



# Assessment of Intratumoral Heterogeneity in Isolated Human Primary High-Grade Glioma: Cluster of Differentiation 133 and Cluster of Differentiation 15 Double Staining of Glioblastoma Subpopulations

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## Abstract

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**BACKGROUND:** Gliomas are the most common primary brain tumors, representing 50–60% of malignant primary brain tumors. Gliomas are highly heterogeneous with marked inter- and intratumoral diversity. Gliomas heterogeneity is a challenging issue in the development of personalized treatment. The simplest method for studying heterogeneity is using ex vivo cell cultures; in our case, the cell lines were isolated from patient with glioblastomas.

**AIM:** Here, we reported distinct cell subpopulations heterogeneity in glioblastoma cells.

**METHODS:** Human glioblastoma cells isolation is conducted by enzymatic method with combination of collagenase I, hyaluronidase, and trypsin enzyme in proportional amount from patient. Immunostaining was performed to assess glial fibrillary acidic protein (GFAP), Ki-67, isocitrate dehydrogenase-1 (IDH-1) status, and program death ligand-1 (PD-L1) expression. Primary glioblastoma cell line was characterized by flow cytometry (fluorescence-activated cell sorting) analysis based on cluster of differentiation (CD) 133 and CD15 marker expression. U87MG and CGNH-89 cell lines were used as control. Distinct subpopulation analysis was performed by double staining of CD133 and CD15 in isolated primary glioblastoma cell line and its comparative control cells.

**RESULTS:** Our isolated glioblastoma cells morphology was adherent cells which were able to form spheres depending on environment. Immunostaining confirmed GFAP, Ki-67, IDH-1 mutants, and PD-L1 expression. Our isolated glioblastoma cells expressed CD133 and CD15, coexpressed CD133/CD15 in different patterns. The highest subpopulation in primary glioblastoma was CD133<sup>+</sup>/CD15<sup>+</sup>.

**CONCLUSION:** Glioblastoma cells can be isolated using enzymatic methods. Isolated glioblastoma cells consist of four different subpopulations distinguished by CD133/CD15 double staining. Intratumoral heterogeneity exists and directly or indirectly depends on their microenvironment.

## Introduction

In adults, gliomas account for 29% of all brain tumors; 80% of primary malignant brain tumors occurring in patients 65–84 years of age [1]. Gliomas are highly heterogeneous, infiltrative, and diffuse with different degrees of invasiveness [2]. Based on the World Health Organization (WHO) classification 2016, the glioma is divided into two subtypes: (1) Non-infiltrative and (2) infiltrative (diffuse). Diffuse types must be enforced using biomolecular parameters.

Some cancers have been reported to have a small cell population of growth sustaining and tumorigenic

abilities. Such cells, known as cancer stem cells (CSCs) or cancer initiating cells, have been identified in certain kinds of tumors including gliomas [3], [4]. Intratumoral heterogeneity originated from genetic, epigenetic, and niche in addition to environmental effects and produced marked diversity within cancer cell populations. Gliomas heterogeneity is a challenging issue in the development of personalized treatment strategies. Even within cluster of glioma cells, there is a different of phenotypical and morphological states, as reflected by differences in gene expression, motility, metabolism, proliferation, metastatic potential, and drug resistance ability.

Cluster of differentiation (CD)-15 or trisaccharide 3-fucosyl-N-acetylglucosamine, known as

stage-specific embryonic antigen 1 (SSEA1), is strongly expressed in many types of pluripotent stem cells and neural stem cells (NSCs) in the adult brain [5]. CD15 was recently proposed to be a CSC marker derived from brain tumors [5]. CD133, which was originally detected in neuroepithelial stem cells of mice, is a cell surface marker expressed on human NSCs [3], [4], hematopoietic stem cells, and endothelial progenitor cells [6]. It is most frequently used as a representative CSC marker, in many cancers including gliomas [5].

The tumor spheres studied here indicated the existence of CD133<sup>-</sup> and CD15<sup>-</sup> glioma stem cells (GSCs), indicating that all these cells were immature. The data could imply intrinsic relationships between NSCs and GSCs, suggested that GSCs might retain some NSCs characteristics [7]. However, since normal tissue stem cells and CSCs could have some similar properties, further studies should focus on the differences, including the molecular genetics and epigenetics, between CD133<sup>+</sup> and/or CD15<sup>+</sup> normal tissue stem cells and CD133<sup>+</sup> and/or CD15<sup>+</sup> GSCs. Understanding such differences may help to elucidate the tumorigenesis and establishment of novel therapies for glioblastoma.

CD133 and CD15 were chosen since CD133 may not detectable in fresh glioblastomas specimens, meanwhile, CD15 expressed in cells with tumor initiation capacity; in glioblastomas, CD15 is an enrichment marker of stem cells in CD133-negative tumors (CD15<sup>+</sup> cells from CD133<sup>-</sup> tumors able to form neurospheres colonies and highly tumorigenic *in vivo*). Double staining of CD133 and CD15 could define cell subpopulations in human primary high-grade glioma (HGG). In the current study, we isolated glioma cells from a patient and expanded the cells with spheres culture, finally, the cells were characterized by the CD133/CD15 using flow cytometry and compared to malignant gliomas U87MG cells and CGNH-89 cells [8].

## Materials and Methods

### **Preparation of human glioblastoma cell medium**

#### *Transport medium*

A medium for transporting clinical specimens to the laboratory for examination, we used transport medium cocktail that based on phosphate-buffered saline (PBS) 1 (Gibco, 1740576), 1% antibioticantimycotic (Ab-Am) (Gibco, 1772653), 1% amphotericin B (Amp B) (Gibco, 15290026), 1% gentamicin (Gibco, 15750060), Nanomycopolitine (Gibco, LX16010).

#### *Glioma medium (CGNH sphere, isolated HGG)*

BrainPhys Neuronal Medium (Stem Cell Technology, 05796) supplemented 5% fetal bovine

serum (FBS) (Gibco, 10270106), 20 ug/ml recombinant human epidermal growth factor (EGF) (BioLegend, 585508), 20 ug/ml recombinant human b-FGF (BioLegend, 571508), 20 ug/ml recombinant human LIF (593906), 2% N2 (Stem Cell Technology, 07152), 2% Neurocult (Stem Cell Technology, 05711), 1 ug/ml heparin (Stem Cell Tech., 07980), 1% Ab-Am (Gibco, 1772653), 1% Amp B (Gibco, 15290026), 0.1% gentamicin (Gibco, 15750060), and 1% Nanomycopolitine (Gibco, LX16010).

#### *CGNH attached and U87 medium*

The complete growth medium used to expand CGNH and U87MG cells were RPMI 1640 Medium (Biowest, L0500-500), 10% FBS (Biowest, S1810-500), 1% Ab-Am (Gibco, 1772653), 1% Amp B (Biowest), 1% gentamicin (Gibco, 15750060), and Nanomycopolitine (Gibco, LX16010).

### **Tissue sample preparation for Ex vivo study**

The tissue sample was from a male patient, 47 years old, with HGG (WHO grade IV) malignant brain tumor. The informed consent used the guidelines approved by the Institutional Ethics Committee, Faculty of Medicine, Universitas Padjadjaran – Dr. Hasan Sadikin Hospital, Bandung, Indonesia. Tissue was then washed using PBS 1× (Gibco 1740576), then was placed in transport medium and transferred to the laboratory to be processed immediately.

### **Immunohistochemical staining for in vitro glial fibrillary acidic protein (GFAP), Ki-67, isocitrate dehydrogenase-1 (IDH-1) mutant, and program death ligand-1 (PD-L1) study**

Resected specimens were fixed with 10% natural buffer formalin and embedded in a paraffin block. Immunostaining of the section for GFAP, Ki-67, IDH-1 R132H (arginine 132 into histidine) mutant, and PD-L1 was performed using the streptavidin-biotin method. Sections (4 μm thick) were deparaffinized with xylene, rehydrated, and incubated with fresh 0.3% hydrogen peroxide in methanol for 30 min at room temperature. After rehydration through a graded ethanol series, the specimen was washed in PBS. After a blocking treatment with 5% skimmed milk for 30 min, the specimens were then incubated with the anti-GFAP polyclonal antibody (SKU 040 Biocare), anti-Ki-67 monoclonal antibody (MIB-1, SKU 325 Biocare; Clone Sp6), anti-IDH-1 R132H mutant monoclonal antibody (sc-373816 Santa Cruz), and anti-PD-L1 monoclonal antibody (Ab 205921 Abcam; Clone 28-8) at a dilution of 1:100, 1:100, 1:200, and 1:200, respectively, in PBS containing 1% bovine serum albumin at 4°C overnight. They were then washed with PBS and incubated in secondary antibody for 30 min at room

temperature. Staining was performed using a Histofine SAB-PO (M) kit (Nichirei, Tokyo, Japan). The chromogen was a 3.3–0.02% solution containing 0.0055% H<sub>2</sub>O<sub>2</sub> in a 50 mM ammonium acetate-citric acid buffer, pH 6.0. The specimens were lightly counterstained with hematoxylin. Negative controls were prepared by substituting normal mouse serum for each primary antibody, as no detectable staining was evident. Each of IHC expression calculated as percentage of cytoplasmic and membranous staining of the cells at the central layer of cell layers of cancer cell nest in three consecutive high-power fields. For IDH-1, R132H mutant if stained was negative or < 10% categorized as negative and if > 10% as positive. PD-L1 IHC results were defined on the tumor proportion score as follows based on distributions as 0 (negative), 1 (<20%), 2 (20–50%), 3 (50–80%), 4 (>80%); score for intensity as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong); results from distribution x intensity as histoscore low (0–4), moderate (4–6), and high (6–12).

### ***Ex vivo primary glioblastoma cells isolation and characterization***

The tumor tissue was washed with PBS 1× on a Petri dish and then was cut it into small pieces using surgical scalpel. The sample fragments were washed again with PBS 1× (Gibco, 1740576), inserted into 50 ml falcon tube (TPP, 91050) containing the mixed enzyme solution of 4 mg/ml collagenase Type I (Gibco, 10114532), 1 mg/ml hyaluronidase (Sigma, 515397), trypsin 0.1%, and then rocked 5 h, 300 rpm, 37°C, 5% CO<sub>2</sub> followed with 16 h incubation, 37°C, 5% CO<sub>2</sub>. Sample was filtered by 70 μm cell strainer (Corning, 431752) then centrifuged at 1600 rpm for 10 min. Supernatant was discarded, the pellet was washed with PBS 1× and centrifuged at 1600 rpm 5 min 2×. The pellet was then resuspended with the complete growth medium. The cell suspension was next incubated at 37°C, 5% CO<sub>2</sub> [9]. Glioma cells at passage 3, CGNH-89, and U87 cell line (ATCC® HTB-14™) were characterized using flow cytometry (Macsquant Analyzer 10) for positive and negative markers of glioblastoma cells at density 1 × 10<sup>5</sup> cells/tube. The cells were then stained with PerCP/Cyanine5.5 anti-human CD15 (SSEA-1) antibody (BioLegend, 323020) and APC anti-human CD133 antibody (BioLegend, 372806) according to the manufacturer protocol. The experiments and surface marker measurements were performed in triplicate [10], [11].

### ***Ex vivo proliferation assay***

Glioma cells were cultured in glioblastoma growth medium; the medium was replaced every 3 days. 3 × 10<sup>5</sup> cells were seeded on T25 flask and cultured in complete medium until reaching confluence 80% around 3 days for proliferation assay. Briefly, cultured cells were detached using trypsin (Gibco, 25200072), then incubated for 1–3 min at 37°C, complete medium was added to neutralize

trypsin and the detached cells were centrifuged (MPW-2000) at 1600 rpm for 5 min at room temperature. The cell pellet was resuspended with trypan blue (Sigma, 25200072) and diluted in 1:1 dilution. Then, cells were counted using a hemocytometer (Neubauer, 17849). Population doubling (PD) was counted at every passage with the formula:  $PD = [\log_{10}(NH) - \log_{10}(NI)] / \log 10$

NI is the inoculum cell number and NH is the cell harvest number. The cumulative PD index for each passage was obtained by adding the PD of each passage to the PD of the previous passages. The PD time (PDT) was determined by the formula:  $PDT = t(\text{time}) / PD$  (in days) [8].

### ***Data analysis***

Statistical analysis was performed using the Stat View (ver. 5.0, SAS Institute Inc., NC). Statistically significance was considered at  $p < 0.05$ .

## **Results**

### ***Morphological characteristics of ex vivo isolated primary HGG cells***

The glioma cells were isolated from resected brain tumor of patient with HGG; the cells were isolated by enzymatic method as described previously. The tissues cells, separation of cells, and adherent morphological characteristic are shown in Figure 1a-c. The cells showed neurosphere-like formation (Figure 1d); clonal formation of neurospheres in EGF proved that our isolated cells are likely a cancer forming cells (CSCs) [10].

### ***PD time assay, characteristics of U87 and CGNH-89 cells***

PDT assay was used to evaluate the proliferation capacity of isolated glioblastoma cells. As shown in Figure 1e, the PDT of isolated cells decreased by increasing of the cell passages. The PDT of isolated HGG in Passage 3 was 5.14 days compared to Passage 5 that has lower PDT, 3.35 days. Morphological characteristic of adherent gliomas U87MG cells shown in Figure 1f, adherent CGNH-89 cells shown in Figure 1g, and spheres CGNH-89 cells shown in Figure 1h.

### ***In vitro immunohistochemical analysis of glioma tissue***

IHC analysis confirmed the expression of GFAP, Ki-67, IDH-1 R132H mutant, and PD-L1 in HGG (Figure 2a-d). GFAP was expressed in >80% cells; GFAP expression showed that the glioblastoma is originated from glial cells. Ki-67 expressed in >10% cells confirmed

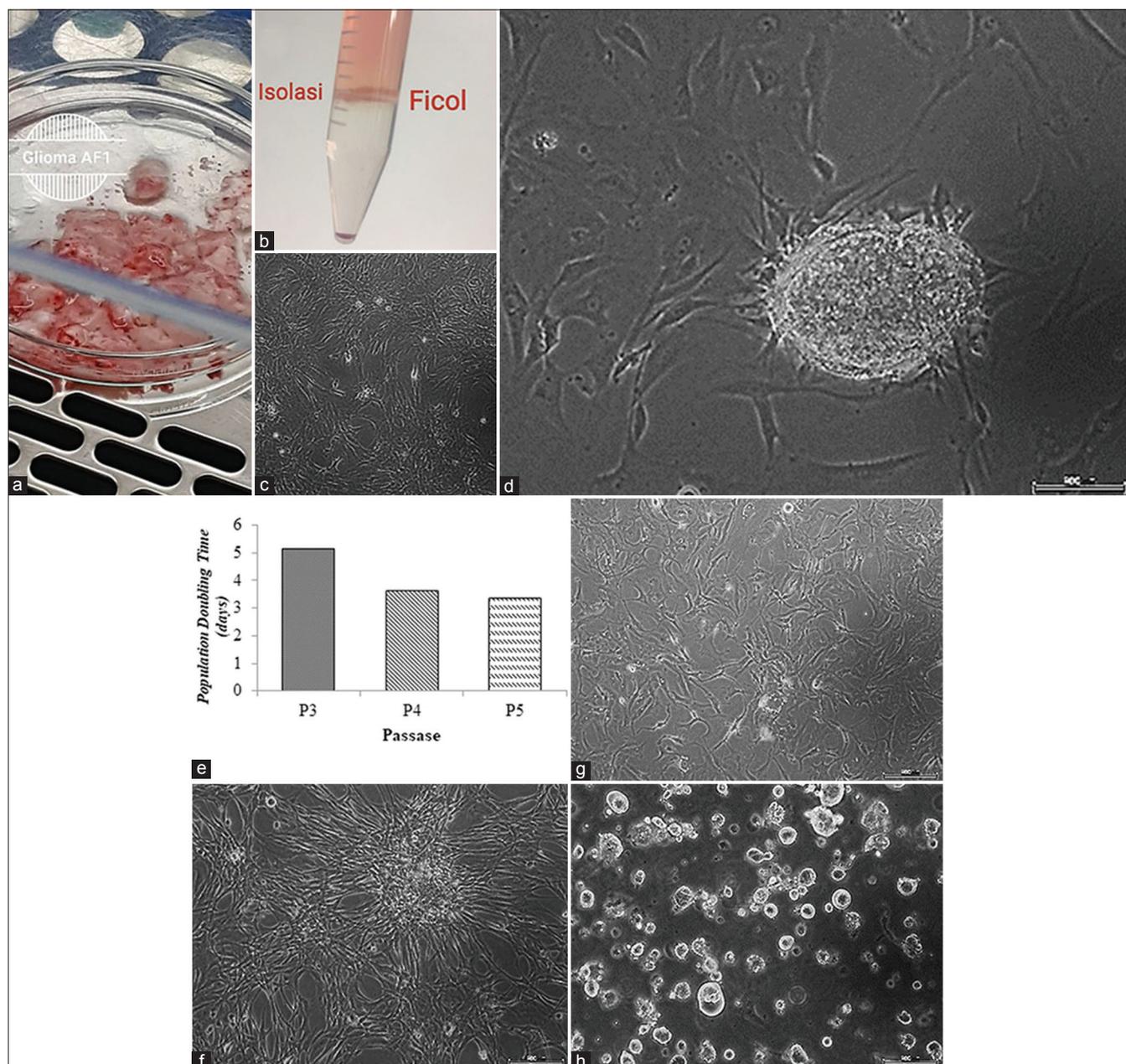


Figure 1: The isolated primary glioblastoma tissues (a), process for primary glioblastoma isolation (b), and adherent morphological characteristic (c). The isolated primary high-grade glioma glioblastoma shown as stem cells sphere morphology (d); with population doubling time count in days for primary glioblastoma cells (e). Adherent morphology of U87MG cells (f), adherent morphology of CGNH-89 cells (g), and spheres morphology of CGNH-89 cells (h)

the proliferation activity of isolated tumor since it is an important marker for proliferation in clinical practice [12]. Expression of IDH-1 R132H mutant confirmed that the tumor is likely a secondary, transformed from lower grade tumor. PD-L1 was expressed in high amount, >80% area of the tumor. Its expression was closely correlated to the pathological grade of the glioma which was confirmed the HGG of isolated tumor [13].

#### Characteristics of ex vivo isolated primary human HGG cells

Flow cytometric analysis was conducted to define the biological properties of glioma cells using CD133 and CD15 markers compared to U87MG and CGNH-89 cells.

CD133 is the marker for GSCs. CD15 is the marker for neural precursor and some immature astrocytes (NSCs). All cells expressed putative GSCs CD133 ( $65.73 \pm 1.12\%$  for isolated primary HGG,  $76.19 \pm 1.42\%$  for U87MG cells,  $62.52 \pm 1.71\%$  for adherent CGNH-89, and  $77.69 \pm 0.44\%$  for sphere CGNH-89) and NSCs CD15 ( $33.56 \pm 1.92\%$  for isolated primary HGG,  $22.52 \pm 0.34$  for U87MG cell,  $22.14 \pm 0.65\%$  for adherent CGNH-89, and  $51.17 \pm 0.54\%$  for spheres CGNH-89) (Figure 3).

#### Subpopulation of ex vivo isolated human primary HGG and control cells

Double staining was performed to examine coexpression of CD133 and CD15 in isolated primary

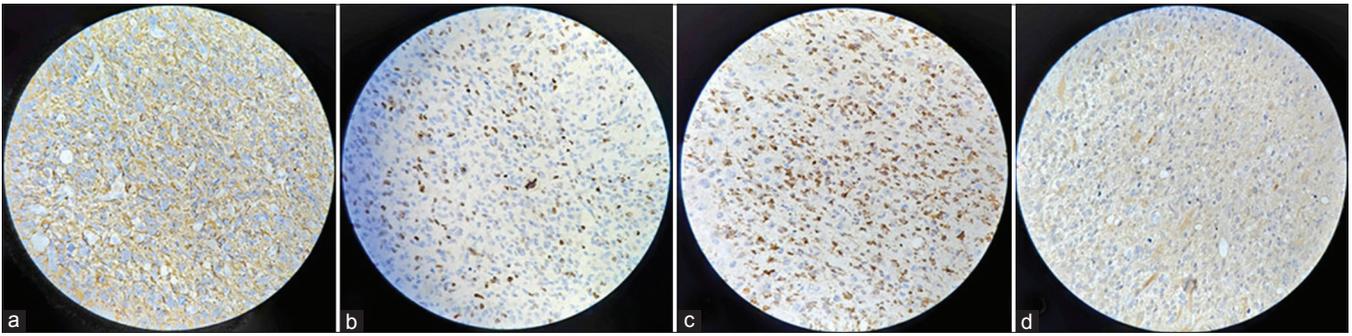


Figure 2: Immunoprofile of glioblastoma tissue. The tissues were positively stained with glial fibrillary acidic protein (a), Ki-67 (b), isocitrate dehydrogenase-1 mutants (R132) (c), and program death ligand-1 antibody (d) (20x).

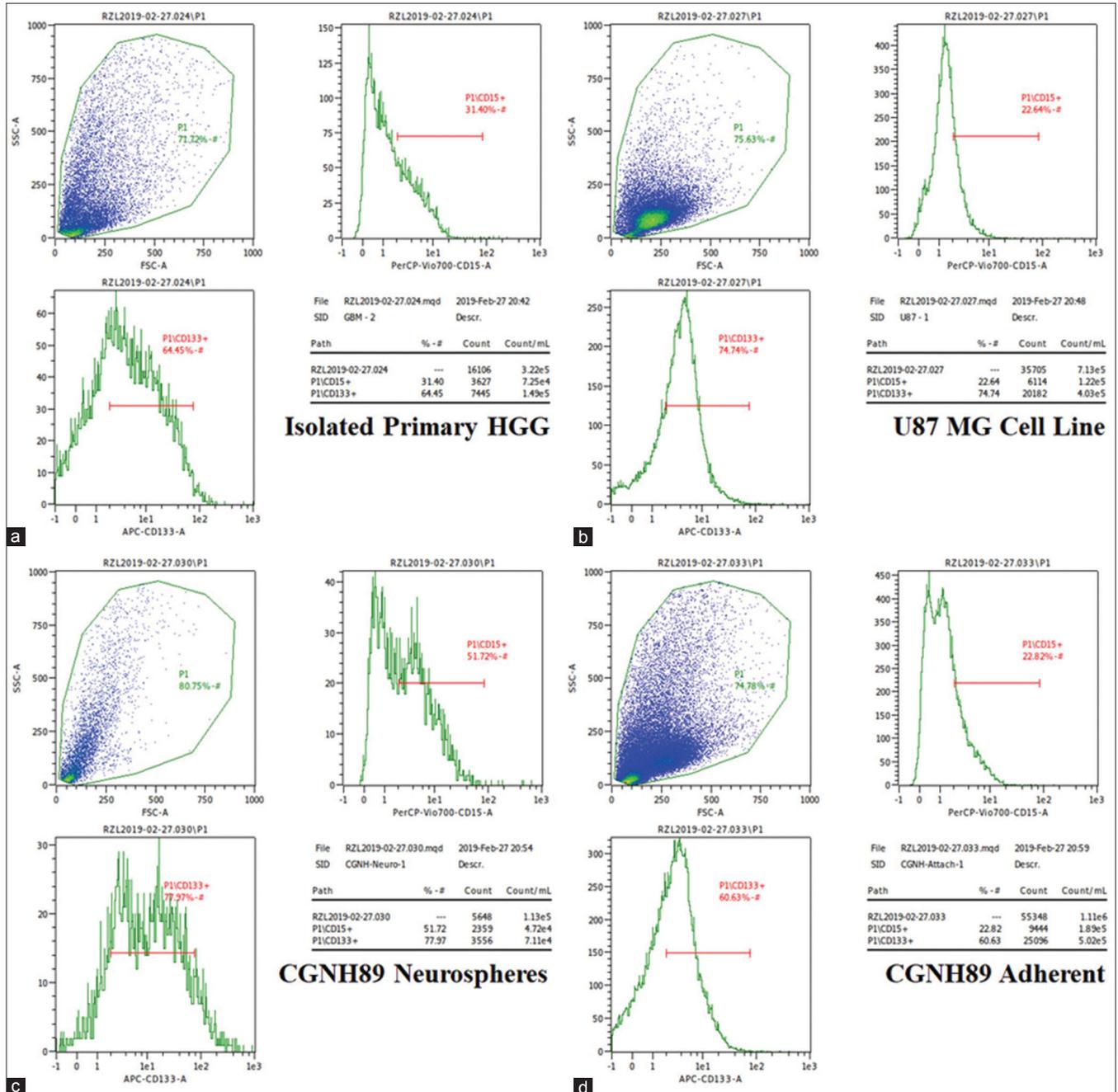


Figure 3: Density plot of cluster of differentiation (CD)133 and CD15 in isolated primary glioblastoma (a), U87MG (b), adherent CGNH-89 (c), and spheres CGNH-89 (d)

human HGG cells and comparative cells. Both cells coexpress CD133 and CD15 in different proportion

(Figure 4). The data showed that the main subpopulation in isolated HGG and sphere CGNH is CD133<sup>+</sup>/CD15<sup>+</sup>

where the highest subpopulation in U87MG cells is CD133<sup>+</sup>/CD15<sup>-</sup>. The highest population for CD133<sup>-</sup>/CD15<sup>-</sup> shown in Figure 5.

### Discussion

Immunostaining of glioma tissue showed the protein expression of GFAP, Ki-67, IDH-1 R132H mutant, and PD-L1. GFAP immunoreactivity indicates that the tumor is astroglial lineage [14]. GFAP is absent in the primitive neuroepithelial cells, oligodendrocytes, ganglion cells, vascular endothelium, meningeal cells, fibroblasts, and other mesenchymal elements [14]. Ki-67 was expressed in >10% cells and confirmed the proliferation activity of isolated tumor cells since Ki-67 is an important marker for cell proliferation in clinical

practice [12]. Expression of IDH-1 R132H mutant suggests that the tumors are likely secondary tumors [15]. The expression of PD-L1 is tightly related to the pathological grade of glioma. This protein will bind to PD-1 protein on B-cells, T-cells, natural killer T-cell, and dendritic cells and is responsible to immune suppression process. The highly expression of PD-L1 in the tumor sections confirmed the high grade of isolated tumor [13].

CD15, known as SSEA-1, expressed as a trisaccharide 3-fucosyl-N-acetylglucosamine, is highly expressed in many types of NSCs in the adult brain [7]. CD15 was proposed to be a marker of tumor-initiating cells (TIC) in human glioblastoma. The use of CD15 as the marker for TIC because it fulfills the functional criteria such as being highly tumorigenic *in vivo* can give rise to both CD15<sup>+</sup> and CD15<sup>-</sup> cells, thereby establishing a cellular hierarchy and has self-renewal and multilineage differentiation potentials [16]. This study showed that isolated HGG and its comparative

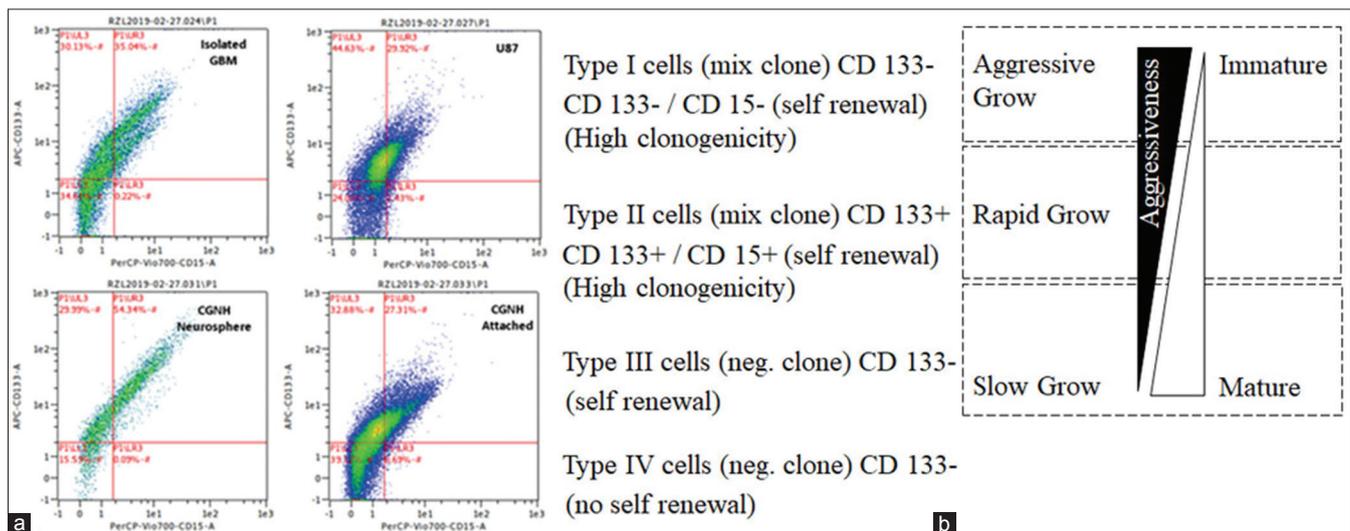


Figure 4: Analysis of isolated primary glioblastoma and comparative cell line by coexpression of cluster of differentiation (CD) 133/CD15 (a). Schematic lineage model of Type I, II, and III cells (comprising CD133<sup>-</sup> and CD133<sup>+</sup>) in glioblastoma applying with coexpression of CD133 and CD15 to define distinct intratumoral subpopulations (b) [10], [20]

	CD133-/CD15+	CD133+/CD15-	CD133+/CD15+	CD133-/CD15-
<b>U87 Cell Line</b>	1,27%	45,88%	30,05%	22,80%
<b>CGNH89 (adherent)</b>	0,75%	35,43%	26,20%	37,62%
<b>CGNH89 (spheres)</b>	0,15%	29,92%	54,56%	15,37%
<b>Isolated primary HGG</b>	0,31%	29,37%	37,27%	33,05%

Figure 5: Subpopulations from lineage model of Type I, II, and III cells (comprising cluster of differentiation [CD] 133<sup>-</sup> and CD133<sup>+</sup>) in isolated primary glioblastoma cells and standard cell line by applying double staining of cluster of differentiation (CD) 133 and CD15 (see detail in text)

cells express CD15. The highest expression of CD15 was in CGNH neurosphere,  $51.17 \pm 0.54$ . This result indicates that neurosphere expresses more CD15 and CD133 than adherent cells. Ahmed *et al.* stated that CD133<sup>-</sup> population are adherent cells [16].

Flow cytometry analysis showed the expression of NSCs marker, CD133. Besides, being a marker for NSCs, CD133 is also a marker for endothelial progenitor cells and hematopoietic stem cells [10], [17]. The proportion of CD133<sup>+</sup> is  $65.73 \pm 1.12\%$  in isolated HGG. In CGNH-89 cell, we cultured CGNH-89 in two different condition, adherent and spheres culture. The results showed that sphere CGNH had high CD133<sup>+</sup> expression ( $77.69 \pm 0.44$  for sphere CGNH and  $62.52 \pm 1.71$  for adherent CGNH). CD133<sup>+</sup> has capability for unlimited self-renewal and has good ability to initiate and drive brain tumor *in vivo* [9] and CD133 positivity also has high correlation with survival and chemoresistance in gliomas [18]. However, one study also found that there are CD133-negative (CD133<sup>-</sup>) CSCs in gliomas that exhibit similar property as CD133<sup>+</sup>CSCs [19]. Our findings also confirmed successful CD133<sup>+</sup> isolation from secondary glioma since Ahmed *et al.* reported unsuccessful isolation from secondary glioma and stated that there are no CSCs from secondary glioma [16].

Double staining of CD133 and CD15 defined four different cell subpopulations in isolated glioma and its comparative cells. The result of this study is in agreement with the study that conducted by Kahlert *et al.* (2012) that separated three different subpopulations, CD133<sup>-</sup>/CD15<sup>-</sup>, CD133<sup>+</sup>/CD15<sup>+</sup>, and CD133<sup>-</sup>/CD15<sup>+</sup>; concluding that all subpopulations of the cells had the ability to form neurospheres, with CD133<sup>-</sup>/CD15<sup>+</sup> has lower clonogenicity [10]. These findings correspond to CSCs lineage hierarchy proposed by Chen *et al.* (2010) that postulated tumor stem cells lineage in glioblastoma. Type I cells (CD133<sup>-</sup>) are able to give rise to aggressive tumors that consist both of CD133<sup>+</sup> and CD133<sup>-</sup> cells; the Type II cells are positive for CD133 and generate rapidly growing, invasive grafts that comprise also a mixture of CD133<sup>+</sup> and CD133<sup>-</sup> cells; and Type III cells (CD133<sup>-</sup>) form confined slowly growing tumor masses [20]. In addition, the existence of a fourth more differentiated CD133<sup>-</sup> cell population with non-tumorigenic properties and limited proliferation capacity under neurosphere conditions was proposed. According to the model, Type I, II, and III cells are lineally related and represent discrete stages of differentiation. Type I cells are the least and type III cells the most differentiated, whereas type II cells represent an intermediate stage, as shown in Figure 4a and b.

Our results showed that isolated primary glioblastoma cell line and its comparative cell lines have a small proportion of CD133<sup>-</sup>/CD15<sup>+</sup> (<2%). These subpopulations could be categorized as more differentiated population (the fourth type) that has minimal clonogenic potential [10], [20]. The double negative subpopulations CD133<sup>-</sup>/CD15<sup>-</sup> are

categorized as Type I cells since it has self-renewal and strong clonogenicity [10]; it was dominant in adherent CGNH89 ( $37.62 \pm 1.72\%$ ). The double-positive CD133<sup>+</sup>/CD15<sup>+</sup> could be categorized as Type II cell, since it has a good renewal capacity; its dominant in spheres CGNH-89 ( $54.56 \pm 0.41\%$ ) and isolated primary glioblastoma cells ( $37.27 \pm 1.97\%$ ). It can be concluded that we confirmed the presence of Type I cells, Type II cells, and more differentiated population based on Chen *et al.* classification [20]. Somehow, we observed an additional subpopulation of CD133<sup>+</sup>/CD15<sup>-</sup> that was prominent in U87MG cells ( $45.88 \pm 1.09\%$ ); results summarized in Figure 5 and Table 2.

Double staining of CD133/CD15 can also separate three different subclasses of human glioblastoma cells for their stem cells identity; however, in this study, another subpopulations that have not been reported before (CD133<sup>+</sup>/CD15<sup>-</sup>) need to be added and reclassified in a novel hierarchy [10], [16], [19], [21]. Surprisingly, modification of the cellular microenvironment from adherent to become spheres could modify the characteristics of subpopulations, suggesting how important the environment milieu is.

## Conclusion

Our isolated HGG cells could be separated into four different subpopulations by CD133/CD15 double staining. Intratumoral heterogeneity does exist and directly or indirectly depends on their environment. This intratumoral heterogeneity study serves as a foundation for future study in this area and offers insights into the cell type-dependent distribution and disease outcomes.

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