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Immunophenotypic Characterization of Human Glioblastoma Stem Cells: Correlation With Clinical Outcome

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ABSTRACT

Recently, glioma stem cells have been identified as the main cause of glioma propagation and recurrence and a number of several cell markers have been indicated as putative GSC markers. In the present work, a retrospective study to evaluate the prognostic potential of ability to generate GSCs in our series of 15 glioblastoma patients is described. β-tubulin III, nestin, CD133, GFAP, and SOX-2 marker expression, both in primary GBM cultures and in respective glioblastoma stem cells (GSCs), was evaluated by flow cytometric analysis. Our results demonstrated various expression levels of these markers in both cell cultures; of note, only those cells expressing SOX-2 at greater than 30% levels were able to produce in vitro neurospheres. Moreover, statistical analysis revealed that the GSCs generation negatively affected overall survival (OS) ($P = 0.000$) and progression-free survival (PFS) ($P = 0.001$). In addition, a very poor OS ($P = 0.000$) and PFS ($P = 0.000$) were observed among patients whose tumors expressed Ki67, evaluated by immunohistochemistry, and showed the ability to generate in vitro GSCs. Overall, the results suggest that in vitro GSCs generation associated to the expression of Ki67 and SOX-2 may be useful to identify patients at risk of disease progression. J. Cell. Biochem. 116: 864–876, 2015.

KEY WORDS: GLIOBLASTOMA MULTIFORME; CANCER STEM CELL; SOX-2 EXPRESSION; OVERALL SURVIVAL; PROGRESSION FREE SURVIVAL

Glioblastoma multiforme (GBM) is the most common and malignant primary brain tumor. The peak incidence is in middle adult life. In particular, the mean age for the occurrence is 56 to 60 years old [Raysi Dehcordi et al., 2012]. Despite a multimodal aggressive treatment, involving surgery, radiotherapy, and chemotherapy, the vast majority of patients die within two years [Wen and Kesari, 2008; Clarke et al., 2010; Raysi Dehcordi et al., 2012]. According to the results of the European Organization for Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada (NCIC), there is a significantly increased overall survival (OS) in patients who received surgery followed by radiotherapy with concomitant and adjuvant temozolomide treatment, in whom OS could be increased from 12.1 to 14.6 months. The median progression-free survival (PFS) is about 6.9 months with radiochemotherapy, and 5 months with radiotherapy alone [Stupp et al., 2005]. Nevertheless, 3–5% of the patients survives for more than 36 months and they are referred to as "long-term survivors" [Krex et al., 2007].

For years, parallelisms have been made between stem cell biology and oncology, especially because of the growing evidence that genes, with important roles in stem cell biology, also play a role in tumorigenesis. Cancer stem cells (CSCs) are tumor cells displaying normal neural stem cell properties including maintained proliferation, self-renewal, and differentiation ability as well as tumor-initiating cell features [Clarke et al., 2006]. Since their discovery in acute myeloid leukemia in 1997 [Bonnet and Dick, 1997], CSCs have been the object of intense researches and they have been described in many tumors [Reya et al., 2001], including brain cancers [Stiles and

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Rowitch, 2008; Gilbert and Ross, 2009]. Glioma stem cells are among the first CSCs to be described for solid tumors [Singh et al., 2004] and it seems that their number may be proportional to the degree of malignancy, with low-grade gliomas having few stem cells and secondary GBM, in the incipient phase, having fewer glioma stem cells than primary GBM [Sampetrean and Saya, 2013].

In accordance with the CSCs definition [Clarke et al., 2006], glioma stem cells are cells that sustain and give rise to the initial glioma; therefore a few glioma stem cells may be sufficient to initiate and propagate a local recurrence. In this perspective, the excision of the lesion should be as aggressive as possible. This concept is in agreement with results obtained in one of our previous studies, where we noted that the extent of surgical resection is the most relevant factor affecting survival [Raysi Dehcordi et al., 2012].

Nowadays, in order to obtain an exeresis that is as radical as possible, thereby eliminating infiltrating cells, neurosurgeons use specific safeguards in the operating room, such as operating microscope, neuronavigation (IGS), intraoperative carmustine wafer implantation and 5-aminolevulinic acid-photodynamic assisted resection (ALA-PDD-assisted resection). Moreover, it seems that glioblastoma stem cells (GSCs) have a crucial role in the radio and chemo resistance that characterizes GBM [Stopschinski et al., 2013]. The first demonstration that the analysis of GSCs may predict survival was made by Zeppernick et al. (2008), who showed that both the proportion of CD133+ cells and their organization in clusters are significant prognostic factors.

The cancer stem cell phenotype, still the subject of intense study, is strongly heterogeneous and variable among glioblastomas; no universal CSC markers exist and the CSCs may stain positive for stem cell markers and usually negative for early and late differentiation neuronal markers [Gursel et al., 2011]. Of all the markers identified so far, our attention has been focused on CD133, β-tubulin III, nestin, GFAP, and SOX-2 expression in GBM primary cultures and in GSCs generated from them.

CD133 or Prominin 1 (PROM1) is a glycoprotein expressed in hematopoietic stem cells, endothelial progenitor cells, neural stem cells, and brain tumors [Jin et al., 2013] and has been proposed as a marker to identify CSCs in solid tumors located in the brain, prostate, liver, lung, and colon [Stopschinski et al., 2013]. Its use as a marker for stemness phenotype, however, has become quite controversial [Wang et al., 2008] due to recent studies showing that CD133 negative cells can be highly tumorigenic in vivo [Gunther et al., 2008]. Further investigation on the functional role of CD133 should definitely be carried out.

In a more recent prospective study, authors demonstrated that GSCs generation and $CD133^{+}/Ki67^{+}$ co-expression are two considerable prognostic factors of disease progression and poor clinical outcome [Pallini et al., 2008]. Moreover, Zhang et al. (2008) showed that co-expression of CD133 and nestin had more powerful prognostic value than just single markers [Zhang et al., 2008; Dahlrot et al., 2013]. To the contrary, several authors have reported that no significant difference exist in OS according to the expression of CD133 in patients with GBM [Joo et al., 2008].

β-tubulin III is a microtubule protein, mainly expressed in cells of neuronal origin and its expression has also been revealed in normal cells and is overexpressed in many cancers including gliomas [Tsourlakis et al., 2013]. In gliomas, β-tubulin III expression seems to be correlated with an increased malignancy, high proliferative rates and poor prognosis [Draberova et al., 2008; Katsetos et al., 2009], with β-tubulin III positive cells ranging from 20% to 90% as a typical feature of undifferentiated cells [Gambelli et al., 2012].

SOX-2 is a member of the SOX transcription factors family, responsible for supporting many cellular processes, such as maintaining stem cell features of embryonic stem cells and pluripotent stem cells [Dahlrot et al., 2013; Herreros-Villanueva et al., 2013] and is overexpressed in several types of human cancer, including high grade gliomas [Basu-Roy et al., 2012; Cox et al., 2012; Leis et al., 2012; Herreros-Villanueva et al., 2013]. Herreros-Villanueva et al. (2013) have demonstrated a strong relationship between sphere-forming capacity and SOX-2 expression level in pancreatic cancer cells lines. Of note, they show that *sox-2* knockdown in cancer cell suppresses their sphere formation ability, suggesting that SOX-2 overexpression is necessary for the generation of CSCs in pancreatic cancer [Herreros-Villanueva et al., 2013]. Recent studies, performed on human gliomas, demonstrate that the SOX-2 presence is not a prognostic marker. Indeed, Wan and colleagues (2011) have observed that there is no correlation between the SOX-2 expression and clinical survival [Wan et al., 2011] and, furthermore, that SOX-2 expression does not correlate with the malignancy grade of astrocytic tumors [Phi et al., 2008]. In undifferentiated GSC lines, SOX-2 expression is very variable, ranging between 46% to 95% [Gambelli et al., 2012].

An immature neural stem cell marker for CSCs detection is nestin, a cytoskeletal protein expressed during development of the central nervous system. It is up-regulated in cancer and promotes CSCs features, tumor cell proliferation, migration, and invasion [Dahlrot et al., 2013]. Moreover, several authors demonstrate that the high presence of nestin in positive cells is correlated, in gliomas, with the degree of malignancy [Maderna et al., 2007; Strojnik et al., 2007; Tomita et al., 2013], whereas other researchers have observed that nestin expression in astrocytoma samples is associated with poor survival [Wan et al., 2011; Arai et al., 2012].

Glial fibrillary acidic protein (GFAP) is a protein involved in the structure and function of the cytoskeleton, and is commonly used as an astrocytes marker: its expression is increased following brain damage or during degeneration of the central nervous system [Middeldorp and Hol, 2011]. However, several researchers have demonstrated that neurospheres from GBM express typical neural stem cell markers including the GFAP [Bleau et al., 2008; Gunther et al., 2008]. Gursel et al. (2011); in a comprehensive review, report that GFAP was expressed in undifferentiated glioblastoma tumor sphere as well as nestin and SOX-2, known stemness markers [Gursel et al., 2011]. Prestegarden and colleagues (2010) have shown that the percentage of GFAP+ cells is not related to patients survival rate [Prestegarden et al., 2010]. In agreement with these evidences, Rieske et al. claim that GSCs show a multilineage antigenic phenotype (GFAP + /beta tubulin III + /nestin +) such as normal neural progenitors [Rieske et al., 2009].

As the literature provides poor and heterogeneous information about the link between the biology and clinical behaviour of GBM, we conducted a retrospective study to evaluate the prognostic potential of GSC markers in our series of GBM patients. In particular,

we performed an immunophenotypic characterization of both primary GBM cultures and their respective GSCs, along with an evaluation of the GBM ability to generate neurospheres in vitro, which was then correlated to clinical outcome and Ki67 positivity.

MATERIALS AND METHODS

PATIENTS SELECTION

Our study population consisted of 15 consecutive patients with a suspected diagnosis of primary glioblastoma multiforme (Grade IV in accordance with World Health Organization ,WHO, established guidelines), undergoing a complete surgical resection at the Operative Unit of Neurosurgery, San Salvatore Hospital in L'Aquila from June 2010 to June 2011.

The tumors diagnoses and immunochemical analysis of Ki67 were performed in San Salvatore Hospital in L'Aquila by histopathology analysis.

The median age at surgery was 64 years old (range 36–75); 10 patients were male and 5 female. Data were collected retrospectively and patients with aphasia, deep lesions (thalamus or midline), multiple or recurrent tumors, partial or subtotal exeresis as well as those who were either uncooperative or underwent biopsy, were excluded from the study. After surgery, the patients included in the study were subjected to radio and chemotherapic treatment.

PATIENT TREATMENT

All patients included in this study underwent a gross-total surgical exeresis in accordance with our fluorescence-guided tumor resection protocol (ALA-PDD assisted resection).

Preoperatively, high resolution and contrast-enhanced magnetic resonance images (MRIs) were acquired for each patient. Additionally, when necessary, patients underwent preoperative functional MRIs. MRI images were used for image-guided neuronavigation system, which allowed the surgeon to localize the tumor more accurately and choose a safe surgical corridor by which to approach the tumor. The neuronavigation provided data regarding orientation during the entire surgical procedure, including details of structures adjacent or deep to the area of dissection as well as details about the lesion in question (such as the amount of remaining tumor, position of deeply located—encased or displaced—neural and vascular structures, etc.).

In the postoperative time, the gross-total surgical exeresis was confirmed by MRI with Gadolinum performed within 48 h after surgery. Adjuvant treatment included radiotherapy and chemotherapy. Focal radiotherapy was delivered once daily at 2 Gy per fraction, 5 day/week, for a total of 60 Gy, and was prescribed by the radiation oncologist, according to the guidelines of the International Commission on Radiological Units. Concomitant treatment with temozolomide was prescribed by the oncologist at a dose of 75 mg/m^2 for each day of radiation treatment. Four weeks after radiotherapy, patients received adjuvant temozolomide chemotherapy, 150 to 200 mg/m^2 on days 1 to 5 at 28-day intervals. All patients were followed for tumor evaluation by neurosurgeons and a medium follow-up of 22 months (range 3–36 months) was performed. Follow-up included neurologic and MRI examinations. If tumor recurrence was documented, surgery, additional chemotherapy, and/or additional radiotherapy were considered in a multidisciplinary discussion. Survival was calculated from the date of diagnosis. The disease was considered to have progressed if both the diameter and volume of the tumor increased by $\geq$25% of initial measurements or if a new mass was evident on axial-contrast enhanced T1-weighted magnetic resonance imaging scan [Macdonald et al., 1990].

Functional outcome was evaluated basing on Karnofsky Performance Status (KPS), a general measure of independence status of patients with cancer, indicating a person's ability to work, perform physical activity, and care for the self. A score from 0 (dead) to 100 (healthy) at 10-unit intervals for each level was assigned for each patient before tumor resection, at the discharge and at the last follow-up.

REAGENTS

DMEM (Dulbecco's Modified Eagle Medium, high glucose), DMEM/F12 (1:1), phosphate-buffered saline (PBS), fetal Bovin Serum (FBS), glutamine, penicillin and streptomycin were obtained from EuroClone (Milano, Italy).

Trypsin from bovine pancreas (T1426) was purchased from Sigma Chemical Co. (St. Louis, MO). Primary glioblastoma cultures and GSCs were seeded into a 100 mm sterile petri dishes and flasks acquired by EuroClone. Optical microscope (Eclipse 50i) and inverted microscope (TS100) were purchased from Nikon (Nikon Corporation, Japan). 50X B-27® Serum-Free Supplement was purchased from Life Technology Corporation, EGF (Recombinant human Epidermal Growth Factor) and bFGF (human basic Fibroblast Growth Factor) were acquired from ImmunoTools (Friesoythe, Germany), and Accutase™ was from PAA-GE Healthcare Life Sciences (GE Healthcare Bio-Sciences AB, Sweden). To recover large size neurospheres, we used a cell strainer 100 μm nylon (BD Falcon). For cytofluorimetric analysis, we used a FACSCalibur supplied with CellQuest software (Becton Dickinson, San Jose, CA). The primary antibodies were used for immunophenotypic analysis: anti-β-tubulin III (Alexa Fluor 488, Catalog No. 560338), anti-SOX-2 (PE Mouse, Catalog No. 560291), anti-nestin (Alexa Fluor 647, Catalog No.560393)—all acquired from Becton Dickinson (BD)—anti-GFAP (Glial fibrillary acidic protein, Catalog No. G9269 Sigma–Aldrich) and anti-CD133 (anti-PROM-1, Catalog No. MAB8818, Abnova). Secondary antibody anti-rabbit FITC conjugated (Millipore) was used for detection of GFAP positive cells and anti-mouse PE conjugated (Abcam) was used for detection of CD133 positive cells. For differentiation assay, we used NeuroCult™ NS-A Differentiation Human Kit medium (Stem Cell Technology), poly-L-lysine, primary anti-NF-200 antibody (N5389) and primary anti-O4 antibody (O7139) reveled by anti-mouse secondary antibody, FITC conjugated and DAPI solution (D9542), all acquired from Sigma–Aldrich.

ESTABLISHMENT OF GBM PRIMARY AND GSCs CULTURES

The fresh tumor biopsies of GBM were washed in order to remove adhering blood and visible necrotic portions. The tissue samples were mechanically cut into small pieces by a lancet and enzymatically dissociated by incubation with 0.125% trypsin and 0.125% EDTA (pH 7.4) in DMEM serum free solution [Patru et al., 2010; Prestegarden et al., 2010]. The ratio between GBM tissue weight

and trypsin solution was 1 g/10 ml. The digestion was carried out at 37°C for 15–20 min in a water bath by gentle stirring. The cells obtained were collected by centrifugation and grown in adherent and neurosphere conditions to obtain GBM primary and stem cell cultures, respectively. For adherent cultures the cells were plated in 75 cm^2 tissue culture flasks, suspended in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine (complete medium). All flasks were incubated in sterile conditions at 37°C with 5% CO_2. The complete medium was totally replaced every three days. After reaching 80% confluence GBM primary cultures were expanded.

For neurosphere cultures, a rate of recovered cells after enzymatic digestion, was cultured in serum-free DMEM/F12, a specific medium for growing brain stem cells, augmented with B27 supplement, 20 ng/ml EGF, 20 ng/ml bFGF and with penicillin/streptomycin in order to avoid contamination [Di Tomaso et al., 2010]. Subsequently, these cells were kept in an incubator at 5% CO_2 at 37°C, and the culture medium was replenished once every 3 days by centrifugation for 10 min at 400*xg*. Although the generation time of spheres is very variable in primary cultures, after about 30 days most of GBM cultures formed small cellular aggregates that increased in size over time. GBM cell cultures generating neurospheres in growth medium were called GSCs+, while cell cultures not generating them were named GSCs-.

GSCs cultures were expanded when they reached a size greater than 100 μm by enzymatic dissociation of spheres into a single cell suspension using Accutase™ reagent solution for about 10 min at 37°C. Accutase™ solution ensures a gentle and effective aggregates dissociation and preserves surface proteins and epitopes. The neurospheres were observed under a light microscope (Nikon, Eclipse TS100) and images at several days were acquired.

FLOW CYTOMETRY ANALYSIS OF GBM PRIMARY AND GSCs CULTURES

Primary cell cultures, obtained from digestion of GBM specimens, and the tumor spheres dissociated with Accutase™ solution were examined at third culture passage by FACSCalibur flow cytometry for marker detection (β-tubulin III, nestin, SOX-2, GFAP, and CD133).

The cells were collected, rinsed and permeabilized for 5 min with Triton X-100 at RT for intracellular markers. The single cell suspensions (2×10^5 cells) were washed twice and incubated with selected primary antibodies for 1 h at RT in the dark. For CD133 and GFAP detection, secondary PE and FITC-conjugated antibodies respectively were used for 1 h at RT. The population of interest was gating according to its Forward Scatter (FSC)/Side Scatter (SSC) criteria. 10,000 events were acquired for each sample and analyzed by CellQuest software (BD Biosciences).

GSCs DIFFERENTIATION ASSAY

Neurospheres obtained from primary GBM cultures were grown in NeuroCult™ NS-A Differentiation Human Kit medium, a specific culture medium for human neural and progenitor cells for differentiation in neurons, astrocytes, and oligodendrocytes.

To perform differentiation assay, glass coverslips (Ø = 12 mm), coated with 100 μl poly-L-lysine solution, were incubated in sterile air at RT for 30 min under UV light to enable matrix polymerization. Then, neurospheres were dissociated with the Accutase™ solution and single cells were counted and plated on coverslips in 24 well plates at a density of $1 \times 10^4/cm^2$, as advised in the manufacturer's instructions. Adherent cells were cultured for 35 days in NeuroCult NS-A Differentiation Medium that was replaced every three days.

The coverslips were washed and fixed with 4% formaldehyde solution for 10 min at RT and the cells were permeabilized with Triton X-100 solution. For astrocyte detection we used a primary anti-GFAP antibody; for neuron detection we used a primary anti-NF-200 antibody; oligodendrocytes were incubated with the primary anti-O4 antibody (this marker was detected on the membrane surface, so the permeabilization step was not required). Then, the coverslips were washed and stained with the appropriate secondary antibodies and counterstained with DAPI for nuclear staining. The fluorescent images were acquired by Nikon Eclipse 50i microscope (magnification of 10× and 20×).

STATISTICAL ANALYSIS

A retrospective analysis was performed to evaluate differences of clinical outcome between patients with GSCs generating tumors and those not generating them, using the *t*-Student test. Moreover, dependent variables were submitted to a series of mixed effect repeated measure ANOVA. The Kaplan–Meier statistical method was used to assess correlation with both OS and PFS. The variables that were considered and evaluated as most likely to be related to total survival times were: in vitro ability to generate GSCs and immunochemical expression of Ki67 on tumour deparaffinised sections. Correlation between stemness markers expressed by primary cultures and GSCs was tested by calculating the correlation coefficient (Pearson's test). In order to describe the correlation, we used the Salkin scale for the absolute value of the correlation coefficient (r): very strong relationship ($0.80 < r < 1.0$), strong relationship ($0.60 < r < 0.80$), moderate relationship ($0.4 < r < 0.6$), weak relationship ($0.2 < r < 0.4$), and very weak or no relationship ($0.20 < r < 0$) [Mazilu et al., 2014]. The significance level was set at $P \leq 0.01$.

RESULTS

IMMUNOPHENOTYPIC CHARACTERIZATION OF HUMAN GBM PRIMARY CULTURES

The phenotype of GBM primary cell cultures, obtained from tumor tissue specimens, was evaluated by flow cytometric analysis at third culture step using antibodies directed against the antigens β-tubulin III, nestin, GFAP, CD133, and SOX-2. The marker expressions (percentage values) of 15 GBM primary cultures are shown in Figure 1A–E. The percentage of β-tubulin III was between 0.7% and 99% (average value 77.36%); few primary cultures showed β-tubulin III values lower than 53% (0.7–53.09%) (Fig. 1A). The percentage of nestin+ cells ranged between 74% and 99% and only two primary GBM cultures showed an expression of 39.73% and 42.25% (Fig. 1B). The GFAP marker was fairly expressed in primary GBM cultures and we found that positive cell percentages never exceeded 60% of the whole cell population (average value 34.40%), their presence being very variable between samples (Fig. 1C). Several

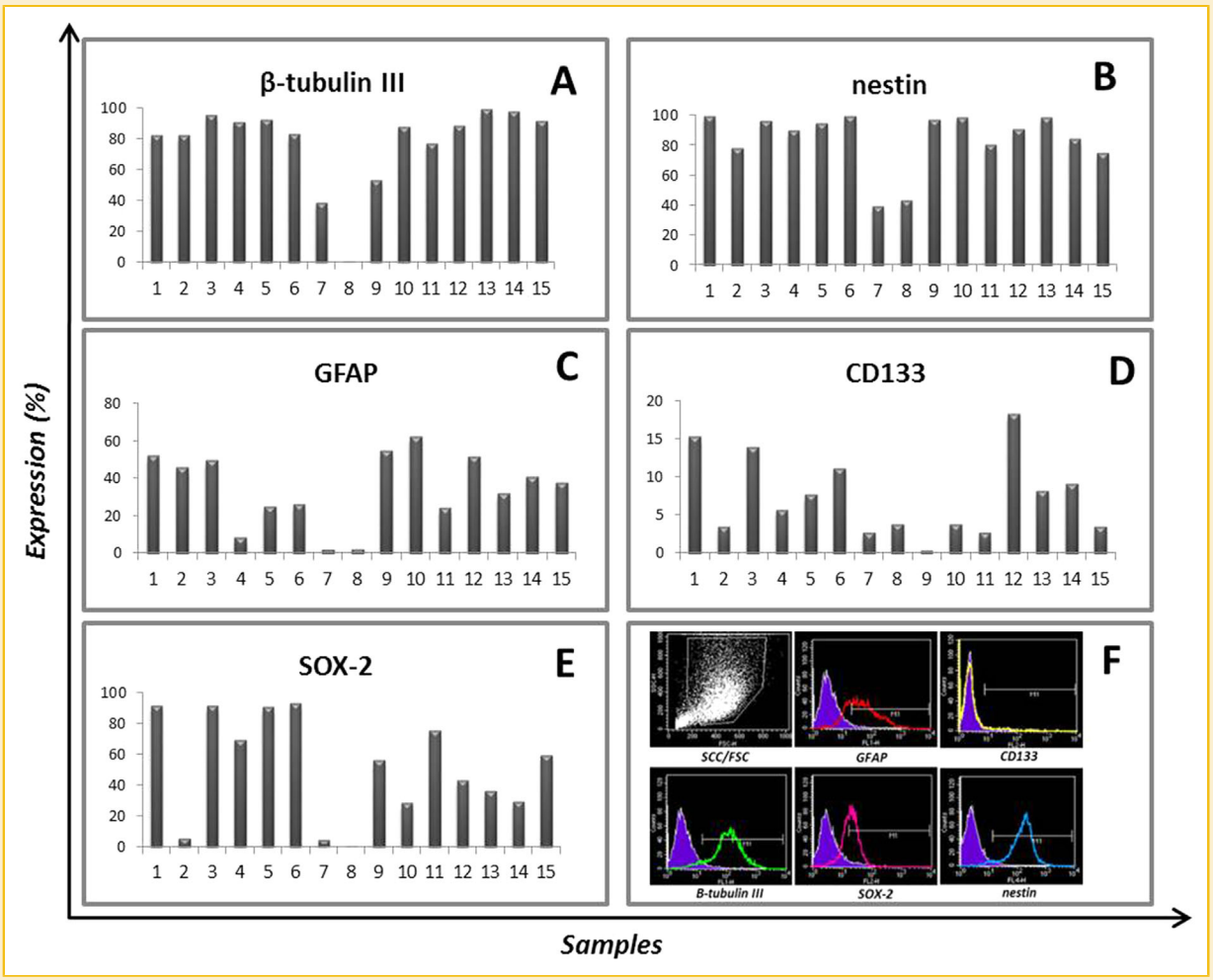

Fig. 1. Marker expression (percentage values) of (A) β-tubulin III, (B) nestin, (C) GFAP, (D) CD133, and (E) SOX-2 was detected in 15 human GBM primary cultures. Flow cytometric analysis was evaluated for each patient at third culture step. (F) Representative flow cytometric profiles of one GBM primary culture maintained in DMEM 10% FBS.

studies reported that CD133 positive cells represent up to 50% of freshly dissociated tumor cells [Singh et al., 2004; Yuan et al., 2004; Gilbert and Ross, 2009]. Patru et al. (2010); moreover, demonstrated that only a few in vitro cultured cells expressed the CD133 antigen, thus suggesting a rapid down-regulation in vitro [Patru et al., 2010]. A recent work (Kase, 2013) demonstrated a wide variability of CD133+ cells in GBM patients being from 0.5% to 82% [Kase et al., 2013], and Pallini et al. (2011) have reported studies of 37 and 44 GBM patients which CD133 expression ranging between 0.5% and 10%. Our results showed that all DMEM 10% cultured cells expressed low and not homogeneous levels of CD133 (0.47–18.27% with an average value of 7.37) (Fig. 1D). The expression levels of SOX-2 were extremely heterogeneous (range: 0.24–91.62%; average value: 51.56%) and just five GBM cultures showed SOX-2 expression levels $\leq$30% (range: 0.24–29.54%, average value: 13.67%) (Fig. 1E). Moreover, Figure 1 F shows a representative flow cytometric profiles of these markers in one primary GBM culture in DMEM 10% FBS.

MORPHOLOGY AND IMMUNOPHENOTYPIC ANALYSIS OF HUMAN GSCs OBTAINED FROM GBM SPECIMENS

To investigate if GBM cells recovered from enzymatic digestion of tumor samples were able to generate floating aggregates, were cultured in DMEM/F12 added with B27 supplement, EGF, and bFGF and analyzed by flow cytometry. The neurospheres' generation time was very different for each cell culture. For instance, some cell cultures produced spheres after just 10 days, others after 30 days. The morphology analysis revealed that the neurospheres were cell aggregates of different sizes floating in the medium (Fig. 2A–D) or semi-adherent growing cells (Fig. 2E and F).

We observed that not all GBM specimen have generated neurospheres. In particular, in 5 GBM cases, the cells derived by tumor digestion, even though maintained in the conditioning medium for up to 60 days, did not generate neurospheres, remaining in suspension as individual cells. Of interest, the ability of generate in vitro neurospheres was detected only when the corresponding primary GBM cultures showed the highest values of SOX-2 levels (>30%).

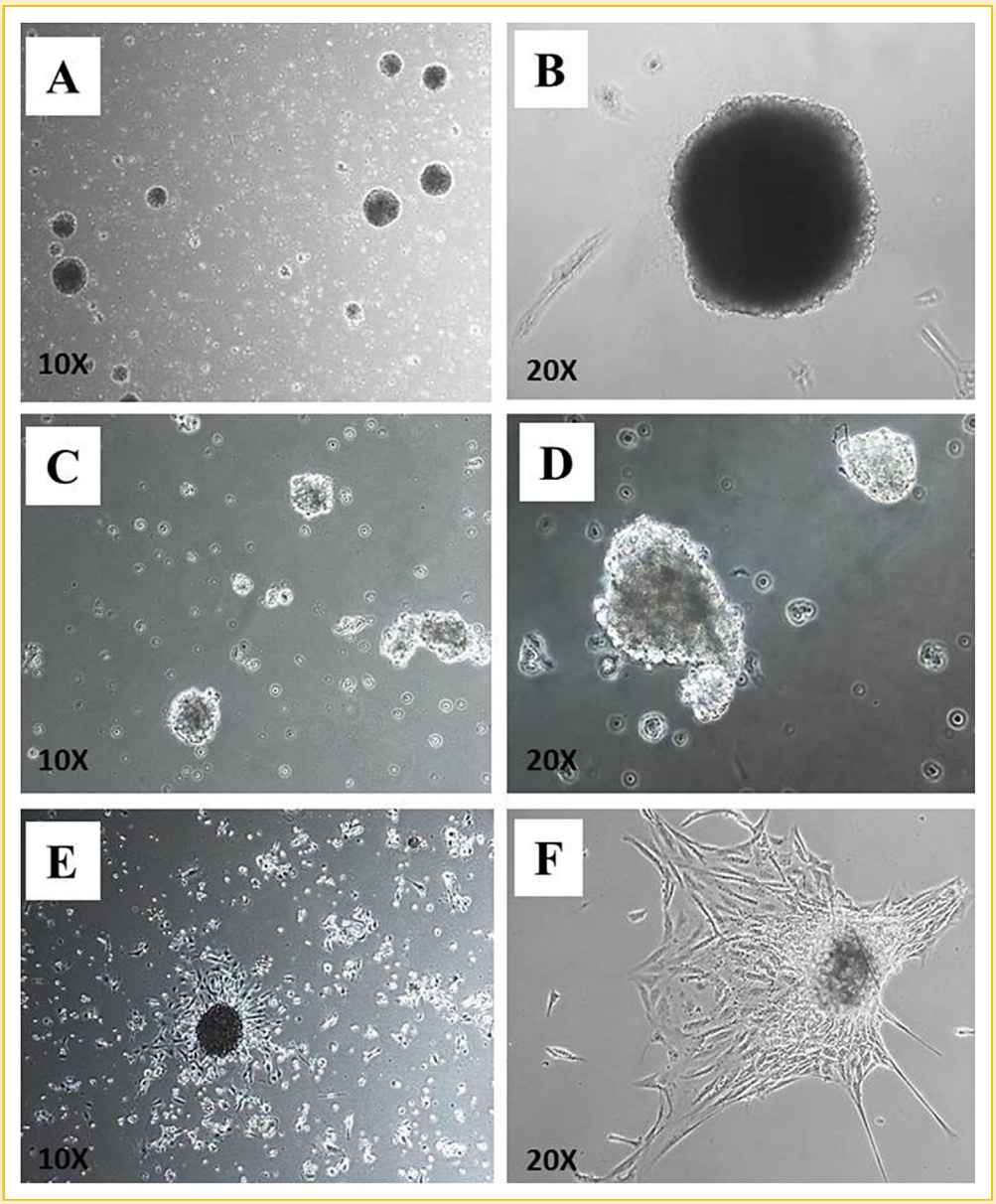

Fig. 2. Representative images of neurosphere cultures, obtained after GBM tissue sample digestion and grown in defined DMEM/F12 (1:1) supplemented with B27, EGF, and bFGF for at least 30 days, were taken by a phase contrast microscopy. Tumor spheres appeared as thick floating spherical cell aggregates (A and B), less dense aggregates with undefined shape (C and D) and aggregates semi-adhering to the culture plate (E and F) (Magnification at 10× and 20×).

All the GSC cultures were examined by immunophenotypic analysis evaluating β-tubulin III, nestin, GFAP, CD133, and SOX-2 markers expression. The flow cytometry analysis of neurospheres, after 30 culture days, showed 92% of cells expressing β-tubulin III (Fig. 3A), 99% nestin positive cells (Fig. 3B), 33.60% cells expressing GFAP (Fig. 3C), 14% of CD133 positive cells (Fig. 3D), and 88.75% SOX-2 positive cells (Fig. 3 E). All percentages were considered as average values. Figure 3F shows representative flow cytometric profiles of one neurosphere culture in the conditioning medium after 30 culture days.

The obtained results indicated that percentage values of analysed markers in the neurosphere cultures were higher when compared to GBM primary cultures. In particular, the percentage of CD133+ cells in neurosphere samples was twice as higher than respective DMEM 10% cells.

In this work we also investigated the probable correlations between expressed levels of β-tubulin III, nestin, GFAP, CD133, and SOX-2 in both primary GBM and neurosphere cultures, as reported in Table I.

EVALUATION OF NEUROSPHERE CULTURES' MULTIPOTENCY FOR NEURONS, ASTROCYTES, AND OLIGODENDROCYTES DIFFERENTIATION

In order to confirm the stemness features of neurospheres generated from primary GBM cultures, the cells were differentiated in the three

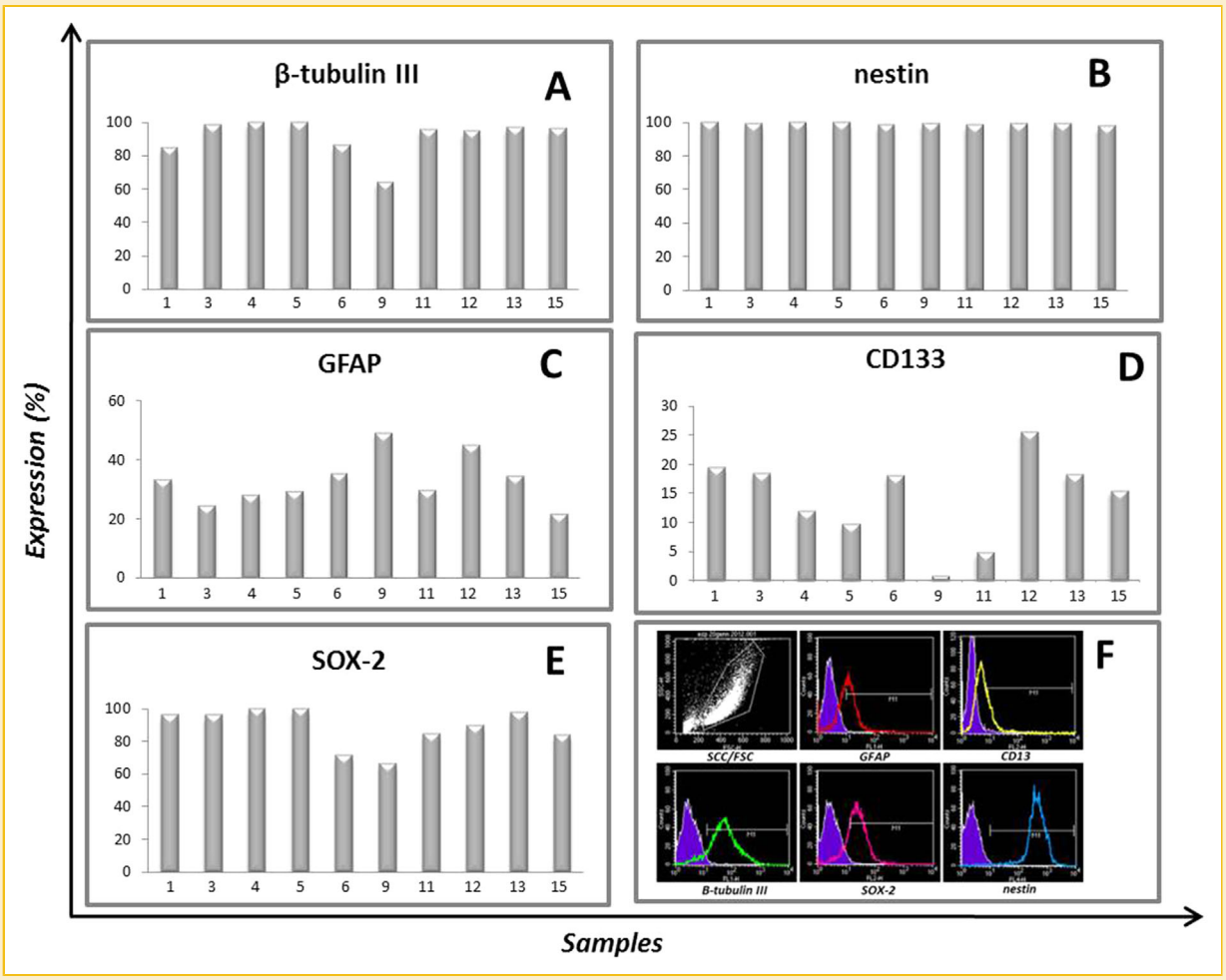

Fig. 3. Marker expression (percentage values) of (A) β-tubulin III, (B) nestin, (C) GFAP, (D) CD133, and (E) SOX-2 in neurospheres obtained from 10 primary GBM cultures and maintained in stem cell conditioning medium for at least 30 days. (F) Representative flow cytometric profiles of one neurosphere culture in conditioning medium (DMEM/F12 + B27 + EGF + bFGF) after 30 culture days.

neural lineages (neurons, astrocytes, and oligodendrocytes) through the specific medium Neurocult™ (35 days), as previously described. Figure 4 shows representative images of a GSCs culture differentiated into: astrocytes (Fig. 4 panel A), neurons (Fig. 4 panel B), and oligodendrocytes (Fig. 4 panel C). As expected and reported in the manufacturer instructions, we observed that, under specific conditions, the neurospheres differentiated into neurons and few oligodendrocytes, whereas a larger amount of cells differentiated into GFAP+ astrocytes. These data confirmed the stemness features of in vitro generated neurospheres.

TABLE I. Correlation Between Several Markers Expressed on Primary GBM and Neurosphere Cultures

Marker versus marker		Primary GBM cultures	Neurosphere cultures
β-tubulin III	nestin	r = 0.766 (P = 0.000)	r = 0.108 (P = 0.766)
nestin	GFAP	r = 0.662 (P = 0.007)	r = 0.182 (P = 0.618)
SOX-2	nestin	r = 0.666 (P = 0.007)	r = 0.526 (P = 0.119)
SOX-2	β-tubulin III	r = 0.505 (P = 0.055)	r = 0.767 (P = 0.010)
SOX-2	CD133	r = 0.450 (P = 0.093)	r = 0.402 (P = 0.249)
β-tubulin III	GFAP	r = 0.499 (P = 0.058)	r = −0.711 (P = 0.021)
β-tubulin III	CD133	r = 0.423 (P = 0.116)	r = 0.386 (P = 0.271)
nestin	CD133	r = 0.430 (P = 0.110)	r = 0.030 (P = 0.935)
CD133	GFAP	r = 0.323 (P = 0.240)	r = 0.087 (P = 0.818)
SOX-2	GFAP	r = 0.181 (P = 0.520)	r = −0.554 (P = 0.097)

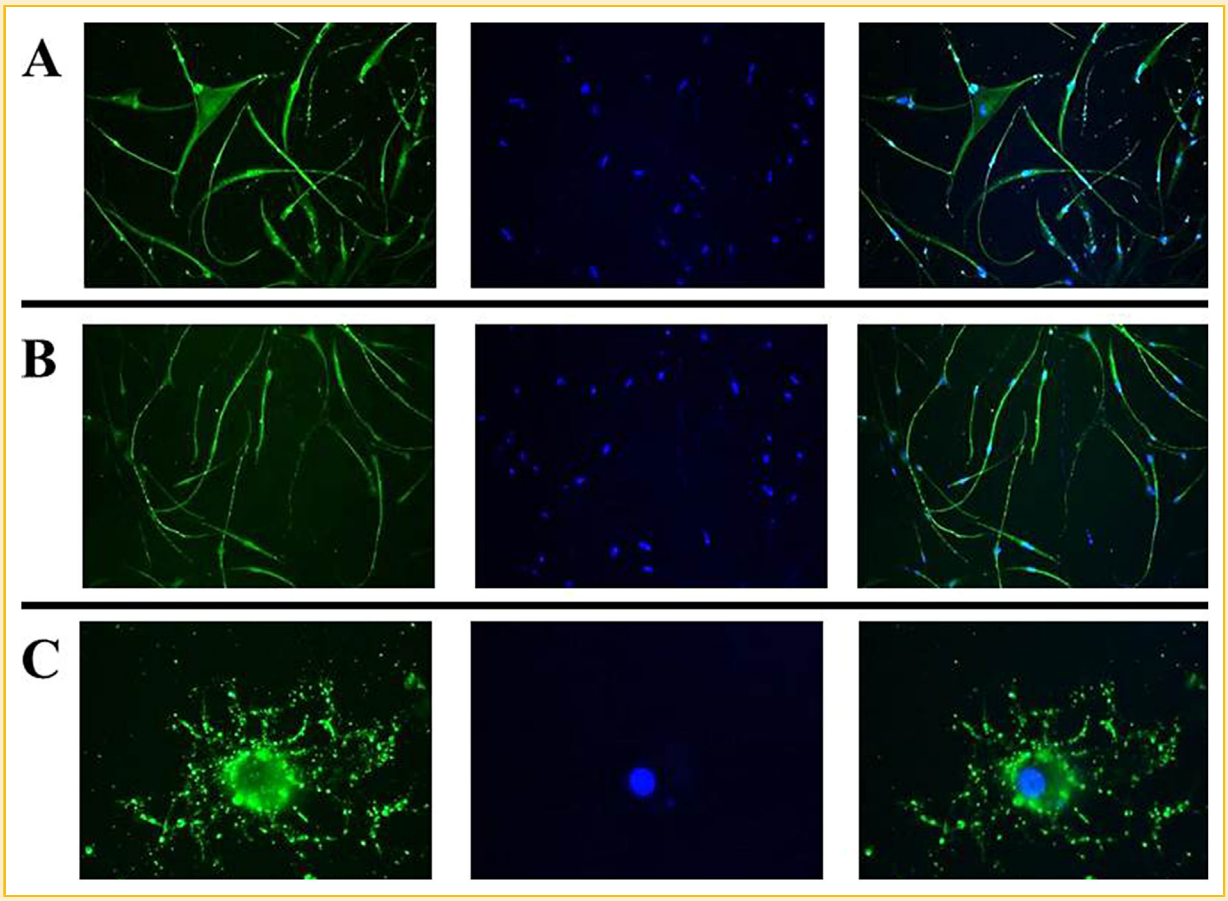

Fig. 4. Immunofluorescence of neurospheres obtained from GBM primary cultures differentiated in (A) astrocytes and stained with GFAP antibody at 10× magnification, (B) in neurons and stained with NF200 antibody at 10× magnification, and (C) in oligodendrocytes after staining with O4 antibody at 40× magnification. Cell nuclei counterstained with DAPI (blue).

CORRELATION BETWEEN GSCS GENERATION AND GSCs + /KI67+ WITH CLINICAL OUTCOME

All the samples collected in the operating room from the explanted tumor and subjected to histological examination confirming GBM (WHO grade IV) were processed for neurosphere generation assay and evaluated by immunophenotypic analysis, as previously described. Tumour biopsies were also evaluated for Ki67 positivity, known as a proliferative marker. In particular, 10 out of the 15 GBM patients were able to generate GSCs and 7 patients expressing Ki67 generated GSCs in vitro.

Median OS for all patients from the time of diagnosis (OS) was 17 months (range 8–29.5) with a 1-year and 2-year probability of survival of 66.6% and 26.6%, respectively. Median PFS was 12 months, with a 1-year probability of PFS of 53.3%.

Statistical analysis revealed that the generation of GSCs (GSCs+) negatively affected both OS ($P = 0.000$) (Fig. 5A) and PFS ($P = 0.001$) (Fig. 5B) as opposed to OS and PFS in patients that not generate GSCs (GSCs-) (Fig. 5C and D, respectively). The GSCs+ patients had a median OS of 11 months (Fig. 5A) and a median PFS of 9 months (Fig. 5B) when compared with 25 months of OS (Fig. 5C) and 17 of PFS (Fig. 5D) among those GSC- patients. In addition, we proved that the ability to generate GSCs and Ki67 expression (GSCs+/Ki67+) was associated with a very poor OS ($P = 0.000$) (Fig. 6A) and PFS ($P = 0.000$) (Fig. 6B). Moreover, subjects $GSC^+/Ki67^+$ had a median OS of 11 months (Fig. 6A) and a median PFS of 9 months (Fig. 6B) compared with 27 months of OS (Fig. 6C) and 17 of PFS (Fig. 6D) among those without either generation of GSCs or expression of Ki67 (GSCs-/Ki67-).

The patients' KPS have all been reported before surgery, at the discharge and at the last follow-up (Fig 7). Statistical study showed that within the group of GSCs+ patients quality of life was worse than GSCs- patients. In particular, in GSCs+ group there were statistically significant differences between preoperative KPS values and scores recorded at the discharge and at the last follow-up (*P-value ≤ 0.01). In GSCs- group there were statistically significant differences between KPS scores in preoperative time and scores at the discharge (#$P \leq 0.01$), and between KPS scores recorded at the discharge and at the last follow-up (§$P \leq 0.01$). We also detected a significant difference when compared KPS score at the last follow-up in GSCs+ group to KPS score at the last follow-up in GSCs- group (◆$P \leq 0.01$).

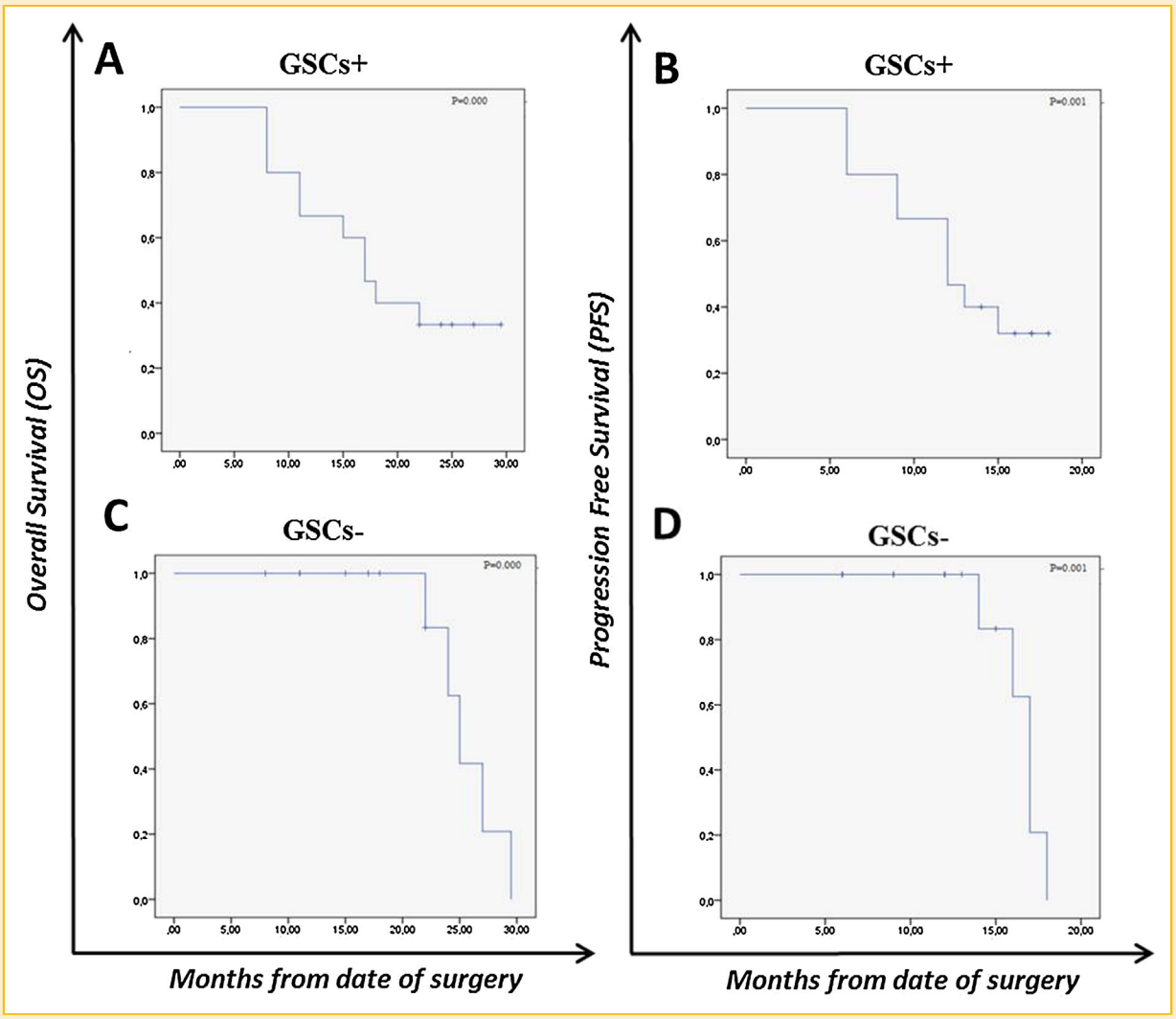

Fig. 5. Kaplan–Meier estimate of Overall Survival (OS) related to the presence of GBM stem cells (GSCs +) (A) and to the absence of them (GSCs-) (C). Kaplan–Meier estimate of Progression-Free Survival (PFS) related to the presence of glioma stem cells (GSCs +) (B) and to their absence (GSCs-) (D).

CORRELATION BETWEEN SOX-2 EXPRESSION LEVELS AND PATIENTS SURVIVAL

Based on previous results on SOX-2 expression in GBM primary cultures, we observed that only those expressing SOX-2 at levels higher than 30% were able to generate in vitro neurospheres. Also, patients with a lower expression of SOX-2 not generated GSCs ensuring a better clinical outcome in OS and PFS parameters. Figure 8 shows a strong and negative correlation between SOX-2 expression levels ($r = -0.75$) from 15 primary GBM cultures in DMEM 10% and patients survival (months) ($P = 0.001$). Altogether, these findings demonstrate that the ability to generate GSCs can negatively affect the clinical outcome.

DISCUSSION

Glioblastoma, the most common malignant primary brain tumor in adults, is characterized by a high cellular and molecular heterogeneity as well as a high invasiveness [Rath et al., 2013; Stieber et al., 2014]. Recent studies have suggested that GBM is driven and maintained by a subpopulation of clonogenic cells, the glioblastoma stem cells, which seem to play a crucial role in GBM biology [Rath et al., 2013]. These cells contribute to its resistance to both chemotherapy and radiotherapy through the potential activation of DNA damage checkpoint responses and the increase in DNA repair capacity [Dahlrot et al., 2013]. Other authors have demonstrated that in vitro growth of GSCs represents a significant predictor of clinical outcome [Pallini et al., 2008; Zeppernick et al., 2008]. Although it has recently been demonstrated that GSCs have tumorigenic potential in addition to enhanced resistance mechanisms [Singh et al., 2004], the real link between the biological features of brain tumor stem cells and their clinical behavior has yet to be established.

In the present work, 15 GBM biopsies were enzymatically digested in order to obtain primary cultures, and these cells were maintained both in DMEM 10% and in neurospheres conditioning medium

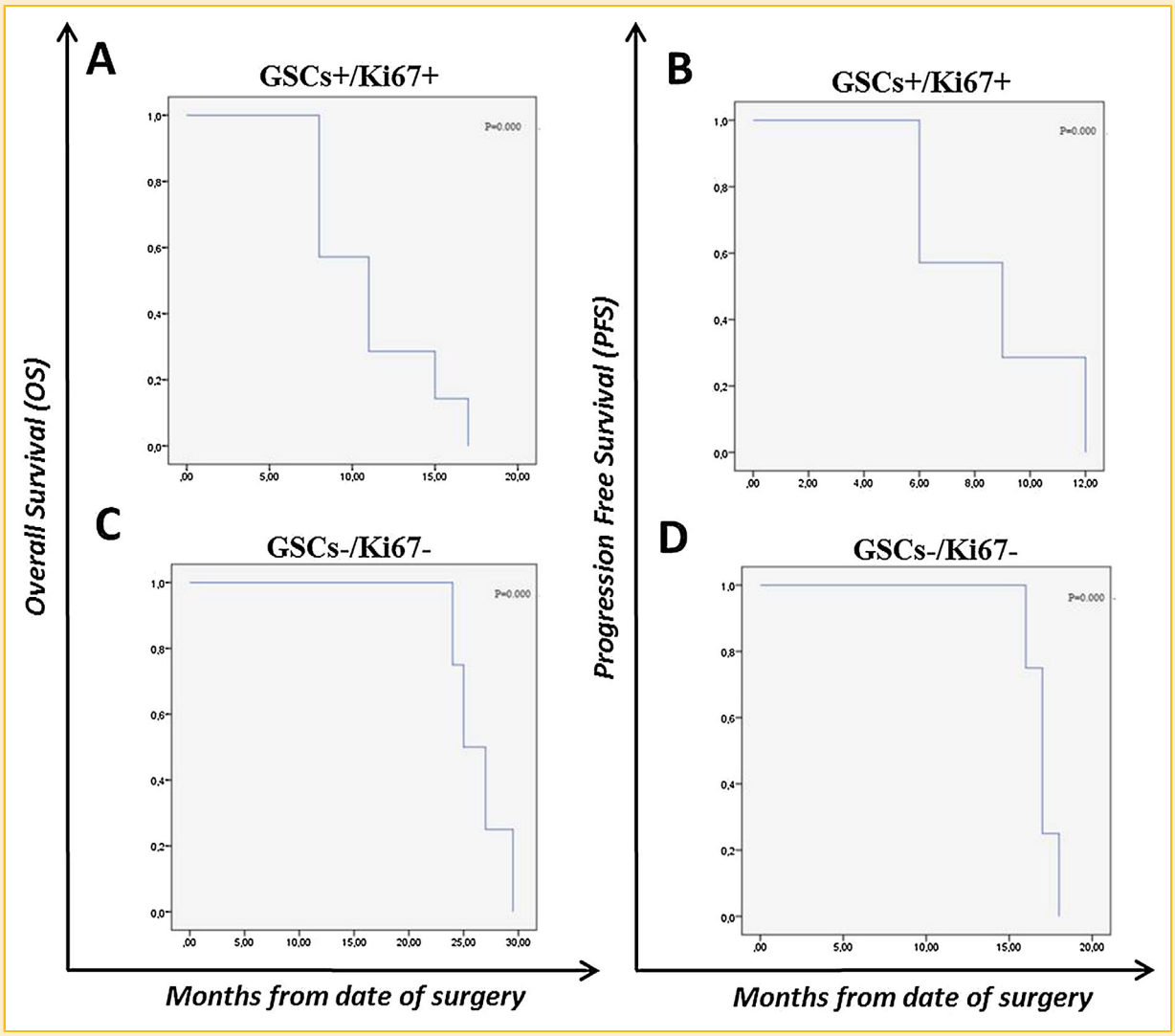

Fig. 6. Kaplan–Meier estimate of Overall Survival (OS) related to the presence of GBM stem cells and with Ki67 positivity (GSCs+/Ki67+) (A) and to the absence of them (GSCs-/Ki67-) (C). Kaplan–Meier estimate of Progression-Free Survival (PFS) related to the presence of glioma stem cells and with Ki67 positivity (GSCs+/Ki67+) (B) and to their absence (GSCs-/Ki67-) (D).

(DMEM/F12 + B27 + EGF + bFGF). We performed cytofluorimetric analysis for detection of β-tubulin III, nestin, GFAP, CD133, and SOX-2 markers both in cultured DMEM 10% cells and in GSCs obtained from them. In primary GBM cultures from 15 patients, we revealed variable expression levels of these markers, in accordance with the well-known heterogeneity of glioblastomas. These data showed a strong correlation between β-tubulin III versus nestin ($r = 0.766$; $P = 0.000$), nestin versus GFAP ($r = 0.662$; $P = 0.007$), and SOX-2 versus nestin ($r = 0.666$; $P = 0.007$) in primary GBM cultures confirming that nestin levels were matched with some malignancy markers.

In our 15 GBM samples, we also evaluated the presence of CD133+ cells and we detected that primary GBM cells showed a low percentage value of CD133 (average value 7.37%). This result is in agreement with a previous work of Pallini et al. (2008) in which CD133+ cells were ranged between 0.5%–10% [Pallini et al., 2008]. The neurosphere generation from GBM specimens was evaluated in suitable culture condition, as described in Method's section. The flow cytometry experiments, designed to detect the stemness markers, showed fairly homogeneous levels of β-tubulin III, nestin, GFAP, and SOX-2, whereas CD133 expression was variable among GSC samples. A strong positive correlation was observed between SOX-2 versus β-tubulin III expression levels ($r = 0.767$; $P = 0.010$), and a strong negative relationship between β-tubulin III versus GFAP expression ($r = -0.711$; $P = 0.021$).

Gangemi et al. (2009) have demonstrated that, in mouse neural stem cells, the *sox-2* gene, when silenced, causes their exit from the cell cycle and an increased expression of a neuronal marker, indicating the central role of the *sox-2* gene in the maintenance of the proliferative potential of stem and precursor stem cells.

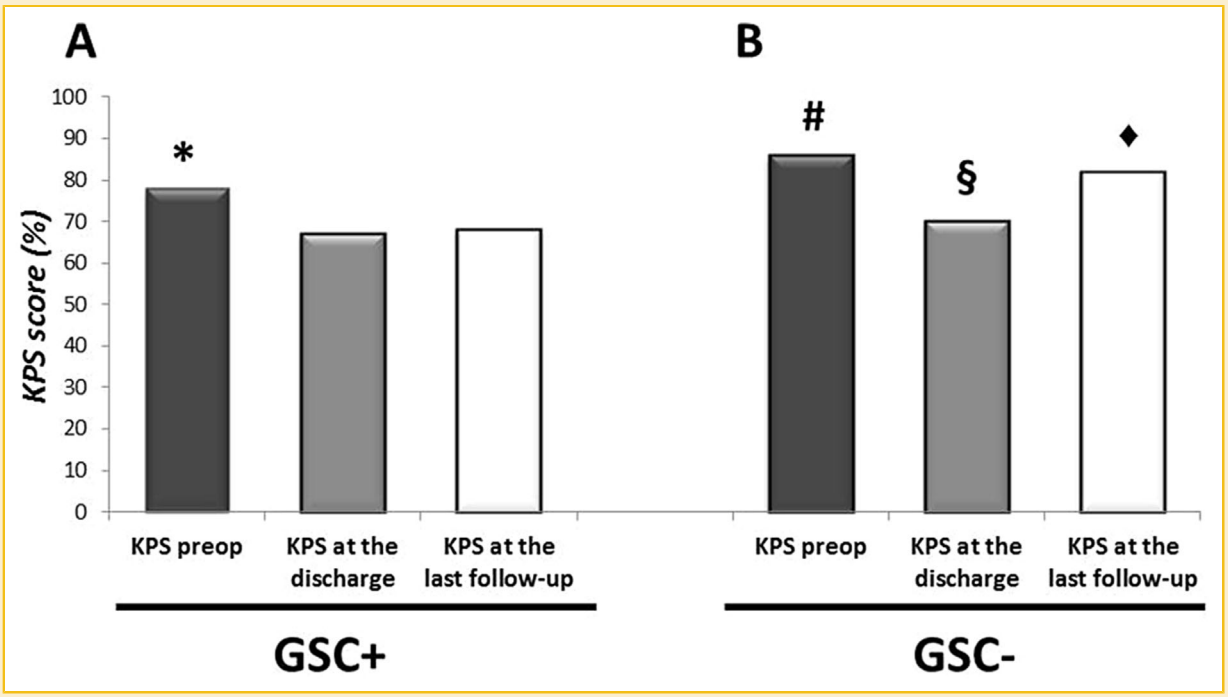

Fig. 7. KPS score in the preoperative period, at the discharge and at the last follow-up of GBM patients. (A) KPS score recorded in GSCs+ group, (B) KPS score recorded in GSC-. (*P-value ≤ 0.01 when compared KPS preoperative in GSCs+ group to KPS at the discharge and at the last follow-up; #$P \leq 0.01$ when compared KPS score in preoperative time in GSCs- group to scores at the discharge; §$P \leq 0.01$ when compared KPS at the discharge to scores at the last follow-up; ◆$P \leq 0.01$ when compared KPS score at the last follow-up in GSCs+ to KPS score at the last follow-up in GSCs-).

Furthermore, they also evaluated *sox-2* knockdown in human tumor-initiating cells (TICs) and observed similar results, such as loss of tumorigenicity, the cell proliferation arrest and exit from cell cycle in *sox-2* silenced cells that become progressively more mature and differentiated [Gangemi et al., 2009].

Interestingly, within the array of 15 GBMs analysed, 10 cultures were able to form GSCs spheres and the immunophenotypic results suggested a link between the ability to generate neurospheres and the SOX-2 expression in primary GBM cell cultures. Specifically, only those cells that expressed SOX-2 at greater than 30% levels were able to produce in vitro neurospheres and in patients with a lower SOX-2 expression we also detected an increase of total survival, OS, and PFS clinical parameters.

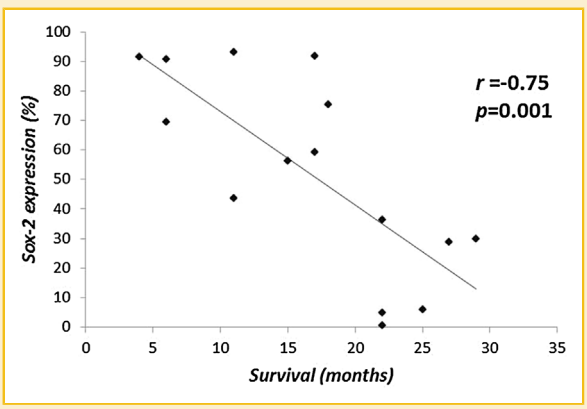

Fig. 8. Correlation between SOX-2 expression levels of DMEM 10% GBM primary cultures and patients survival (months).

The ability to generate GSCs and the simultaneously expression of Ki67 in our tumor lesions seem to be prognostic factors of tumor recurrence and poor survival, whereas Dahlort found no association between Ki67 staining and the clinical outcome of GBM [Dahlrot et al., 2013]. In addition, we noted that patients whose GBM generated GSCs in vitro (GSC+), presented an unfavourable outcome with significantly shorter OS and PFS in comparison with subjects whose GBM did not generate GSCs (GSC-). In addition, in our series, we evaluated that the GSCs generation and the simultaneous histological positivity to Ki67 in 7 tumors specimens was an important negative prognostic factor for both OS and PFS. These data suggest that the detection of Ki67 positivity within the group of tumors generating GSCs may be useful to identify subjects at risk of disease progression.

Finally, because GBM is almost invariably fatal within a few time and because a curative treatment is unlikely to be discovered in the near future, in recent years greater emphasis has been placed on measures of outcome other than duration of survival. KPS scale is a widely used as a measure of health related quality of life and it has been found it correlates significantly with survival. Although this

study had only the purpose to evaluate how the presence of GSCs can influence the clinical outcome, we observed that GSCs- patients have higher scores in KPS than GSCs+ patients. A future perspective could be to evaluate further data and variables in order to better analyse the possible correlation between the GSCs expression, survival, and quality of life.

In our view, all these data, taken together, establish that not all GBM primary cultures have the ability to generate GSCs in vitro, and this ability can probably predict a clinical outcome. Therefore it could be argued that the presence of GSCs-/SOX-2-/Ki67- cells might be regarded as a new prognostic factor. Hence, it would be necessary to analyze a greater number of primary GBM cultures to increase the series and compare GSCs+/SOX-2+/Ki67+ tumors to GSCs-/SOX-2-/Ki67- tumors in order to evaluate targeted therapeutic strategies capable of improving the clinical outcome of GBM patients.

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REFERENCES

Arai H,Ikota H, Sugawara K, Nobusawa S, Hirato J, Nakazato Y. 2012. Nestin expression in brain tumors: Its utility for pathological diagnosis and correlation with the prognosis of high-grade gliomas. Brain Tumor Pathol 29:160–167.

Basu-Roy U, Seo E, Ramanathapuram L, Rapp TB, Perry JA, Orkin SH, Mansukhani A, Basilico C. 2012. Sox2 maintains self renewal of tumor-initiating cells in osteosarcomas. Oncogene 31:2270–2282.

Bleau AM, Howard BM, Taylor LA, Gursel D, Greenfield JP, Lim Tung, Holland HY, Boockvar EC. 2008. New strategy for the analysis of phenotypic marker antigens in brain tumor-derived neurospheres in mice and humans. Neurosurg Focus 24:E28.

Bonnet D, Dick JE. 1997. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3: 730–737.

Clarke J, Butowski N, Chang S. 2010. Recent advances in therapy for glioblastoma. Arch Neurol 67:279–283.

Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, Visvader J, Weissman IL, Wahl GM. 2006. Cancer stem cells–perspectives on current status and future directions: AACR workshop on cancer stem cells. Cancer Res 66:9339–9344.

Cox JL, Wilder PJ, Desler M, Rizzino A. 2012. Elevating SOX2 levels deleteriously affects the growth of medulloblastoma and glioblastoma cells. PLoS ONE 7:e44087.

Dahlrot RH, Hermansen SK, Hansen S, Kristensen BW. 2013. What is the clinical value of cancer stem cell markers in gliomas. Int J Clin Exp Pathol 6:334–348.

Di Tomaso T, Mazzoleni S, Wang E, Sovena G, Clavenna D, Franzin A, Mortini P, Ferrone S, Doglioni C, Marincola FM, Galli R, Parmiani G, Maccalli C. 2010. Immunobiological characterization of cancer stem cells isolated from glioblastoma patients. Clin Cancer Res 16:800–813.

Draberova E, Del Valle L, Gordon J, Markova V, Smejkalova B, Bertrand L, de Chadarevian JP, Agamanolis DP, Legido A, Khalili K, Draber P, Katsetos CD. 2008. Class III beta-tubulin is constitutively coexpressed with glial fibrillary acidic protein and nestin in midgestational human fetal astrocytes: Implications for phenotypic identity. J Neuropathol Exp Neurol 67:341–354.

Gambelli F, Sasdelli F, Manini I, Gambarana C, Oliveri G, Miracco C, Sorrentino V. 2012. Identification of cancer stem cells from human glioblastomas: Growth and differentiation capabilities and CD133/prominin-1 expression. Cell Biol Int 36:29–38.

Gangemi RMR, Griffero F, Marubbi D, Perera M, Capra MC, Malatesta P, Ravetti GL, Zona GL, Daga A, Corte G. 2009. SOX2 silencing in glioblastoma tumor-initiating cells causes stop of proliferation and loss of tumorigenicity. Stem Cells 27:40–48.

Gilbert CA, Ross AH. 2009. Cancer stem cells: Cell culture, markers, and targets for new therapies. J Cell Biochem 108:1031–1038.

Gunther HS, Schmidt NO, Phillips HS, Kemming D, Kharbanda S, Soriano R, Modrusan Z, Meissner H, Westphal M, Lamszus K. 2008. Glioblastoma-derived stem cell-enriched cultures form distinct subgroups according to molecular and phenotypic criteria. Oncogene 27:2897–2909.

Gursel DB, Shin BJ, Burkhardt JK, Kesavabhotla K, Schlaff CD, Boockvar JA. 2011. Glioblastoma stem-like cells-biology and therapeutic implications. Cancers (Basel) 3:2655–2666.

Herreros-Villanueva M, Zhang JS, Koenig A, Abel EV, Smyrk TC, Bamlet WR, de Narvajas AA, Gomez TS, Simeone DM, Bujanda L, Billadeau DD. 2013. SOX2 promotes dedifferentiation and imparts stem cell-like features to pancreatic cancer cells. Oncogenesis 2:e61.

Jin X, Jin X, Jung JE, Beck S, Kim H. 2013. Cell surface Nestin is a biomarker for glioma stem cells. Biochem Biophys Res Commun 433:496–501.

Joo KM, Kim SY, Jin X, Song SY, Kong DS, Lee JI, Jeon JW, Kim MH, Kang BG, Jung Y, Jin J, Hong SC, Park WY, Lee DS, Kim H, Nam DH. 2008. Clinical and biological implications of CD133-positive and CD133-negative cells in glioblastomas. Lab Invest 88:808–815.

Kase M, Minajeva A, Niinepuu K, Kase S, Vardja M, Asser T, Jaal J. 2013. Impact of CD133 positive stem cell proportion on survival in patients with glioblastoma multiforme. Radiol Oncol 47:405–410.

Katsetos CD, Draberova E, Legido A, Dumontet C, Draber P. 2009. Tubulin targets in the pathobiology and therapy of glioblastoma multiforme. I. Class III beta-tubulin. J Cell Physiol 221:505–513.

Krex D, Klink B, Hartmann C, von Deimling A, Pietsch T, Simon M, Sabel M, Steinbach JP, Heese O, Reifenberger G, Weller M, Schackert G, German Glioma. 2007. Long-term survival with glioblastoma multiforme. Brain 130:2596–2606.

Leis O, Eguiara A, Lopez-Arribillaga E, Alberdi MJ, Hernandez-Garcia S, Elorriaga K, Pandiella A, Rezola R, Martin AG. 2012. Sox2 expression in breast tumours and activation in breast cancer stem cells. Oncogene 31:1354–1365.

Macdonald DR, Cascino TL, Schold SC, Cairncross JG. 1990. Response criteria for phase-Ii studies of supratentorial malignant glioma. J Clin Oncol 8:1277–1280.

Maderna E, Salmaggi A, Calatozzolo C, Limido L, Pollo B. 2007. Nestin, PDGFRbeta, CXCL12 and VEGF in glioma patients: Different profiles of (pro-angiogenic) molecule expression are related with tumor grade and may provide prognostic information. Cancer Biol Ther 6:1018–1024.

Mazilu D, Gudu T, Ionescu R, Opris D. 2014. Statins do not influence long-term rituximab clinical efficiency in rheumatoid arthritis patients. Biomed Res Int 2014:689426.

Middeldorp J, Hol EM. 2011. GFAP in health and disease. Prog Neurobiol 93:421–443.

Pallini R, Ricci-Vitiani L, Banna GL, Signore M, Lombardi D, Todaro M, Stassi G, Martini M, Maira G, Larocca LM, De Maria R. 2008. Cancer stem cell analysis and clinical outcome in patients with glioblastoma multiforme. Clin Cancer Res 14:8205–8212.

Pallini R, Ricci-Vitiani L, Montano N, Mollinari C, Biffoni M, Cenci T, Pierconti F, Martini M, De Maria R, Larocca LM. 2011. Expression of the stem cell marker CD133 in recurrent glioblastoma and its value for prognosis. Cancer 117:162–174.

Patru C, Romao L, Varlet P, Coulombel L, Raponi E, Cadusseau J, Renault-Mihara F, Thirant C, Leonard N, Berhneim A, Mihalescu-Maingot M, Haiech J, Bieche I, Moura-Neto V, Daumas-Duport C, Junier MP, Chneiweiss H. 2010. CD133, CD15/SSEA-1, CD34 or side populations do not resume tumor-initiating properties of long-term cultured cancer stem cells from human malignant glio-neuronal tumors. BMC Cancer.

Phi JH, Park SH, Kim SK, Paek SH, Kim JH, Lee YJ, Cho BK, Park CK, Lee DH, Wang KC. 2008. Sox2 expression in brain tumors: A reflection of the neuroglial differentiation pathway. Am J Surg Pathol 32:103–112.

Prestegarden L, Svendsen A, Wang J, Sleire L, Skaftnesmo KO, Bjerkvig R, Yan T, Askland L, Persson A, Sakariassen PO, Enger PO. 2010. Glioma cell populations grouped by different cell type markers drive brain tumor growth. Cancer Res 70:4274–4279.

Rath BH, Fair JM, Jamal M, Camphausen K, Tofilon PJ. 2013. Astrocytes enhance the invasion potential of glioblastoma stem-like cells. PLoS ONE 8:e54752.

Raysi Dehcordi, De Paulis S, Marzi D, Ricci S, Cimini A, Cifone A, Galzio MG. 2012. Survival prognostic factors in patients with glioblastoma: Our experience. J Neurosurg Sci 56:239–245.

Reya T, Morrison SJ, Clarke MF, Weissman IL. 2001. Stem cells, cancer, and cancer stem cells. Nature 414:105–111.

Rieske P, Golanska E, Zakrzewska M, Piaskowski S, Hulas-Bigoszewska K, Wolanczyk M, Szybka M, Witusik-Perkowska M, Jaskolski DJ, Zakrzewski K, Biernat W, Krynska B, Liberski PP. 2009. Arrested neural and advanced mesenchymal differentiation of glioblastoma cells-comparative study with neural progenitors. BMC Cancer.

Sampetrean O, Saya H. 2013. Characteristics of glioma stem cells. Brain Tumor Pathol 30:209–214.

Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD, Dirks PB. 2004. Identification of human brain tumour initiating cells. Nature 432:396–401.

Stieber D, Golebiewska A, Evers L, Lenkiewicz E, Brons NH, Nicot N, Oudin A, Bougnaud S, Hertel F, Bjerkvig R, Vallar L, Barrett MT, Niclou SP. 2014. Glioblastomas are composed of genetically divergent clones with distinct tumourigenic potential and variable stem cell-associated phenotypes. Acta Neuropathol 127:203–219.

Stiles CD, Rowitch DH. 2008. Glioma stem cells: A midterm exam. Neuron 58:832–846.

Stopschinski BE, Beier CP, Beier D. 2013. Glioblastoma cancer stem cells–From concept to clinical application. Cancer Lett 338:32–40.

Strojnik T, Rosland GV, Sakariassen PO, Kavalar R, Lah T. 2007. Neural stem cell markers, nestin and musashi proteins, in the progression of human glioma: Correlation of nestin with prognosis of patient survival. Surg Neurol 68:133–143;discussion 143–144.

Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO, European Organisation, for R, Treatment of, Cancer Brain, Radiotherapy G, National Cancer, Institute of, Canada Clinical, Trials G. 2005. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352:987–996.

Tomita T, Akimoto J, Haraoka J, Kudo M. Clinicopathological significance of expression of nestin, a neural stem/progenitor cell marker, in human glioma tissue. Brain Tumor Pathol 31:162–71.

Tsourlakis MC, Weigand P, Grupp K, Kluth M, Steurer S, Schlomm T, Graefen M, Huland H, Salomon G, Steuber T, Wilczak W, Sirma H, Simon R, Sauter G, Minner S, Quaas A. betaIII-tubulin overexpression is an independent predictor of prostate cancer progression tightly linked to ERG fusion status and PTEN deletion. Am J Pathol 184:609–17.

Wan F, Herold-Mende C, Campos B, Centner FS, Dictus C, Becker N, Devens F, Mogler C, Felsberg J, Grabe N, Reifenberger G, Lichter P, Unterberg A, Bermejo JL, Ahmadi R. 2011. Association of stem cell-related markers and survival in astrocytic gliomas. Biomarkers 16:136–143.

Wang J, Sakariassen PO, Tsinkalovsky O, Immervoll H, Boe SO, Svendsen A, Prestegarden L, Rosland G, Thorsen F, Stuhr L, Molven A, Bjerkvig R, Enger PO. 2008. CD133 negative glioma cells form tumors in nude rats and give rise to CD133 positive cells. Int J Cancer 122:761–768.

Wen PY, Kesari S. 2008. Malignant gliomas in adults. N Engl J Med 359: 492–507.

Yuan X, Curtin J, Xiong Y, Liu G, Waschsmann-Hogiu S, Farkas DL, Black KL, Yu JS. 2004. Isolation of cancer stem cells from adult glioblastoma multiforme. Oncogene 23:9392–9400.

Zeppernick F, Ahmadi R, Campos B, Dictus C, Helmke BM, Becker N, Lichter P, Unterberg A, Radlwimmer B, Herold-Mende CC. 2008. Stem cell marker CD133 affects clinical outcome in glioma patients. Clin Cancer Res 14: 123–129.

Zhang MY, Song T, Yang L, Chen RK, Wu L, Yang ZY, Fang JS. 2008. Nestin and CD133: Valuable stem cell-specific markers for determining clinical outcome of glioma patients. J Exp Clin Cancer Res 27:85.