Isolation, cultivation and characterization of CD133+ stem cells from human glioblastoma

Isolamento, cultivo e caracterização de células-tronco CD133+ de glioblastoma humano

Lorena Favaro Pavon[1], Luciana Cavalheiro Marti[2], Tatiana Tais Sibov[1], Liza Aya Mabuchi Miyaki[3], Suzana Maria Fleury Malheiros[4], Javier Bustamante Mamani[1], Reynaldo Andre Brandt[5], Guilherme Carvalhal Ribas[5], Jorge Roberto Pagura[5], Marcos Augusto Stavale Joaquim[5], Hallin Feres Junior[5], Lionel Fernel Gamarra[1]

ABSTRACT

Objective: To establish the method of isolation and culture of human glioblastoma neurospheres, and the purification of their stem cells, followed by the process of obtaining tumor subspheres, immunophenotypically characterizing this clonogenic set. **Methods:** Through the processing of glioblastoma samples (n=3), the following strategy of action was adopted: (i) establish primary culture of glioblastoma; (ii) isolation and culture of tumor neurospheres; (iii) purify cells that initiate tumors (CD133+) by magnetic separation system (MACS); (iv) obtain tumor subspheres; (v) study the expression of the markers nestin, CD133, and GFAP. **Results:** The study successfully described the process of isolation and culture of glioblastoma subspheres, which consist of a number of clonogenic cells immunophenotypically characterized as neural, which are able to initiate tumor formation. **Conclusion:** These findings may contribute to a better understanding of the process of gliomagenesis.

Keywords: Glioblastoma; Cell culture; Neoplastic stem cells; Antigens

RESUMO

Objetivo: Estabelecer o método de isolamento e cultivo das neuroesferas de glioblastoma humano, bem como purificação de suas células-tronco, seguido do processo de obtenção de subesferas tumorais, caracterizando imunofenotipicamente esse conjunto clonogênico. **Métodos:** Por meio do processamento de amostras de glioblastomas (n=3), cumpriu-se a seguinte estratégia de ação: (i) estabelecimento da cultura primária de glioblastoma; (ii) isolamento e cultura de neuroesferas tumorais; (iii) purificação das células que iniciam os tumores (CD133+) por sistema de separação magnética (MACS); (iv) obtenção subesferas tumorais; (v) estudo da expressão de marcadores GFAP, CD133 e nestina. **Resultados:** Este estudo descreveu com sucesso o processo de isolamento e cultivo de subesferas de glioblastoma, as quais são constituídas por um conjunto clonogênico de células caracterizadas imunofenotipicamente como neurais, capazes de iniciar a formação tumoral. **Conclusão:** Estes achados poderão contribuir para a compreensão do processo de gliomagênese.

Descritores: Glioblastoma; Cultura celular; Células-tronco neoplásicas; Antígenos

INTRODUCTION

According to the World Health Organization (WHO)[1], central nervous system (CNS) tumors are classified into seven major groups which include primary tumors (neuroepithelial, meninges, paraspinal and cranial nerves, germ cell, sellar region, and hematopoietic) and secondary tumors or metastatic.

Gliomas are tumors that arise from glial cells and include astrocytomas, oligodendrogliomas, oligoastrocitomas (also called mixed gliomas), and

Study carried out at the Instituto Israelita de Ensino e Pesquisa Albert Einstein, Hospital Israelita Albert Einstein – HIAE, São Paulo (SP), Brazil.

[1] Instituto do Cérebro – InCe, Hospital Israelita Albert Einstein – HIAE, São Paulo (SP), Brazil.

[2] Centro de Pesquisa Experimental, Hospital Israelita Albert Einstein – HIAE, São Paulo (SP), Brazil.

[3] Instituto do Cérebro – InCe, Hospital Israelita Albert Einstein – HIAE, São Paulo (SP), Brazil; Faculdade de Enfermagem, Hospital Israelita Albert Einstein – HIAE, São Paulo (SP), Brazil.

[4] Department of Neurology and Neurosurgery, Universidade Federal de São Paulo – UNIFESP, São Paulo (SP), Brazil; Center for Neuro-oncology Hospital Israelita Albert Einstein – HIAE, São Paulo (SP), Brazil.

[5] Neurosurgery, Hospital Israelita Albert Einstein – HIAE, São Paulo (SP), Brazil.

Corresponding author: Lorena Favaro Pavon – Instituto Israelita de Ensino e Pesquisa Albert Einstein – Avenida Albert Einstein, 627/701, 2º subsolo, Piso Chinuch – Morumbi – Zip code: 05651-901 – São Paulo (SP), Brazil – Phone: (55 11) 2151-3727 – E-mail: lorenap@einstein.br

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ependymomas. Gliomas are part of the group of neuroepithelial tumors and account for 31% of primary tumors and 80% of malignant tumors of the CNS. The astrocytoma group corresponds to 76% of the gliomas, and glioblastoma represents 53.7%[2]. Glioblastoma is the most frequent and malignant of the astrocytomas, and despite numerous advances in the diagnosis and treatment of these tumors, the prognosis remains very limited[3,4].

Glioma is also described as the most common human CNS neoplasm,[2] and it is very difficult to treat due to several factors: (i) it is infiltrative; (ii) it is composed of cells with different morphological and functional characteristics expressing various neuronal markers; (iii) it is highly resistant to radiation and chemotherapy processes[5]. These characteristics are probably due to the competence of tumor cells which is similar to the competence of stem cells, explaining high rate of recurrence of the disease or its primary resistance to treatment. Therefore, it is extremely important to search for new approaches referring to the genesis, progression and clinical behavior of brain tumors.

Among the theories that seek to determine the molecular genesis of CNS tumors, one that is gaining many supporters in recent years is the hypothesis of tumor stem cells[6,7]. Recent studies have shown that the onset and progression of some malignant tumors can be determined by a subpopulation of tumorigenic cells with great capacity of self-renewal, called tumor stem cells[5,8].

The first prospect of identification and characterization of tumor stem cells of different phenotypes was reported in human brain tumors with increased expression of the CD133 antigen[5].

Monoclonal anti-CD133 antibodies have been previously used to identify normal human neural stem cells[9]. The brain tumor stem cell has been isolated exclusively by the expression of this antigen (CD133). Three evidence suggested that these $CD133^+$ cells were brain tumor stem cells: (1) they originated clonogenic cell sets (neurospheres); (2) they underwent the process of self-renewal and proliferation; and (3) they differentiated and returned to express the phenotype of the tumor that originated them when implanted in immunodeficient animals.

A study conducted by Uchida et al.[10] described that purified $CD133^+$ cells generated neurospheres in culture, and differentiated into neurons and glial cells that were capable of cell differentiation. Other groups have also verified that, contrary to $CD133^-$ cells, $CD133^+$ cells were capable of inducing brain tumors in *in vivo* models[11,12]. Therefore, these studies strongly suggest that the subpopulation of cells that initiate brain tumors are concentrated in a small fraction of $CD133^+$ cells.

OBJECTIVE

This study aimed at establishing the method of isolation and culture of human glioblastoma neurospheres, and the purification of their stem cells, followed by the process of obtaining tumor subspheres, immunophenotypically characterizing this clonogenic set.

These objectives adopted the following action strategy: (i) establishment of primary cultures of glioblastoma; (ii) isolation and culture of tumor neurospheres; (iii) purification of cells that initiate tumors by selection with CD133 magnetic microbeads; (iv) flow cytometry of the $CD133^+$ tumor cells; (v) obtaining tumor subspheres; (vi) study of the expression of GFAP (acidic fibrillary glial and intermediate filament protein specific for astrocytes in the NS), CD133 (cell membrane glycoprotein highly expressed in glial and neuronal stem cells) and nestin (intermediate filament protein class found in the embryonic development phase of the human brain) markers.

METHODS

This study involved the processing of samples of glioblastoma (n=3) obtained in surgical procedures of the Neuro-Oncology Center of the *Hospital Israelita Albert Einstein (HIAE)*. The samples were donated by patients who signed the free and informed consent form (CEP 687).

The diagnosis of this tumor was based on the findings of magnetic resonance images previously discussed by the Integrated Neuro-Oncology Program and the Laboratory of Pathology of *HIAE*.

As control for the study, immunohistochemistry reactions for GFAP, a biomarker used in the diagnosis of glioblastoma[13] were conducted.

The study followed the strategy of action described below.

Establishment of primary culture of samples of glioblastoma

The fresh tumor samples of glioblastoma were washed, fractionated in PBS (1X), and enzymatically dissociated with 0.3% collagenase. The cells were resuspended in DMEM-LG medium: Dulbecco's Modified Eagle's Medium-low glucose supplemented with 10% Fetal

Bovine Serum and 1% Antibiotic-Antimycotic (100X) and L-Glutamine 200mm (100x), and plated at a density of $5x10^6$ cells (alive) per $25cm^2$, which were cultivated in an incubator (Thermo Fisher Scientific Inc. 3110, Waltham, MA) equipped with 5% CO^2 at 37°C for at least 24 hours. After reaching 80% confluency, glioblastoma cells were analyzed by immunocytochemical assays using CD133 antibody (1:100 Abcam, Cambridge, MA), as indicated by the manufacturer (DAKO, Biogen).

Culture of tumor neurospheres

The cells obtained from the primary culture of tumor cells were resuspended in a culture medium, defined as a medium for growing brain tumor stem cells (CTTC), composed of Dulbecco's Modified Eagle's Medium/F12 (Gibco®), supplemented with N2 (Gibco®), EGF (20ng/mL; Invitrogen), bFGF (20ng/mL; Gibco®), leukemia inhibitory factor (LIF, Chemicon), and B27 (1:50; Life Technologies Corporation), and plated at a density of $2x10^4$ viable cells in 24-well plates. The cells were cultivated in an incubator (3110 Thermo Fisher Scientific Inc., Waltham, MA) equipped with 5% CO^2 at 37°C, and the culture medium was changed every 3 days[14].

Purification of cells that initiate tumors by magnetic separation using the marker CD133 antigen

The colonies of tumor neurospheres were dissociated using StemPro® Accutase® (Invitrogen) Cell Dissociation Reagent. For magnetic marking we used CD133 (MACS®, Miltenyi Biotech) magnetic microbeads. The cells were labeled with CD133/2-PE (Miltenyi Biotech clone AC133), and the separation efficiency of the positive cell fractions was evaluated by FACSARIA (BD Biosciences, San Jose, CA) flow cytometry, and analyzed using the FACSDIVA (BD Biosciences, San Jose, CA) software. This study allowed the separation of $CD133^+$ cell fractions and $CD133^-$ cell fractions,[13] and the $CD133^+$ fraction was analyzed by transmission electron microscopy, following a routine protocol[15].

Formation of tumor subspheres

The $CD133^+$ cells and the $CD133^-$ cells isolated by magnetic separation were suspended in a culture medium called "medium for brain tumor stem cells" and plated at a density of $2x10^4$ live cells in 96-well plates. This method allowed the formation of tumor subspheres composed exclusively of subpopulations of purified $CD133^+$ cells.

Immunophenotypic characterization of tumor subspheres

For immunophenotypic characterization of tumor subspheres, digital multiparameter flow cytometry was used (FACSARIA, Becton Dickinson, San Jose, CA) and the experiments were performed using commercially available monoclonal antibodies: GFAP (clone:51-10C9, BD Pharmingen, San Diego, CA), nestin (clone:AD2; BD Pharmingen, San Diego, CA) and CD133 APC (clone:/2293-C3; Miltenyi Biotec, Bergisch Gladbach, Germany), sheep anti-mouse secondary antibody (PE; Chemicon, Temecula, CA), and isotype-specific monoclonal antibodies. Staining, acquisition and analysis techniques were performed according to the manufacturer's instructions.

RESULTS

The morphological and histological analysis indicated the presence of a highly infiltrative brain tumor tissue, with a gradual increase in cellularity (Figure 1A). The neoplastic cells were predominantly spindle-shaped cells and derived from mature astrocytes (Fig. 1A). The capillary endothelial cells were numerous and swollen (Figure 1A), an effect related to the high vascular proliferation. The presence of GFAP defined the glial lineage of the tumor (Figure 1B). The immunohistochemical analyses (Figure 1B) were strongly positive for GFAP, and the cells were neoplastic, immature-appearing, elongated, and spindle-shaped.

Glioblastoma tissue samples were used for establishing the primary culture of those tumors, which was homogeneous with spindle-shaped cells arranged in multidirectional bundles (Figure 1D). Immunocytochemical tests revealed the expression of the antigenic marker CD133 in the tumor neurospheres of the glioblastoma primary culture (Figures 1D and F).

The cells obtained from the primary culture of these tumors were resuspended in culture medium CTTC, in which the isolation and culture processes of the glioblastoma-derived neurospheres were conducted (Figures 1G and H).

Immunophenotypic characterization was then conducted by flow cytometry tests, evaluating the efficiency of magnetic separation of cell fractions positive for the antigenic marker CD133 in samples of tumor neurospheres (89% of $CD133^+$ cells), standardizing the sorting process for $CD133^+$ cells (Figure 2A). Only these $CD133^+$ cells were capable, in culture, of generating glioblastoma subspheres (Figure 2B, Figures 3A to 3E) which did not happen in compared fractions of $CD133^-$ cells (Figure 3F).

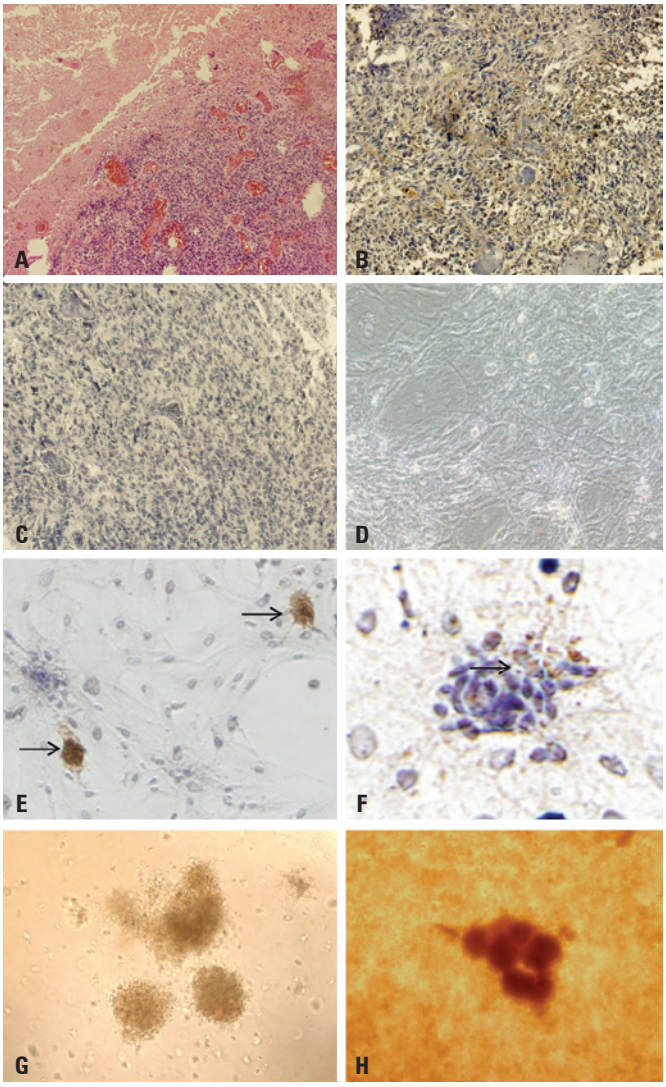

Figure 1. Histological and immunohistochemical tests on samples of glioblastoma obtained in histotechnical paraffin sections. (A) Hematoxylin and Eosin (H/E). (B) GFAP. (C) Negative control PH+ (GFAP). (A, B and C) Magnification: 200X. (D) Primary culture of glioblastoma. (E and F) Immunocytochemistry test for CD133 in primary culture (arrow: expression of the antigenic marker CD133). (G and H) Isolation and growth of glioblastoma tumor neurospheres. (D, E, G and H) Magnification: 400X. (F) Magnification: 600X

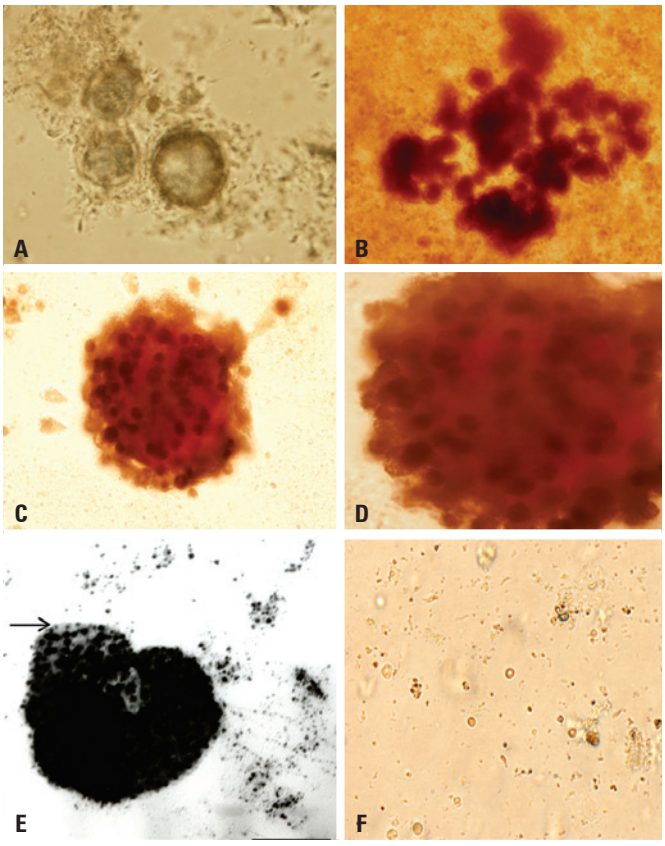

Figure 3. Culture of glioblastoma tumor subspheres obtained after the selection process of CD133$^+$ cells by MACS. (A and B) Magnification: 200X. (C and F) Magnification: 400X. (D) Magnification: 600X. (E) Analysis of the transmission electron microscopy images of the tumor subspheres; arrow: electron-dense points showing the magnetic microbeads conjugated to anti-CD133; bar: 1μm. (F) Negative control for the forming process of tumor subspheres obtained from CD133$^-$ cells

The glioblastoma-derived subspheres were described as cell aggregates or a set of clonogenic cells (Figure 3), which were immunophenotypically characterized showing the pattern of expression of the markers GFAP (87%) and nestin (39%) (Figure 4).

Figure 3E, as evidenced by ultrastructural analysis of transmission electron microscopy, described the presence of electron-dense lumps on the glioblastoma subspheres, pointing to the magnetic beads conjugated to anti-CD133 antibodies.

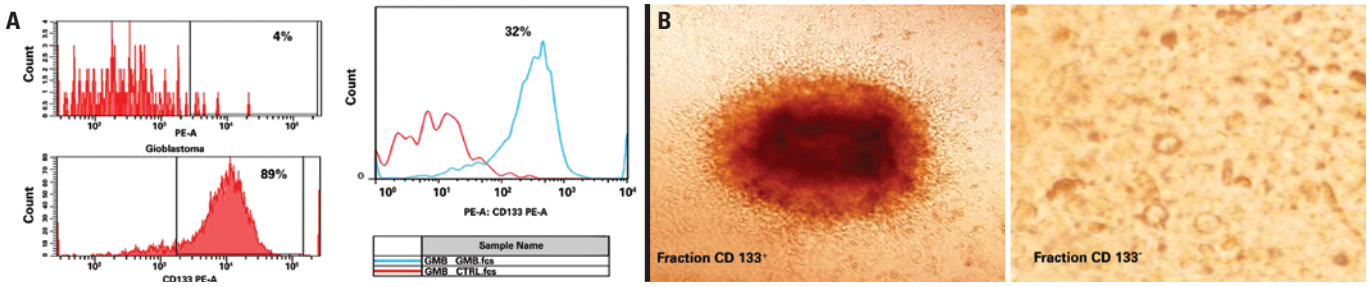

Figure 2. (A) Immunophenotypic characterization by flow cytometry tests, evaluating the efficiency of magnetic separation of cell fractions positive for the antigenic marker CD133 in tumor neurosphere samples (89% of CD133$^+$ cells). (B) Culture of glioblastoma tumor subspheres obtained after the selection process of CD133$^+$ cells, compared with the absence of subspheres obtained from CD133$^-$ fractions (negative control). (B) Magnification: 400X

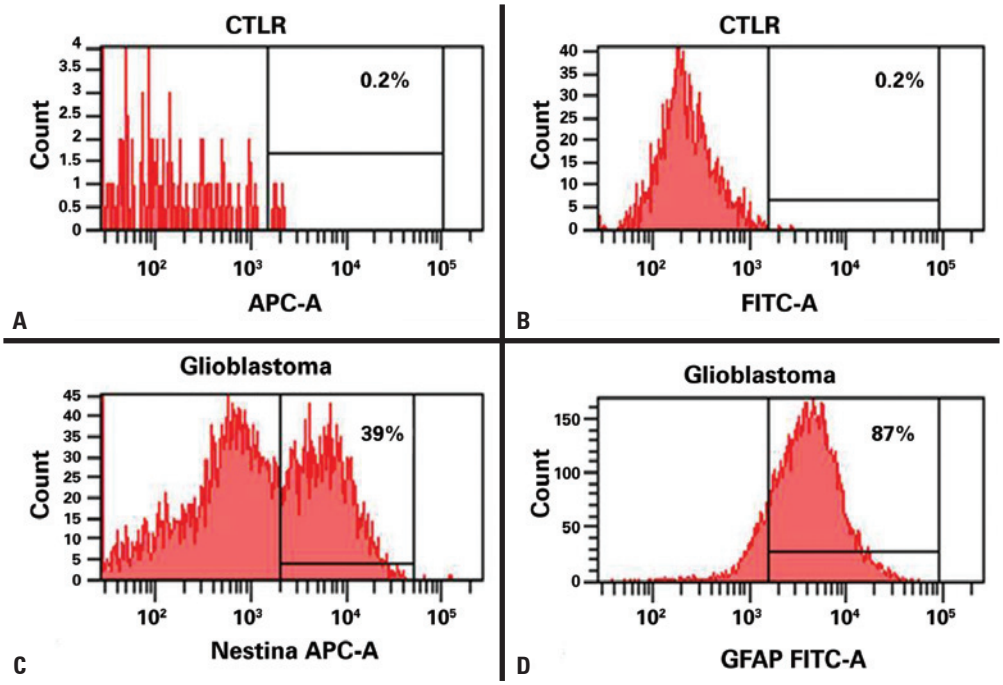

Figure 4. Immunophenotypic characterization by flow cytometry tests showing the pattern of expression of markers GFAP and nestin in glioblastoma tumor subsphere samples

DISCUSSION

Despite recent advances in the treatment of human brain cancer, the cellular and molecular mechanisms by which gliomas begin and are established have not been elucidated. Recent studies have shown that the onset and progression of some malignant tumors can be determined by a subpopulation of tumorigenic cells with great capacity of self-renewal, called tumor stem cells[16,17].

The tumor stem cell hypothesis describes that brain tumors, despite being a heterogeneous mass of cells, are composed of a rare cell population ($CD133^+$), which is capable of initiating the formation of a new tumor or metastasis, and its nature is defined by the formation of neurospheres[6].

The investigation process of brain tumor stem cells remains inconclusive, and their function still cannot be objectively established. To fully understand the biology of brain tumor stem cells, it is highly desirable to establish permanent lines of research in the isolation, culture and purification processes of their stem cells.

Therefore, this study aimed to establish the method of isolation and culture of tumor stem cells, properly classified as glioblastoma. The study was based initially in the primary culture process of the tumor, described as a homogeneous mass of spindle-shaped cells arranged in multidirectional bundles. Immunocytochemical assays, performed in these primary cultures of glioblastoma, revealed the expression of the antigenic marker CD133 in the initial cell aggregates during the formation of possible neurospheres, noting that the isolation and culture processes of glioblastoma tumor neurospheres occurred only after the use of a culture medium suitably supplemented (medium CTTC).

The purification process of the cells that initiate tumors, performed by a magnetic selection method using MACS, attributed to $CD133^+$ cells the ability to generate, in culture, glioblastoma tumor subspheres.

These glioblastoma tumor subspheres were described as cell aggregates or a set of clonogenic cells immunophenotypically characterized by the expression of markers such as GFAP, indicating the glial origin of the tumor cells, and nestin, an intermediate filament characteristic of immature cells, present in high levels in stem cells derived from cell lines of the central nervous system[8].

Obviously, these tumor subspheres also expressed the antigenic marker CD133, present in neural tumor stem cells, whose ultrastructural description revealed the presence of magnetic beads coupled to anti-CD133 antibodies on the glioblastoma tumor subspheres.

In its initial results, this study showed that the isolation and culture processes of glioblastoma subspheres actually give immunophenotypic characteristics of neural stem cells to the tumor cells that constitute them.

The results described in this study are based on recent findings in literature, which characterized the immunophenotypic profile of glioblastoma stem cells,

describing a set of cell markers (CD133, nestin, and GFAP) with tumorigenic properties[18,19].

Parallel studies defined another set of antigenic markers in the characterization process of brain tumor stem cells, such as CXCR4, Sox2, Musachi-1, and Nanog[20].

This study used the molecular marker CD133 as a predictor in the substantiation of the selection process of stem cells. This approach follows previous patterns found in literature, which identified a molecular signature for the $CD133^+$ glioblastoma cells, similar to human embryonic stem cells[21]. These authors determined that these $CD133^+$ cells were glioblastoma stem cells, and also identified a more aggressive subtype of the disease[21], insofar as these cells are highly resistant to radiation and chemotherapy procedures[16].

Other studies have also suggested that the expression of the marker $CD133^+$ could be a molecular indicator of glioblastoma spreading[16,22], which justifies its prognostic value[23] and the recent demand for drugs that act against this subset of cells ($CD133^+$) in order to inhibit or retard the proliferation of highly invasive gliomas[24].

CONCLUSION

This study proposes a process of isolation, characterization and culture of glioblastoma stem cells, which can contribute to the study of the genesis of brain tumors, identifying the cell responsible for the origin and spreading of tumors, and thus elucidating new therapeutic paradigms for neuro-oncology.

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REFERENCES

1. Louis DN, Ohgaki H, Wiestler OD, Cavanee WK, editors. WHO classification of tumors of the central nervous system. 4th ed. Vol. 1. Lyon: IARC Press; 2007.
2. Central Brain Tumor Registry of the United States (CBTRUS).[Internet]. North American Association of Central Cancer Registries. [cited 2012 May 14]. Available from: http://www.cbtrus.org.
3. Chen R, Nishimura MC, Bumbaca SM, Kharbanda S, Forrest WF, Kasman IM, et al. A hierarchy of self-renewing tumor-initiating cell types in glioblastoma. Cancer Cell. 2010;17(4):362-75.
4. Brandes AA, Tosoni A, Franceschi E, Reni M, Gatta G, Vecht C. Glioblastoma in adults. Crit Rev Oncol Hematol. 2008;67(2):139-52.
5. Wen PY, Kesari S. Malignant gliomas in adults. N Engl J Med. 2008;359(5): 492-507.
6. Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, et al. Identification of a cancer stem cell in human brain tumors. Cancer Res. 2004; 63(18):5821-8.
7. Sulman E, Aldape K, Colman H. Brain tumor stem cells. Curr Probl Cancer. 2008;32(3):124-42.
8. Dell'Albani P. Stem cell markers in gliomas. Neurochem Res. 2008;33(12): 2407-15.
9. Setoguchi T, Taga T, Kondo T. Cancer stem cells persist in many cancer cell lines. Cell Cycle. 2004;3(4):414-5.
10. Uchida H, Arita K, Yunoue S, Yonezawa H, Shinsato Y, Kawano H, et al. Role of sonic hedgehog signaling in migration of cell lines established from CD133-positive malignant glioma cells. J Neurooncol. 2011;104(3):697-704.
11. Facchino S, Abdouh M, Chatoo W, Bernier G. BMI1 confers radioresistance to normal and cancerous neural stem cells through recruitment of the DNA damage response machinery. J Neurosci. 2010;30(30):10096-111.
12. Galli R, Binda E, Orfanelli U, Cipelletti B, Gritti A, De Vitis S, et al. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. Cancer Res. 2004;64(19):7011-21.
13. Morrison CD, Prayson RA. Immunohistochemistry in the diagnosis of neoplasms of the central nervous system. Semin Diagn Pathol. 2000;17(3):204-15.
14. Lenkiewicz M, Li N, Singh SK. Culture and isolation of brain tumor initiating cells. Curr Protoc Stem Cell Biol. 2009; Chapter 3:Unit3.3.
15. Pavon LF, Marti LC, Sibov TT, Malheiros SM, Oliveira DM, Guilhen DD, et al. The ultrastructural study of tumorigenic cells using nanobiomarkers. Cancer Biother Radiopharm. 2010; 25(3):289-98.
16. Cho DY, Lin SZ, Yang WK, Hsu DM, Lin HL, Lee HC, et al. The role of cancer stem cells (CD133(+)) in malignant gliomas. Cell Transplant. 2011;20(1):121-5
17. Natsume A, Kinjo S, Yuki K, Kato T, Ohno M, Motomura K, et al. Glioma-initiating cells and molecular pathology: implications for therapy. Brain Tumor Pathol. 2011;28(1):1-12.
18. Tomuleasa C, Soritau O, Rus-Ciuca D, Ioani H, Susman S, Petrescu M, et al. Functional and molecular characterization of glioblastoma multiforme-derived cancer stem cells. J BUON. 2010;15(3):583-91.
19. Niu CS, Li DX, Liu YH, Fu XM, Tang SF, Li J. Expression of NANOG in human gliomas and its relationship with undifferentiated glioma cells. Oncol Rep. 2011;26(3):593-601. doi: 10.3892/or.2011.1308.
20. Ehtesham M, Mapara KY, Stevenson CB, Thompson RC. CXCR4 mediates the proliferation of glioblastoma progenitor cells. Cancer Lett. 2009;18;274(2): 305-12.
21. Yan X, Ma L, Yi D, Yoon JG, Diercks A, Foltz G, et al. A CD133-related gene expression signature identifies an aggressive glioblastoma subtype with excessive mutations. Proc Natl Acad Sci U S A. 2011;108(4):1591-6.
22. Sato A, Sakurada K, Kumabe T, Sasajima T, Beppu T, Asano K, Ohkuma H, Ogawa A, Mizoi K, Tominaga T, Kitanaka C, Kayama T; Tohoku Brain Tumor Study Group. Association of stem cell marker CD133 expression with dissemination of glioblastomas. Neurosurg Rev. 2010;33(2):175-83; discussion 183-4.
23. Pallini R, Ricci-Vitiani L, Montano N, Mollinari C, Biffoni M, Cenci T, et al. Expression of the stem cell marker CD133 in recurrent glioblastoma and its value for prognosis. Cancer. 2011;117(1):162-74.
24. Nakano I, Chiocca EA. Editorial Finding drugs against CD133(+) glioma subpopulations. J Neurosurg. 2011;114(3):648; discussion 648-50. J Neurosurg. 2011;114(3):651-62.