# The biological characteristics of glioma stem cells in human glioma cell line SHG44

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**Abstract.** Gliomas are the most common tumors of the central nervous system (CNS) and a frequent cause of death. The treatment of malignant gliomas is often palliative due to their high recurrence rate. A growing body of evidence suggests that glioma may arise from cancer stem cells (CSC) correlated with neural stem cells (NSC), with the capacity for self-renewal and multipotency. CSCs have been isolated from human gliomas and numerous other solid tumors. It is assumed that a number of established malignant cell lines also contain a rare subpopulation of stem cells. This study was designed to investigate the proportion of CSCs in the human glioma cell line SHG44 and to study the limitations of CD133 immunophenotyping in glioma stem cell research. SHG44 cells were cultured in both serum-containing and serum-free medium. The similar shape in growth curves (in the exponential growth phase) revealed that most cells participated in the population amplification. Time gradient BrdU labeling and monoclonal assay revealed that almost every single cell participated in the division growth (98.82%) and possessed the ability to form clones (96.19%). No significant difference was found in the proportions of $CD133^+$ cells in the serum-containing and serum-free groups (38.25%/37.92%). In addition, no significant difference was noted in the proportions of $CD133^+$ cells among monoclones selected randomly in the serum-containing group. These results suggested that $CD133^-$ cells generate $CD133^+$ cells and have the ability to form clones. Thus, we concluded that most SHG44 cells were CSCs and serum-free medium was not necessary for the generation of CSCs. In this line, $CD133^-$ cells also possessed clonogenic, self-renewal capacities and were also CSCs.

## Introduction

Gliomas are the most frequently occurring primary neoplasm of the central nervous system (CNS), accounting for over 40% of such tumors and 78% of CNS malignant tumors in adults (1). Malignant gliomas are highly recurrent tumors even following surgery, chemotherapy, radiation and immunotherapy. Gliomas consist of heterogeneous populations of cancer cells in phenotypes and possess proliferative potential. It has been suggested that certain cells in general may undergo processes that are analogous to the self-renewal and differentiation of normal stem cells (2).

Accumulating evidence suggests that malignant parenchymal tumors also contain their own stem cells, termed cancer stem cells (CSC). Despite their small quantity, the CSC hypothesis postulates that this subpopulation possesses the ability of infinite proliferation and multipotency, and thus plays a crucial role in the initiation, progression and recurrence of tumors (3-5). Therefore, the tumor is regarded as an aberrant organ, containing a minor portion of CSCs and a major portion of non-CSCs. Although both CSCs and non-CSCs contain oncogenic mutations, the later population lacks the capacity for unlimited self-renewal and multipotency (6,7).

CSCs have been isolated from human gliomas, numerous other parenchymal tumors and established cell lines (6,8-17). Three methods have been introduced to isolate CSCs from parenchymal tumors. First, cancer cells were cultured in serum-free medium similar to that used to culture NSCs; this method was regarded as the most typical. Various authors reported that CSCs from gliomas or glioblastomas were capable of proliferating and forming 'cell spheres' in serum-free medium (10,18-20) and that individual cells from the primary spheres were able to form a new sphere in serial passages, suggesting that these cells were capable of self-renewal. Second, CSCs were isolated based on the expression of CD133 (20), a membrane glycoprotein which is characterized in two independent studies (21,22), and originally identified in neuroepithelial stem cells (23). Its interest as a CSC marker has been on the increase since it appeared that it was capable of identifying a cancer-initiating subpopulation in brain (20) and colon tumors (24). When CSCs were isolated from human brain tumors, $CD133^+$ cells exhibited stem cell properties *in vitro* and initiated tumor growth *in vivo* (8,20). Only the $CD133^+$ cells collected using fluorescence-activated cell sorting (FACS) or immunomagnetic beads were capable of forming spheres (25). Third, the 'side-population cells' (SP cells) were composed of CSCs (26). Stem cell subpopulations have been defined using the fluorescent dyes rhodamine 123 and Hoechst 33342 (27,28).

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SP cells expressed ATP-binding cassette (ABC) transporters, which could rapidly efflux the fluorescent nuclear dye Hoechst 33342. Therefore, the cells were not stained by fluorescent dyes and were isolated by FACS (29).

Although different methods were used in the isolation of CSCs from parenchymal tumors, several studies concluded that only a small portion of the cancer cells were clonogenic, and only these cells were capable of tumor propagation (30). These methods were also used to isolate stem cells from established cancer cell lines. However, controversy arose regarding the proportion of CSCs in cancer cell lines, including the human glioma cell line SHG44, which has been maintained *in vitro* over decades. Huang *et al* reported that the SHG44 cell line contained only 0.021% CSCs using a CD133 magnetic activated cell sorter (MACS) (31). Most studies indicated that serum-free medium was indispensable for the growth of CSCs (32) and that CSCs were composed of $CD133^+$ cells (19,20). Therefore, the present study investigated the proportion of CSCs in the human glioma cell line SHG44, as well as the limitations of CD33 immunophenotyping.

## Materials and methods

*Cell culture*. SHG44 cells (obtained from the Experimental Animal Center of Sun Yat-Sen University, Guangzhou, China) were cultured in serum-containing medium, which was composed of DMEM/F12 (Gibco, Carlsbad, CA, USA) and 10% fetal bovine serum (FBS; Lanzhou Minhai Bio-Engineering Co., Ltd., Lanzhou, China). The cells were harvested, incubated in phosphate-buffered saline (PBS) containing 0.25% trypsin and 0.02% EDTA for 5 min at 37˚C, triturated gently using a Pasteur pipette and washed with culture medium. The suspension was centrifuged at 1,000 rpm for 5 min to collect the cells. The supernatant was removed carefully and completely. The cell pellet was resuspended in fresh serum-free medium, which was composed of DMEM/F12, 20 ng/ml basic fibroblast growth factor (bFGF; Sigma, St. Louis, MO, USA), 20 ng/ml epidermal growth factor (EGF; Sigma), and 20 $\mu$l/ml B27 supplement (Gibco). The suspension was then plated into culture flasks at a density of $1.5x10^4$ cells/ml. Cells were incubated at 37˚C with 95% air, 5% $CO_2$ and 100% humidity. SHG44 cells cultured in serum-free medium were stable and passaged 4-5 times every month.

*Cell morphology and viability*. SHG44 cells were cultured in serum-containing and serum-free medium alternately, and were observed and compared daily using an inverted microscope. The effects on SHG44 cells caused by different media were studied. The viability of SHG44 cells cultured in serum-free medium was determined using the trypan blue dye exclusion assay, and the number of cells was counted using a hemocytometer.

*Growth curve*. A methyl thiazolyl tetrazolium (MTT; Sigma) assay was performed to determine the growth curves of the SHG44 cells cultured in both serum-containing and serum-free medium. SHG44 cells were dissociated, adjusted to a density of $1.5x10^4$ cells/ml with different media, respectively, and seeded into four 96-well plates (approximately 100 $\mu$l per well). Two plates were used for the serum-containing group and the remaining two plates were used for the serum-free group. MTT assay was performed daily (12 wells each group) from the second to the eighth day of incubation. Briefly, 20 $\mu$l MTT (5 mg/ml) was added to each well, and the cells were incubated for 4 h. The culture medium was then removed carefully. On the eighth day, 150 $\mu$l DMSO (Sigma) was added to each well. After shaking thoroughly for 10 min, the absorbance of each well was read by an enzyme immunoassay instrument at a wavelength of 492 nm. Since absorbance is in proportion to the number of living cells in a sample, the MTT assay reflects the extent of cell proliferation. Growth curves were drawn according to the absorbance.

*Time gradient BrdU labeling assay*. To determine the proliferation modality of SHG44 cells, time gradient bromodeoxyuridine (BrdU; Sigma) labeling assay was performed. SHG44 cells were dissociated, adjusted to a density of $1.5x10^4$ cells/ml with different media, respectively, and plated on 6-$cm^2$ culture plates (1.5 ml per plate). SHG44 cells were cultured in BrdU-supplemented medium (with and without serum). The final concentration of BrdU was 6 $\mu$g/ml. The cells were incubated at 37˚C with 95% air, 5% $CO_2$ and 100% humidity. After incubating for 2 h, SHG44 cells from the two groups were fixed, respectively, using 4% paraformaldehyde (PFA) for 20 min every 6 h. Cells were fixed 8 times, each time with 5 culture plates from each group. Therefore, the proliferation time of the cells in the last group was 50 h. Each time, when the cells were fixed well, they were washed 3 times with PBS, and immunostaining was performed as previously described (33,34), with some modification. Briefly, the cells were permeabilized with cold 0.5% Triton X-100 for 15 min at room temperature and washed 3 times with PBS. Endogenous peroxidase activity was quenched in 3% $H_2O_2$ for 10 min at room temperature and washed 3 times with PBS. The cells were then incubated in 2 M HCl for 30 min at 37˚C to denature DNA, followed by incubation in 0.1 M borate saline buffer to counteract HCl, and washed with PBS. The cells were then incubated at 4˚C overnight in primary mouse anti-BrdU monoclonal antibody (1:1200; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China). After washing with PBS, cells were treated with 2-step plus polymer HRP anti-mouse/rabbit IgG detection system (PV9000; Beijing Zhongshan Jinqiao Biotechnology Co.). The cells were then stained with AEC (3-amino-9-ethylcarbazole) (Beijing Zhongshan Jinqiao Biotechnology Co.) and examined under a microscope.

*Monoclonal assay*. Monoclonal assay was performed to determine the self-renewal ability of the targeted cells. It was essential to prepare single-cell suspensions without affecting cell viability significantly. Cell viability was determined using the trypan blue dye exclusion assay. The single-cell suspension of SHG44 cells cultured in serum-containing medium was prepared and adjusted to a density of approximately 100 cells/$\mu$l under a microscope. Cells 1 $\mu$l were added to 10 ml serum-containing and serum-free medium, respectively, and seeded into 96-well plates (approximately 100 $\mu$l per well), which were labeled as serum-containing and serum-free. Wells containing no cells or more than one cell were excluded, and those with only one cell were marked and checked daily under a microscope to observe the clone formation. The same procedures

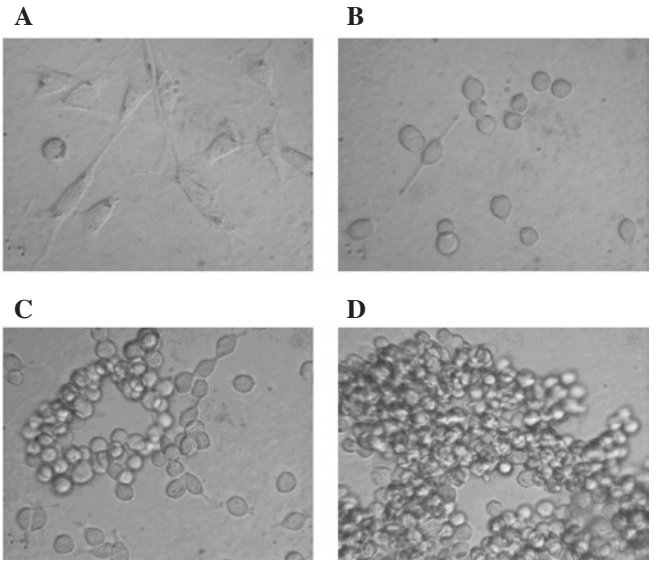


Figure 1. SHG44 cells were cultured in serum-containing/free media. (A) SHG44 cells adhered in serum-containing medium. (B) SHG44 cells adhered loosely in serum-free medium on the first day. (C) Floating cells multiplied in serum-free medium 2 days later. (D) Most cells became cell spheres in serum-free medium 5 days later.

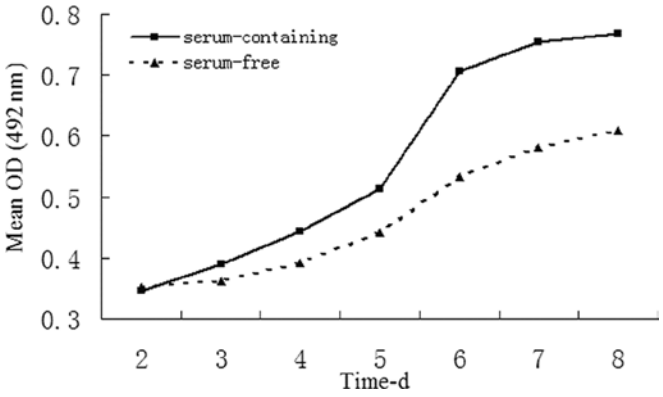


Figure 2. MTT proliferation assay. The growth curves of the serum-containing and serum-free groups were similar in shape, although the absolute value of the serum-containing group was higher.

described above were also performed on SHG44 cells cultured previously in serum-free medium.

*Immunocytochemistry of CD133*. Immunostaining was performed to study the expression of CD133 induced by different media. The single-cell suspension of SHG44 cells previously cultured in serum-containing medium was prepared and adjusted to a density of $1.5x10^4$ cells/ml with serum-containing and serum-free medium and then plated on 6-$cm^2$ culture plates (1.5 ml per plate), respectively. The same procedures described above were also carried out on SHG44 cells previously cultured in serum-free medium. Thus, there was a total of four groups in this assay. The cells were incubated at 37˚C with 95% air, 5% $CO_2$ and 100% humidity. Immunostaining of CD133 was performed on these cells after 3 days of proliferation. The SHG44 cells of these groups were then fixed using 4% PFA for 20 min, followed by washing 3 times with PBS. Endogenous peroxidase activity was quenched in 3% $H_2O_2$ for 10 min at room temperature and washed with PBS. The cells were then incubated at 4˚C overnight in primary goat anti-CD133 polyclonal antibody (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After washing with PBS, cells were treated with 2-step plus polymer HRP detection system for goat primary antibody (PV9003; Beijing Zhongshan Jinqiao Biotechnology Co.). The cells were then stained with AEC (Beijing Zhongshan Jinqiao Biotechnology Co.) and examined under a microscope.

*Expression of CD133 of monoclones*. Immunostaining of CD133 was also performed on clones formed by single SHG44 cells cultured in serum-containing medium. From each monoclonal assay performed by serum-containing medium, 5 monoclones were selected randomly. Each clone was harvested by trypsin and then seeded on the 6-$cm^2$ culture plate filled with serum-containing medium. The cells were then incubated at 37˚C with 95% air, 5% $CO_2$ and 100% humidity. Immunostaining of CD133 was performed on these cells as previously described after proliferating to a moderate cell density.

*Statistical analysis*. Data were processed with the SPSS 16.0 statistical software and expressed as the mean±SD. The Student's t-tests or $\chi^2$ tests were used where appropriate. $P<0.05$ was considered to be statistically significant.

## Results

*SHG44 cells were stable in both serum-containing and serum-free media*. SHG44 cells grew as single cells attached to the culture flask and exhibited polygonal or typical fibroblast-like morphology when they were cultured in serum-containing medium (Fig. 1A). In serum-free medium, SHG44 cells attached to the culture flask, but adhered loosely on the first day. Cells exhibited globular or excrescent morphology (Fig. 1B). The cells began to float and form small cell spheres in the following two days, but there were still many adherent cells (Fig. 1C). The floating cells increased as the adherent cells decreased. On the fifth day, most cells floated and formed cell spheres, which became larger (Fig. 1D). SHG44 cells were stable for long-term culture in serum-containing and serum-free medium.

When SHG44 cells cultured in serum-free medium were reseeded in serum-containing medium, the shape of the cells was the same as those cultured previously in serum-containing medium. Therefore, SHG44 cells grew both in serum-containing medium as single cells attached to the culture flask and in serum-free medium as floating spheres. When the medium was changed, the two growth patterns alternated. The alteration ability did not decrease after the process was repeated a number of times.

*Majority of SHG44 cells participated in the population expansion*. The MTT proliferation assay revealed that the growth curves of the serum-containing and serum-free group were similar in shape (Fig. 2), although the absolute value of the serum-containing group was higher. The two groups exhibited an exponential growth pattern.

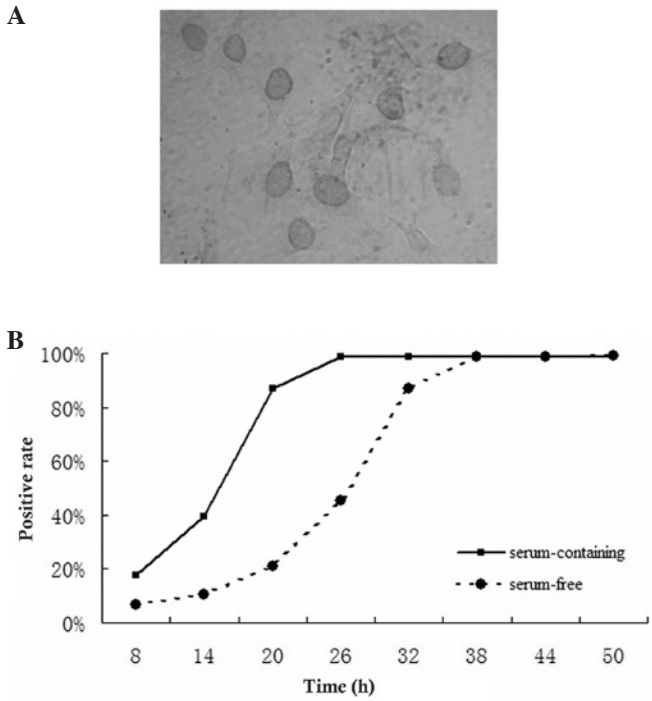


Figure 3. BrdU labeling assay. (A) Results of immunocytochemistry of BrdU. (B) The proportion of $BrdU^+$ cells increased as BrdU exposure time increased. The extreme proportions of $BrdU^+$ cells in the serum-containing and serum-free groups were similar and almost 100%, indicating that almost every cell was involved in the division growth.

Time gradient BrdU labeling assay revealed that the proportion of $BrdU^+$ cells increased as the BrdU exposure time increased. When the BrdU exposure time was 8 h, the proportion of $BrdU^+$ cells in the serum-containing group was 17.50±1.00%, and that of the serum-free group was 6.60±0.40%. When the time increased to 38 h, the proportions increased to 98.78±0.43% and 98.86±0.44%, respectively (P>0.05; Fig. 3A and B). Therefore, the extreme proportions of $BrdU^+$ cells in the serum-containing and serum-free groups were similar and almost 100%, indicating that almost every cell participated in the division growth.

If only a small portion of the stem cells contributes to the formation of a glioma sphere, there must be three destinies for the original non-stem cells: to die, to persist without involvement in sphere formation, or to proliferate in a monolayer form. However, it was difficult to find dead cells or debris in the serum-free cultures, which were common in the primary cultures of neural stem cells. Of note, 4 days after passage, almost all of the cells were involved in the formation of floating spheres; neither a monolayer nor quiescent single cells were observed.

*Majority of SHG44 cells possessed self-renewal ability.* In the clonogenic analysis, when the cells used to perform monoclonal assay had previously been cultured in serum-containing medium, the clone formation rate of the serum-containing group was 96.31±2.10%, and that of the serum-free group was 95.98±2.27%. When the cells were cultured in serum-free medium previously, the clone formation rates were 96.28±3.72 and 96.08±3.75%, respectively. The clone formation rates of these four groups were similar (P>0.05) and almost 100%, which indicated that most SHG44 cells possessed self-renewal ability; the characteristic used to define CSCs.

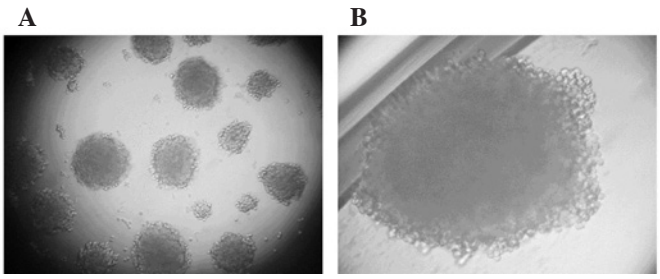


Figure 4. The morphology of monoclones formed in serum-free medium. In serum-free medium, (A) several cell spheres or (B) a typical cell sphere were observed in each labeled well.

The shape and proliferation characteristics of clones correlated with the medium in 96-well plates rather than the medium in which single cells were previously cultured. The amplification speed of monoclones formed in serum-free medium was lower than that in serum-containing medium. In serum-containing medium, 78.50% (168/214) of single cells divided into two cells at 24 h. There were approximately 64 cells of each clone 5 days later, and >100 cells on the seventh day. On the fifteenth day, each clone exceeded a quarter of the bottom of the well and the cells appeared more tense. In serum-free medium, only 10.84% (22/203) of single cells divided into two at 24 h, and the rate increased to 80.30% (163/203) at 48 h. There were 20-30 cells of each clone on the fifth day, and approximately 60 cells on the seventh day. On the seventeenth day, several cell spheres or a typical cell sphere were observed in each labeled well (Fig. 4A and B).

*Limitations of CD133 immunophenotyping in glioma stem cell research.* SHG44 cells previously cultured in serum-containing medium were reseeded in serum-containing and serum-free medium, respectively. Immunostaining of CD133 was performed following 3 days of cell proliferation. The proportion of $CD133^+$ cells in the serum-containing group was 38.25±1.28%, and that in the serum-free group was 38.24±1.81%. When the same procedures were performed on SHG44 cells previously cultured in serum-free medium, the proportions were 37.82±0.89% and 37.92±1.21%, respectively. The proportions of $CD133^+$ cells of these 4 groups were similar (P>0.05; Fig. 5A). Whether the cells were previously cultured in serum-free medium or reproliferated in serum-free medium, the rate of $CD133^+$ cells in the serum-free group was no higher than that in the serum-containing group.

Monoclones were selected randomly in the serum-containing medium, and these clones were also formed by single cells previously cultured in serum-containing medium. Immunostaining of CD133 was performed on the clones after they were reseeded in serum-containing medium and proliferated to a moderate cell density. The proportion of $CD133^+$ cells in the clones was 38.26±1.32% (P>0.05; Fig. 5B). As a result, the proportion of $CD133^+$ cells in the SHG44 line was approximately 38%. It indicated that these single cells contained approximately 38% of $CD133^+$ cells and 62% of $CD133^-$ cells in the monoclonal assay. However, when immunostaining of

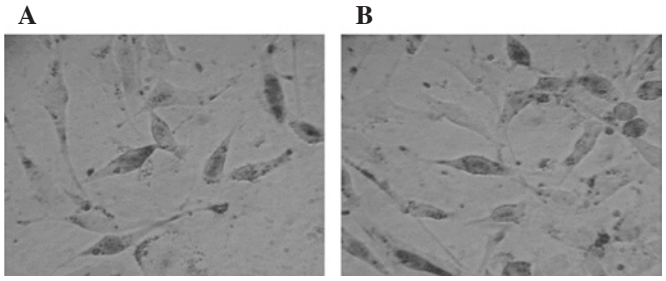


Figure 5. Expression of CD133 in SHG44 cells. (A) The results of immunocytochemistry of CD133. No significance was found in the proportions of $CD133^+$ cells cultured in different media. (B) The results of immunocytochemistry of CD133 of every SHG44 cell monoclone, which was formed in serum-containing medium.

CD133 was performed on the monoclones, we found neither 100% $CD133^+$ clones nor 100% $CD133^-$ clones. Thus, we concluded that i) both $CD133^+$ and $CD133^-$ cells were capable of forming clones, ii) the descendants of the $CD133^-$ cells were a mixture of both $CD133^-$ and $CD133^+$ cells, and iii) $CD133^-$ cells possessed clonogenic, self-renewal capacities and were also CSCs.

## Discussion

The human glioma cell line SHG44 has been widely used in the study of glioma for decades. This cell line was relatively stable although it has been cultured long-term *in vitro*. In our study, SHG44 cells were stable when cultured in serum-containing and serum-free medium, respectively, and two growth patterns were capable of alternating. This conversion ability did not decrease after the process was repeated numerous times.

The growth curves revealed that the majority of SHG44 cells participated in the population expansion, whether these cells were cultured in serum-containing or serum-free medium. When the BrdU exposure time was 38 h in the time gradient BrdU labeling assay, the proportion of $BrdU^+$ cells in the serum-containing and serum-free groups was similar and almost 100%, indicating that almost every cell was involved in the division growth. For this reason, we assumed that most SHG44 cells were CSCs.

Without any classification based on a specific marker, a single cell clone culture of SHG44 cells was performed using different media. Almost every viable SHG44 cell was capable of self-renewal and generated a clone. The similar clone formation rates also revealed that serum-free medium may only provide a different growth environment for SHG44 cells and these cells were stable when cultured in different media, as mentioned previously. Thus, we assumed that most SHG44 cells were CSCs and that serum-free medium was dispensable for CSCs in this cell line.

The controversy regarding the culture medium is ongoing. Investigators have considered CSCs to be unable to maintain their characteristics without serum-free medium. As a result, CSCs would become non-tumorigenic and begin to differentiate in serum-containing medium (32). However, results of certain studies have shown that a subpopulation of cells exhibited CSC characteristics and maintained the stability of the cell line in serum-containing medium (35-37). In serum-free medium, non-CSCs possessed stemness and tumorigenic properties through gene mutation (38). Thus, the CSCs isolated from the cell lines cultured *in vitro* did not reflect the real conditions *in vivo*.

Serum-free medium containing mitogens (EGF and/or bFGF) has been reported to selectively favor the proliferation of CSCs rather than non-CSCs (10,19,35). The present study has shown that most SHG44 cells proliferated in both serum-free and serum-containing media, although at different velocities. If serum-free medium has a selective effect on CSCs, most SHG44 cells have been selected. In serum-free medium, viable cells formed an attached monolayer at an early stage. Therefore, it is illogical to assume that CSCs merely adopt the appearance of a floating sphere as differentiation is generally irreversible. The suspension or attachment state did not appear to distinguish different populations with different abilities for self-renewal. Furthermore, every viable single SHG44 cell produced a clone in both serum-free and serum-containing media. Serum-free medium, however, was not required to stimulate clone formation and individual cell expansion. The current prevailing opinion is that only a small portion of cells possess unlimited self-renewal capacity (39). However, there are several inconsistencies to this idea, as listed below.

Stem cells are capable of undergoing both symmetrical and asymmetrical cell division and resulting in both stem and non-stem cells (30). If the cell line maintains a rare, but stable subpopulation of stem cells, the stem cells should divide symmetrically. The model is based on two premises. First, most cells proliferate and participate in the population expansion, as shown in our results. Second, the cycle of stem cells occurs at an equal speed to that of non-stem cells since the stem cells are generally quiescent. If the stem cells always divide symmetrically, then it is necessary to establish the origins of these assumed non-stem cells. In contrast to stem cells, non-stem cells are thought to possess a limited amount of self-renewal capacity (30). In addition, descendants of a non-stem cell eventually die. Thus, the SHG44 line would only contain a stem cell subpopulation. However, no great loss of cells was observed in the SHG44 cultures.

Another inconsistency is that 0.021-1.5% of stem cells in the SHG44 line (31) is lower than 0.5-30% of stem cells in glioma specimens (19,35). The establishment of the glioma cell line involved successive sub-cloning to achieve a highly homogeneous tumorigenic cell population. It is likely that this purification process increased the proportion of stem cells.

Previous studies have reported that certain conditions, such as attachment, use of serum-containing medium, and withdrawal of mitogens (EGF/bFGF) were essential to induce CSCs to differentiate into committed non-stem cells (35). Considering that the SHG44 cell line is known to be cultured in the form of an attached monolayer in serum-containing medium, the manner in which the small portion of stem cells maintained their undifferentiated state under this selection pressure is noteworthy. These controversies may arise from shortcomings in the definition, identification and isolation of CSCs. In our opinion, most SHG44 cells were CSCs, which seldom gave rise to committed non-stem cells in both the serum-containing and serum-free media.

Although the biological function of CD133 is not well understood, antibodies to CD133 epitopes have been widely

used to purify hematopoietic stem cells (40). CD133 is now extensively used as a surface marker to identify and isolate CSCs in malignant brain tumors as well as established cell lines (40-43). Although widely used, the validity of CD133 was debated, as recent data revealed that $CD133^+$ and $CD133^-$ cells shared similar stemness and tumorigenic properties in glioma (44-46). In the present study, for the SHG44 cell line, the limitations of CD133 immunophenotyping in glioma stem cells were also verified. First, our study revealed that the proportion of $CD133^+$ cells was 38.10±1.26%. The different culture media had no effect on the expression of CD133 in this cell line. If CSCs were composed of $CD133^+$ cells and serum-free medium was indispensable for CSCs, it is difficult to explain the similar proportions of $CD133^+$ cells in the different media observed in our study. In addition, when the immunostaining of CD133 was performed on randomly selected monoclones, it was of note that the proportion of $CD133^+$ cells in these clones was 38.26±1.32%, rather than 100% $CD133^+$ clones or 100% $CD133^-$ clones. The proportion of $CD133^+$ cells in SHG44 monoclones was also similar to that in the SHG44 cell line. Thus, we confirmed that $CD133^-$ cells also possessed the clonogenic self-renewal abilities and that CD133 could not be used as a surface marker for CSCs in SHG44 cell lines.

In addition, most SHG44 cells possessed the characteristics of CSCs, participating in the formation of floating cell spheres in serum-free medium, and nearly every single cell possessed clone-forming ability in serum-containing/free media. No significant difference was observed in the proportions of $CD133^+$ cells between the serum-containing and serum-free groups (38.25/37.92%). $CD133^-$ cells generated $CD133^+$ cells and were capable of self-renewal.

In conclusion, the current prevailing opinion is that only a small portion of cells are $CD133^+$ cells (20,44). The rate of $CD133^+$ cells in SHG44 in our study was relatively high. However, this observation can be explained by gene mutation or enriching of $CD133^+$ cells in the serial passage *in vitro* (38,47). Although CD133 is essential for stem cells to survive or maintain their primitive state *in vivo* (8), this was not the case for the SHG44 cell line in our study. In contrast to the study *in vivo*, there is no strong factor inducing the primitive CSCs to die or differentiate in the culture system, otherwise, no stem cells would remain in the cell line after a long period of time. Thus, the expression of CD133 is likely altered in the glioma cells, without the loss of their ability for self-renewal, clonogenicity and tumorigenicity (36). The expression of CD133 may also be affected by the environment (48). Thus, for the limitations of CD133 immuno-phenotyping in glioma stem cells, other accurate markers should be explored in the future.

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