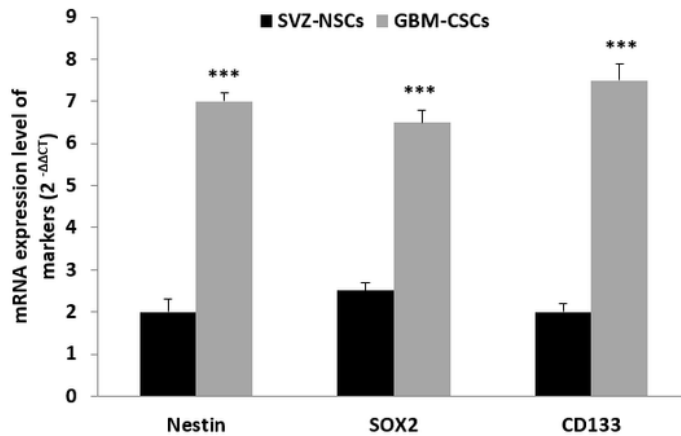
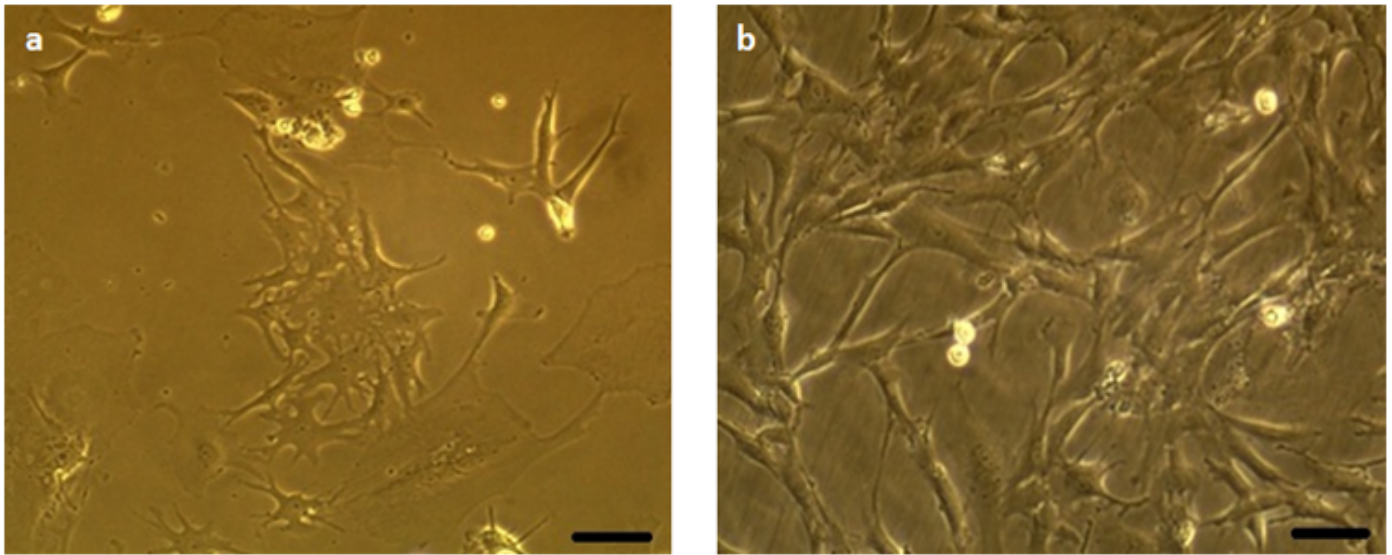


Figure 1

Primary culture of adult glioblastoma cells. (A) Tissue of the human glioblastoma. (B) Morphology of cancer stem cell derived from human glioblastoma tissue. Scale bar of the left image: 50 μm , Scale bar of the right image: 100 μm . (C) Neurosphere derived from a single isolated CD133 positive (left) and nestin positive (right) cell cultured in NSC medium. Scale bar of the right image: 100 μm (D) Specific antigens

expression of neurospheres derived from glioblastoma including nestin, SOX2 and CD133 through real-time RT-PCR analysis. ***P < 0.001, significant intergroup differences versus control (SVZ-NSCs).

A



B

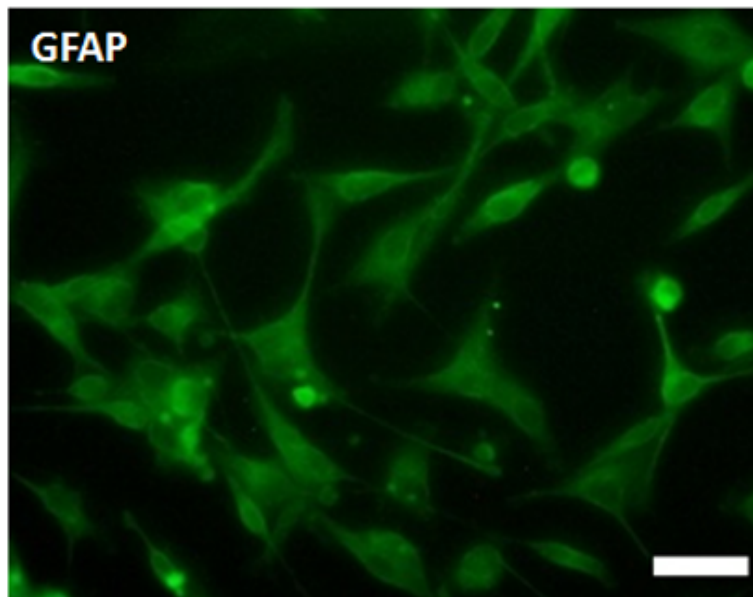


Figure 2

Morphology of the Astrocyte Types. (a) Astrocytes with epithelioid morphology, (C) Astrocytes with fibroblastic morphology, (D) Confirmation of cultured astrocytes through expression of GFAP protein in the astrocyte, scale bar: 100 μ m.

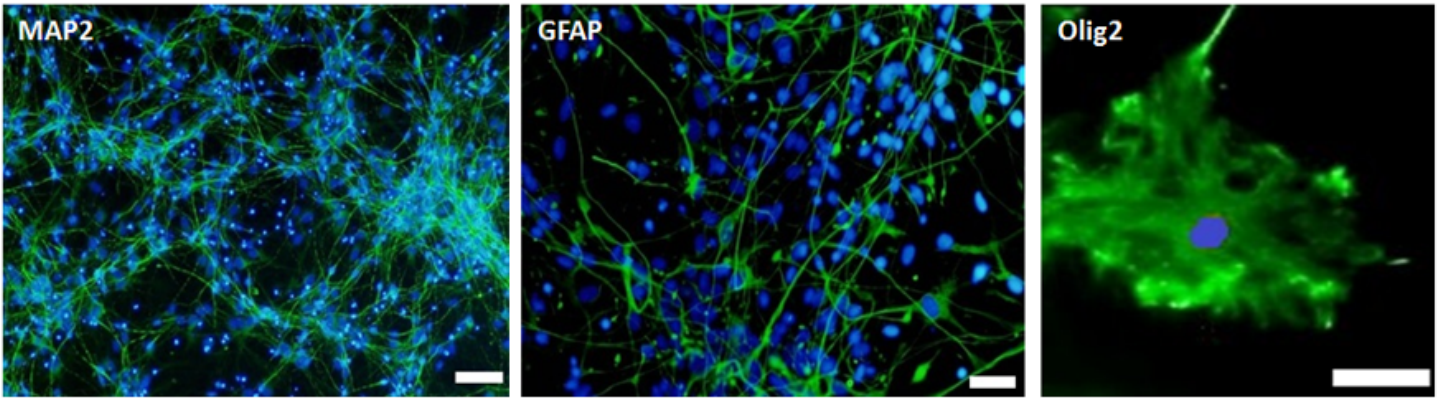


Figure 3

Differentiation of neurospheres derived from glioblastoma. Neurospheres differentiated to neuron (MAP2, left), scale bar: 50 μm , astrocyte (GFAP, center), scale bar: 50 μm and oligodendrocyte (Olig2, right), scale bar: 100 μm

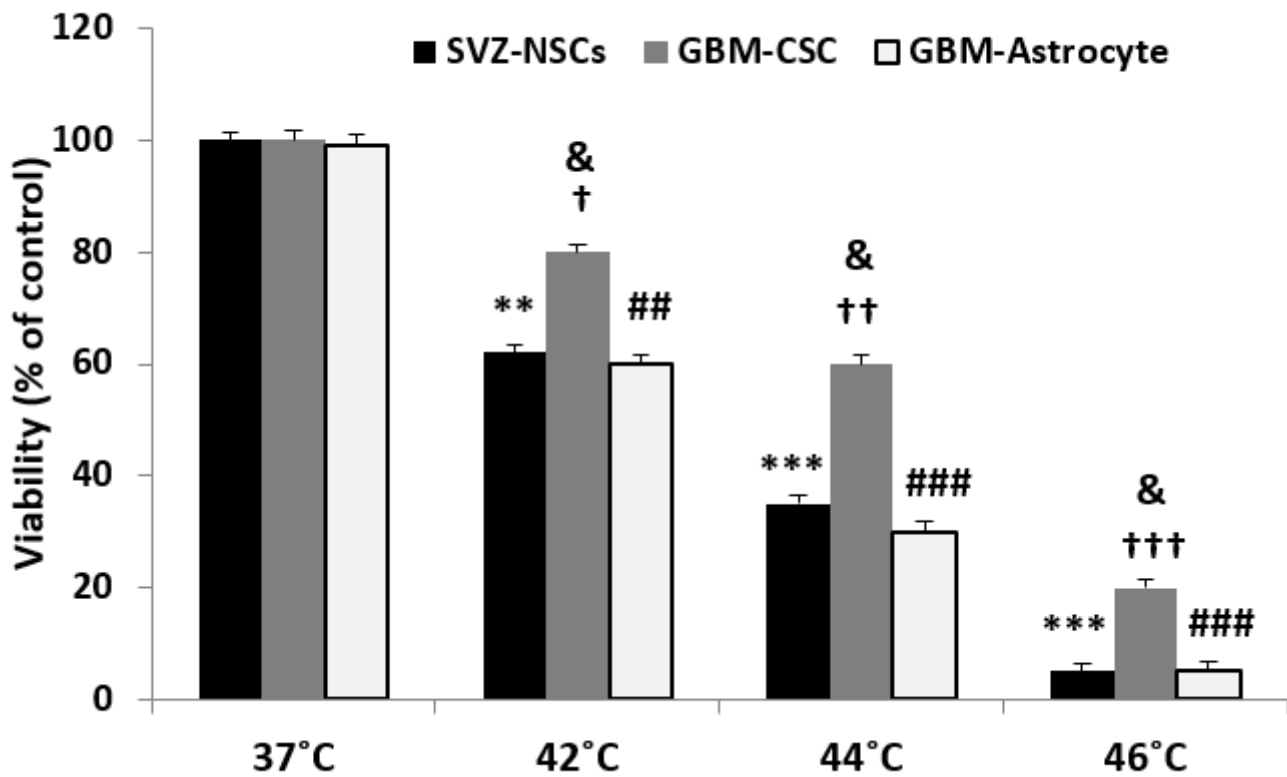
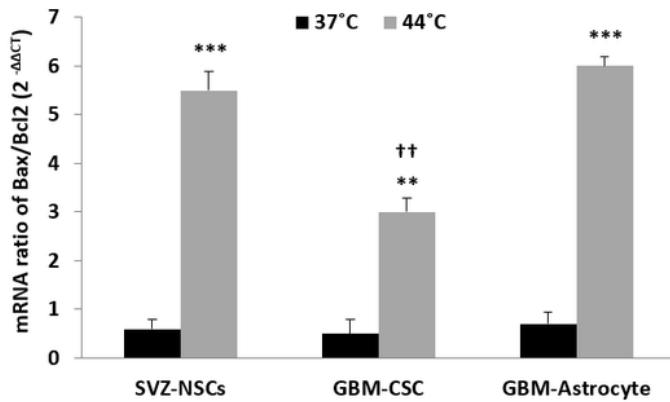


Figure 4

The survival rate of glioblastoma cells after hyperthermia at different temperatures. Hyperthermia with 42 44°C for 1 h and 1 time inhibited the growth of glioblastoma cells in a temperature dependent manner. **P < 0.01, ***P < 0.001, †P < 0.05, ††P < 0.01, †††P < 0.001 and ##P < 0.01, ###P < 0.001, significant

intergroup differences versus control (temperature 37°C). &P < 0.05 intergroup differences versus SVZ-NSCs.

A



B

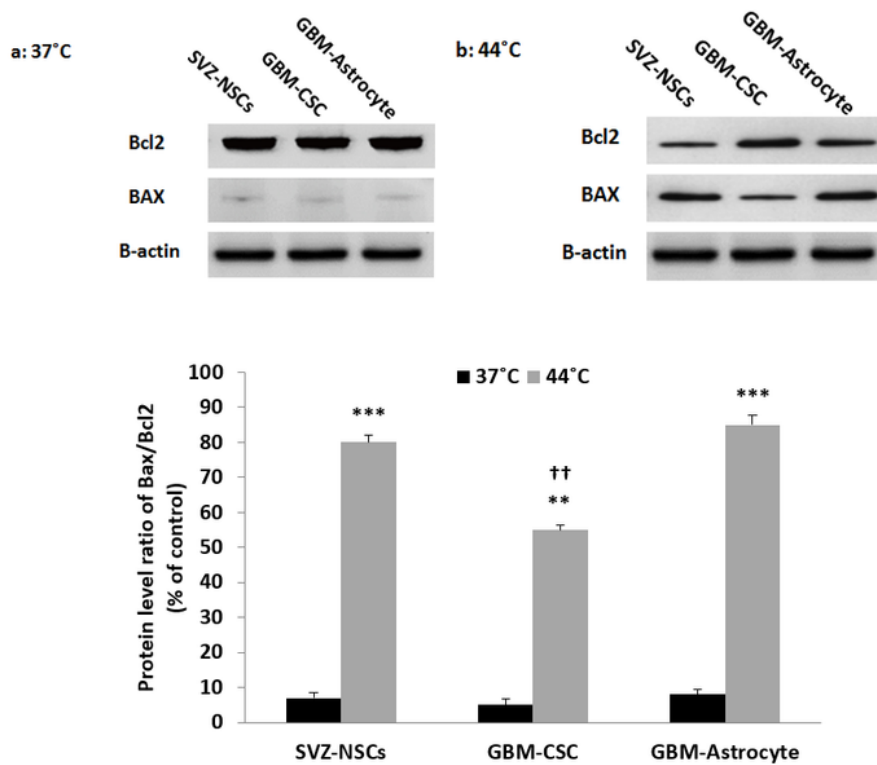


Figure 5

mRNA and proteins expression of anti-apoptotic and apoptotic in glioblastoma cells. Apoptotic mRNA and proteins rate were evaluated in glioblastoma cells in the presence of hyperthermia using real time PCR analysis (A) and western blot assay. The densities of Bax and Bcl-2 bands were calculated, and their

ratio to β -actin was calculated. **P < 0.01, ***p < 0.001, significant intergroup differences versus control (temperature 37°C). ††P < 0.05 intergroup differences versus SVZ-NSCs.