

Cancer Stem Cell Analysis and Clinical Outcome in Patients with Glioblastoma Multiforme

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Abstract Purpose: Cancer stem cells (CSC) are thought to represent the population of tumorigenic cells responsible for tumor development. The stem cell antigen CD133 identifies such a tumorigenic population in a subset of glioblastoma patients. We conducted a prospective study to explore the prognostic potential of CSC analysis in glioblastoma patients.

Experimental Design: We investigated the relationship between the *in vitro* growth potential of glioblastoma CSCs and patient death or disease progression in tumors of 44 consecutive glioblastoma patients treated with complete or partial tumorectomy followed by radiotherapy combined with temozolomide treatment. Moreover, we evaluated by immunohistochemistry and immunofluorescence the prognostic value of the relative presence of CD133⁺ and CD133⁺/Ki67⁺ cells in patient tumors.

Results: *In vitro* CSC generation and the presence of $\geq 2\%$ CD133⁺ cells in tumor lesions negatively correlated with overall ($P = 0.0001$ and 0.02 , respectively) and progression-free ($P = 0.0002$ and 0.01 , respectively) survival of patients. A very poor overall ($P = 0.007$) and progression-free ($P = 0.001$) survival was observed among patients whose tumors contained CD133⁺ cells expressing Ki67. Taking into account symptom duration, surgery type, age, *O*⁶-methylguanine-DNA methyltransferase promoter methylation, and p53 status, generation of CSCs and CD133/Ki67 coexpression emerged as highly significant independent prognostic factors, with an adjusted hazard ratio of 2.92 (95% confidence interval, 1.37-6.2; $P = 0.005$) and 4.48 (95% confidence interval, 1.68-11.9; $P = 0.003$), respectively.

Conclusions: The analysis of CSCs may predict the survival of glioblastoma patients. *In vitro* CSC generation and presence of CD133⁺/Ki67⁺ cells are two considerable prognostic factors of disease progression and poor clinical outcome.

The cancer stem cell (CSC) theory postulates that tumors arise from the neoplastic transformation of normal stem or progenitor cells or from the dedifferentiation of more differentiated cells (1–5).

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Germinal brain regions for normal neural primitive cells are preferentially located in the subventricular zone, the dentate gyrus, and the subcortical white matter. According to the CSC hypothesis, migration of transformed stem cells from such regions may lead to the development of gliomas in different areas of the brain (6, 7). Normal and malignant primitive neural cells often express the transmembrane glycoprotein CD133, whose expression increases considerably in neural tumor tissues (1, 2). Previous reports suggested that tumorigenic cells in glioblastoma are confined into the CD133⁺ population (1, 2). These initial findings have been revisited in light of recent studies showing that CD133⁻ cells isolated from human and mouse gliomas are tumorigenic (8–11). However, tumor xenografts generated in immunocompromised mice by CD133⁺ cells showed higher resistance to radiation and chemotherapy, suggesting that CD133⁺ cells could be a more aggressive tumorigenic population (12, 13). The complexity of the stem cell compartment in glial tumors is confirmed by the demonstration that CD133⁺ cells isolated from human glioma coexpress the glial fibrillary acid protein (GFAP), a marker for differentiated glial cells (14). Although the stem cell compartment of glioblastoma may be not entirely defined by CD133 expression, Zeppernick et al. (15) have shown that both the proportion of CD133⁺ cells and their topological organization

Translational Relevance

In this study, we were able to detect proliferating cells in the cancer stem cell compartment of glioblastoma using immunohistochemical techniques. Glioblastoma tumor cells showing neural stem cell phenotype and proliferating activity (CD133⁺/Ki67⁺) were found to represent highly significant prognosticator for poor clinical outcome. The presence of CD133⁺/Ki67⁺ in glioblastoma did relate with the *in vitro* generation of long-term cultures of glioblastoma stem cells. The growth kinetic of glioblastoma stem cells *in vitro* also related with the clinical progression of parental tumor. These results strongly support the view that glioblastoma stem cells play a key role in tumor growth and resistance to therapy. The availability of considerable numbers of these tumorigenic cells may allow high-throughput analyses of new compounds for the development of stem cell – targeted therapeutic strategies to improve the clinical outcome of patients suffering for glioblastoma.

in clusters were significant prognostic factors for glioma patients, thus providing clinical support to the claim that these cells play an important role in tumor growth and resistance to therapy.

Glioblastoma stem and progenitor cells are highly resistant to conventional chemotherapeutic drugs, including temozolomide (12, 16). Moreover, these cells are relatively radioresistant and could be the source of tumor recurrence after radiation (13). The chemo-radioresistance of such tumorigenic cells may be responsible for the poor clinical outcome of glioblastoma patients and makes crucial the study of glioblastoma stem cells in the clinical setting. We therefore designed a prospective study to determine whether we could identify subgroups of glioblastomas with different intrinsic and prognostic features based on phenotypic and functional analysis of glioblastoma stem cells. Our working hypothesis is that the coexpression of CD133 and Ki67 by glioblastoma cells may relate to the generation of CSC lines and that CD133/Ki67 coexpression along with CSC generation may influence the clinical outcome.

Materials and Methods

Enrollment of patients, diagnosis, and tumor characterization. We harvested tumor tissue samples from 89 consecutive patients with primitive brain tumor undergoing complete or partial surgical resection at the Institute of Neurosurgery, Catholic University School of Medicine, in Rome, Italy, between December 2003 and November 2005. All the patients provided written informed consent according to the research proposals approved by the Ethical Committee of the Catholic University School of Medicine. Patients were eligible for the study if a diagnosis of glioblastoma multiforme was established histologically according to the WHO classification (17). The 44 patients with glioblastoma were 35 to 77 y old at the time of diagnosis (median age, 57 y); 28 were men and 16 were women. Tumor tissue samples were obtained by resection before the initiation of treatment with radiation and chemotherapy. Control frontal and temporal brain specimens were obtained from five patients undergoing epilepsy surgery.

Immunohistochemistry was done on deparaffinized sections using the avidin-biotin-peroxidase complex methods (ABC-Elite kit, Vector

Laboratories; ref. 18). The following primary antibodies were used: anti-p53 monoclonal antibody (DO-7, Dako), anti-epidermal growth factor receptor (EGFR) monoclonal antibody (EGFR.25, Novocastra), anti-Ki67 monoclonal antibody (MIB-1, Dako), and anti-CD133/1 monoclonal antibody (Miltenyi Biotec). Tumors were considered p53 deficient if immunoreaction stained the nuclei of at least 5% of cells (19, 20). Tumors showing moderate-to-strong immunostaining for EGFR in >20% of cells were considered EGFR positive (21).

Immunofluorescence analyses were done on 6- μ m-thick paraffin-embedded sections of human glioblastoma tumor samples. Dewaxed sections were treated for 10 min in a microwave oven in 0.1 mol/L citrate buffer (pH 9). Then, sections were exposed overnight at 4°C to 200 μ L of specific antibodies against CD133/2 (293C3, mouse IgG2b, Miltenyi Biotec) diluted 1:5. Following exposure to primary antibodies, sections were treated with goat anti-mouse immunoglobulins conjugated with Rhodamine Red (R-6393, Molecular Probes, Inc.). After washing in PBS, sections were postfixed with 2% paraformaldehyde for 20 min at 37°C and successively incubated with 50 mmol/L NH₄Cl for 30 min at room temperature. Then, sections were first permeabilized with 0.1% Triton X-100 and secondary exposed to Ki67 antibody (MIB-1, mouse IgG1, κ , Dako) diluted 1:50. Ki67-stained sections were treated with FITC-conjugated anti-mouse (Molecular Probes). Counterstaining was done using 4',6-diamidino-2-phenylindole (Vector Laboratories). Confocal analysis was used to acquire fluorescence stainings (Eclipse TE 2000, Nikon). The CD133/1 and CD133/2 antibodies were validated with several positive and negative controls. Although CD133/1 was more specific for cytoplasmic staining of CD133, both antibodies recognized the same cellular population. The percentages of CD133⁺ and CD133⁺/Ki67⁺ cells were evaluated independently by three pathologists (M.M., G.S., and L.M.L.) who were unaware of the clinical data. For each slide, a minimum of 10 no superimposing fields was examined at high-power magnification (\times 400) counting at least 2,000 tumor cells in areas devoid of necrosis, hemorrhage, and abundant new vessel formation. Differences between the extreme counts of the three pathologists never exceeded 2%. Interobserver agreement was reached in the first analysis in 90% of cases; for the remaining cases, a consensus was reached by a joint review of the slides.

O⁶-methylguanine-DNA methyltransferase (MGMT) promoter methylation patterns were studied by methylation-specific PCR using primers specific for methylated and unmethylated DNA (22) on genomic DNA extracted from paraffin-embedded tissue using QIAamp DNA Mini kit (Qiagen). The annealing temperature was 60°C. DNA from normal lymphocytes treated with SssI methyltransferase (New England Biolabs) was used as a positive control for methylated and unmethylated alleles of MGMT. PCR products were separated onto 3% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

Patient treatment. The treatment plan included radiotherapy to limited fields (2 Gy per fraction, once a day, 5 d a week, 60 Gy total dose) and concomitant temozolomide (75 mg per square meter of body surface area per day) for 7 d a week from the first to the last day of radiotherapy followed by five cycles of adjuvant temozolomide (at 200 mg per square meter of body surface area on days 1 to 5) given at 4-wk intervals. 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, if a new lesion was evident on axial contrast-enhanced T1-weighted magnetic resonance imaging scan, or if the patient's neurologic condition worsened and required an increased dose of steroids (23).

In vitro and in vivo glioblastoma stem cell analysis. Glioblastoma stem cells were isolated from 2.5 to 5 g surgical specimens through mechanical dissociation of the tumor tissue and cultured at clonal density in a serum-free medium supplemented with EGF and basic fibroblast growth factor. This procedure has been used extensively for normal and tumoral neural tissues to obtain the formation of exponentially growing neurospheres generated by single clones that

maintain an undifferentiated state (1, 3). CSC growth potential was evaluated as the time required for dissociated tumor specimens to generate 3×10^6 cells as tumorigenic glioblastoma spheres. To determine the expression of CD133, glioblastoma spheres were dissociated, labeled with CD133/1-PE antibody (Miltenyi Biotec), and analyzed by flow cytometry using a FACScan (Becton Dickinson). Differentiation assays were done after plating CSCs for 14 d on a poly-lysine-coated round glass coverslips in the absence of EGF and fibroblast growth factor and in the presence of 5% serum. For immunostaining of undifferentiated or differentiated CSCs, cells were fixed with 4% paraformaldehyde and stained with antibodies directed against CD133/1 (Miltenyi Biotec), nestin (Santa Cruz Biotechnology), GFAP (Dako), and β -tubulin III (Chemicon). Appropriate secondary antibodies were used. In this condition, the vast majority of neurosphere-derived cells express nestin or CD133 but not astrocytic or neuronal markers. To confirm the tumorigenic activity of glioblastoma CSCs, 10^5 neurosphere cells resuspended in 4 μ L of serum-free DMEM were implanted either s.c. in HDS-athymic nude mice (Harlan) or i.c. into severe combined immunodeficient mice (Charles River). Generation of CSCs was defined by the following criteria (2, 3): formation of primary spheres *in vitro*, capacity of self-renewal on clonogenic and population analysis, ability to coexpress astrocytic as well as neuronal phenotypic markers under serum stimulation *in vitro*, and generation of tumors in immunodeficient mice.

Statistical analysis. Continuous variables were compared with the use of Wilcoxon two-sample test. Contingency tables were analyzed by χ^2 and Fisher's exact test. Progression-free and overall survival curves

were estimated by the Kaplan-Meier method and compared with use of the two-sided log-rank test. The Cox proportional hazards model was fitted to assess the prognostic value of the CSC generation, CD133 expression, CD133/Ki67 coexpression, and potential prognostic factors (24, 25) by generating three different models that considered both the overall and the progression-free survival. Correlation analysis between variables was done before including them into each model. The results are reported as two-sided *P* values with 95% confidence intervals (95% CI). Correlation analysis was done by using the Spearman's rank correlation coefficient. Analyses were done with the use of Statistical Analysis System software (SAS Institute, Inc.).

Results

The major clinical and biological characteristics of the 44 consecutive glioblastoma patients included in the study are shown in Supplementary Tables S1 and S2. All the samples were processed for sphere generation assay and analyzed by immunohistochemistry and immunofluorescence. Tumor-derived neurospheres were subsequently evaluated for CD133 expression, *in vitro* differentiation potential, and *in vivo* tumorigenic activity (Fig. 1; Supplementary Fig. S1). Fourteen of the 44 glioblastomas (32%) generated CSCs (Supplementary Table S2). In 13 patients, we analyzed multiple specimens from different areas of the tumor. All the samples analyzed from each

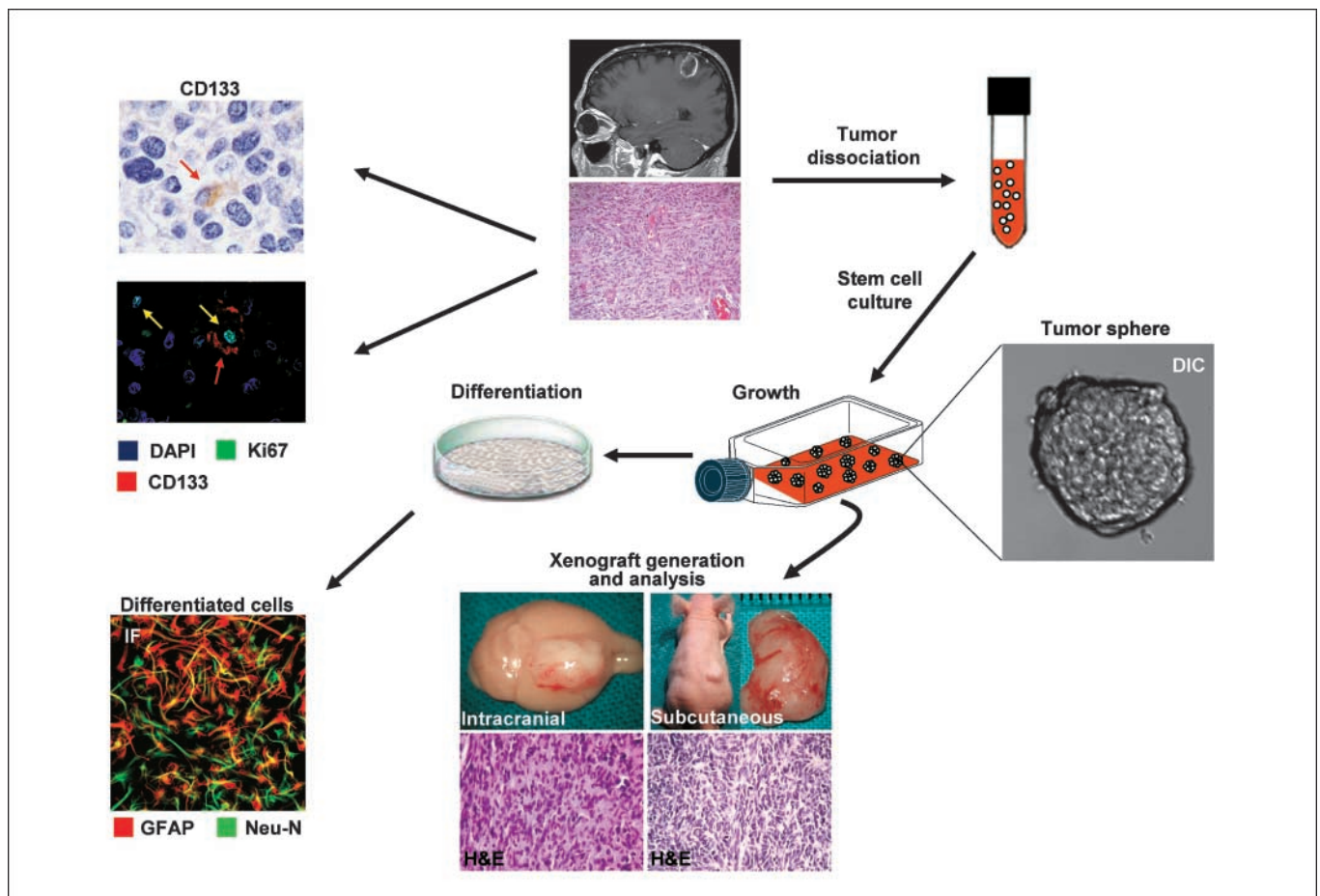


Fig. 1. Isolation and characterization of glioblastoma stem cells. Samples are analyzed by H&E staining, immunohistochemistry, and immunofluorescence. Red arrows, CD133⁺ cells; yellow arrows, Ki67⁺ cells. Heterotopic and orthotopic xenografts resemble the original tumor. Glioblastoma stem cells can be induced to differentiate and express both astrocytic (*GFAP*) and neuronal (*Neu-N*) markers, as visualized by immunofluorescence. DAPI, 4',6-diamidino-2-phenylindole.

Table 1. CSC analysis and patient survival according to CSC generation, CD133, and CD133⁺/Ki67⁻

Outcome variable	No. CSC generation (n = 30)	CSC generation (n = 14)	CD133 < 2% (n = 25)	CD133 ≥ 2% (n = 19)	CD133 ⁺ /Ki67 ⁻ (n = 34)	CD133 ⁺ /Ki67 ⁺ (n = 10)
Overall survival						
Median duration (mo)	14.0 (11.0-18.0)	8.0 (4.0-11.5)	14.0 (11.0-18.0)	10.5 (7.5-12.5)	12.25 (11.0-16.0)	6.75 (3.0-14.0)
Rate at 1 y (%)	56.7 (38.9-74.4)	21.4 (5.2-44.8)	56 (34.8-72.7)	31.6 (10.7-52.5)	50.0 (33.2-66.8)	30.0 (1.6-58.4)
HR for death	1.00	3.6 (1.7-7.4)	1.00	2.2 (1.1-4.2)	1.00	2.7 (1.2-5.9)
Progression-free survival						
Median duration (mo)	9.0 (7.0-12.0)	3.5 (2.0-6.0)	10.0 (6.0-12.0)	5.0 (3.0-7.0)	8.0 (6.0-11.0)	2.5 (1.0-6.0)
Rate at 6 mo (%)	70 (53.6-86.4)	14.2 (0-32.6)	68.0 (49.7-86.3)	31.6 (10.7-52.5)	61.7 (45.4-78)	20.0 (0-44.8)
HR for progression and death	1.00	3.4 (1.7-6.8)	1.00	2.1 (1.1-4.0)	1.00	3.1 (1.4-6.5)

NOTE: Numbers in parenthesis are 95% CI.

single tumor gave equal results. All the long-term CSC lines generated from the 14 tumors were tumorigenic in immunocompromised mice. Tumor xenografts showed histologic features that recapitulated the cytoarchitecture of the parental tumor, including necrosis and perinecrotic palisades. In accordance with their multipotent nature, these CSC lines were able to produce a progeny of neural cells with glial morphology

and expressing astrocytic and neuronal markers. In the parental tumors, median CD133 expression of glioblastoma cells was 1.0% (range, 0.5-10.0%), with a mean of 2.2 ± 2.3% (Supplementary Fig. S2). With similar procedures, the percentage of CD133⁺ cells in control brain tissues was consistently lower than 0.01%. Double immunofluorescence analysis revealed that 10 of 44 patients coexpressed CD133 and Ki67,

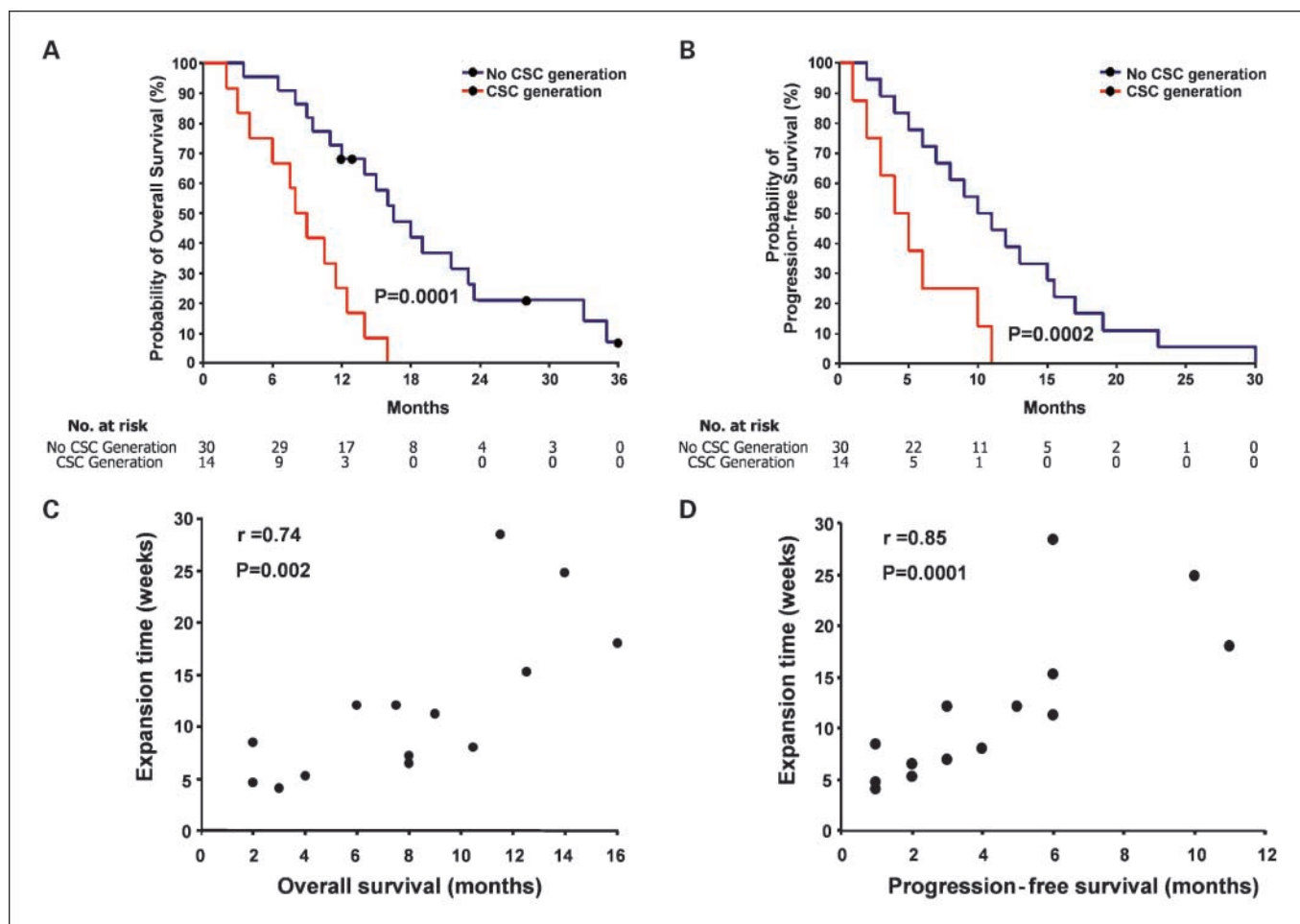


Fig. 2. Kaplan-Meier estimates of overall and progression-free survival according to CSC generation and Spearman's rank correlation analysis between CSC growth and overall or progression-free survival. Probability of overall (A) and progression-free (B) survival in patients with tumors generating or nongenerating CSCs. Correlation between time required for CSC expansion and overall (C) or progression-free (D) survival.

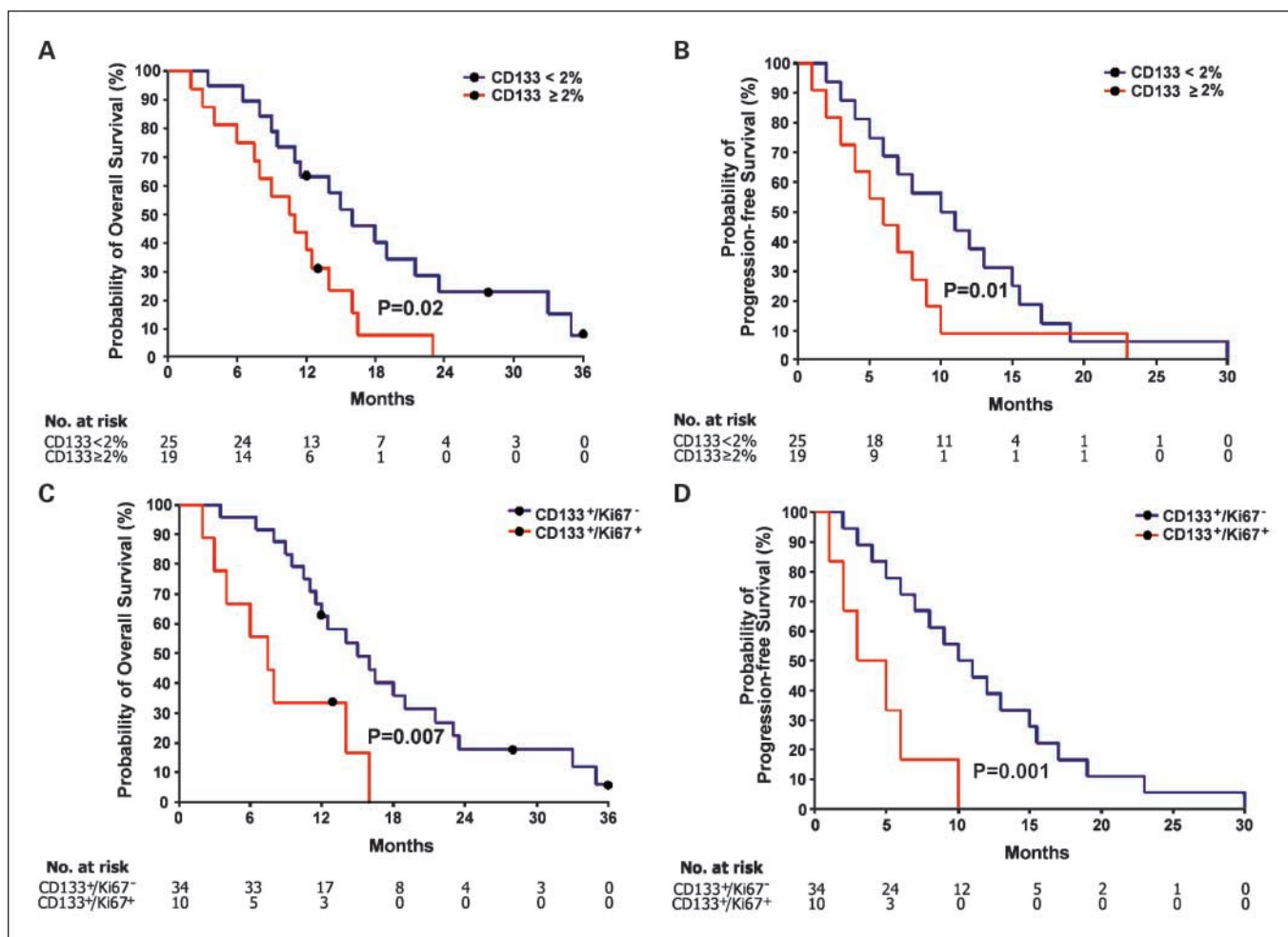


Fig. 3. Kaplan-Meier estimates of overall and progression-free survival according to CD133 expression and coexpression of CD133 and Ki67. Probability of overall and progression-free survival stratified by CD133 expression (A and B) or by the presence of CD133⁺/Ki67⁺ cells (C and D).

ranging from 0.5% to 3% (Supplementary Fig. S2). A higher CD133 expression significantly associated with tumors generating CSCs ($P = 0.006$, Wilcoxon two-sample test), and a significant correlation was found between the CD133/Ki67 coexpression and the generation of CSCs; 8 of the 10 patients with tumors coexpressing both CD133 and Ki67 generated CSCs *in vitro* ($P = 0.0006$; Supplementary Table S2).

In univariate analyses, the generation of CSCs ($P = 0.0005$), CD133/Ki67 coexpression ($P = 0.01$), partial surgery ($P = 0.02$), and symptom duration ≤ 3 months before the beginning of the treatment ($P = 0.01$) were associated with a higher risk of death. There was not a direct correlation between the absolute percentage of CD133⁺ cells and patient survival ($P = 0.11$). However, CD133 expression was significantly associated with overall survival of patients ($P = 0.02$) when a threshold of 2% of CD133⁺ cells was considered based on the mean CD133 expression observed in all the series. Hazard ratio (HR), median survival, and survival rate for the CSC-related factors are reported in Table 1.

In our series, Karnofsky performance status and Ki67 expression were not significantly associated with survival. Such a lack of prognostic value seems to depend on the presence of patients with long-term survival. For example, three patients

showed low Karnofsky performance status and long overall survival (cases no. 7, 11, and 18 in Supplementary Table S1). All of them had their tumor located in the parietal lobe; two of them were secondary glioblastoma carrying neurologic disorders from previous surgery that affected their Karnofsky performance status. Similarly, Ki67 lacked prognostic value due to four patients who survived >28 months and whose tumors showed a wide range of cell proliferation (cases no. 4, 7, 8, and 17 in Supplementary Table S1). These patients were also younger relative to mean age value; two of them were secondary glioblastoma.

The median overall survival among patients with tumors generating CSCs was 8 months (95% CI, 4.0-11.5) compared with 14 months (95% CI, 11.0-18.0) among those without generation of CSCs ($P = 0.0001$; Table 1; Fig. 2A). The median progression-free survival was 3.5 months for glioblastoma generating CSCs and 9 months for glioblastoma not generating CSCs ($P = 0.0002$; Table 1; Fig. 2B). The analysis of CSC expansion from the time of tumor dissociation allowed us to evaluate the possible connection between CSC growth and the prognosis of the 14 patients whose tumors were able to generate CSCs *in vitro*. Such analysis indicated a strong correlation between the time required for cell expansion and

both overall ($P = 0.002$) and progression-free ($P = 0.0001$) survival (Fig. 2C and D), suggesting that a higher proliferative activity of tumorigenic cells is associated with a poorer prognosis. All patients included in these correlation analyses experienced the survival or disease-free survival event.

Patients having tumors with $\geq 2\%$ CD133⁺ cells had a median overall survival of 10.5 months (95% CI, 7.5-12.5) compared with 14 months (95% CI, 11.0-18.0) of those with $< 2\%$ CD133 cells ($P = 0.02$; Fig. 3A). The median progression-free survival was 5 months for glioblastoma with $\geq 2\%$ CD133 cells and 10 months for glioblastoma with $< 2\%$ CD133 cells ($P = 0.01$; Fig. 3B). Although eight tumors expressed 2% CD133⁺ cells, a similar inverse correlation between CD133 expression and survival was observed by comparing glioblastoma patients with $> 2\%$ and $\leq 2\%$ CD133⁺ cells ($P = 0.03$).

The expression of Ki67 on CD133⁺ cells was associated with a very poor overall and progression-free survival (Fig. 3C and D; Table 1). Patients having tumors expressing CD133⁺/Ki67⁺ cells had a median overall survival of 6.75 months (95% CI, 3.0-14.0) compared with 12.25 months (95% CI, 11.0-16.0) of those without proliferating CD133⁺ cells ($P = 0.007$; Table 1; Fig. 3C). The median progression-free survival was 2.5 months for glioblastomas with CD133/Ki67 coexpression and 8 months for glioblastomas without CD133⁺/Ki67⁺ cells ($P = 0.001$; Table 1; Fig. 3D). These observations confirmed that the fraction of CD133⁺/Ki67⁺ cells in the tumor lesion directly correlated with tumor recurrence and patient death.

By the multivariate analysis (Table 2), CSC generation, the expression of $\geq 2\%$ CD133⁺ cells, and CD133/Ki67 coexpression emerged as significant independent prognostic factors along with partial surgery and symptom duration. Age resulted as a significant favorable prognostic factor when analyzed together with the CSC generation or the CD133 expression but not when the same analysis was done with the CD133/Ki67 coexpression. For the CSC generation, the adjusted HR was

2.92 (95% CI, 1.37-6.2; $P = 0.005$) for death and 3.6 (95% CI, 1.69-7.68; $P = 0.0009$) for progression. For the CD133/Ki67 coexpression, the adjusted HR for death was 4.48 (95% CI, 1.68-11.9; $P = 0.003$), whereas the adjusted HR for progression was even higher (6.33; 95% CI, 2.48-16.2; $P = 0.0001$).

Discussion

In this study, the basic biological variables of glioblastoma CSCs revealed their potential prognostic value. Both the ability to grow *in vitro* and the presence of CD133⁺/Ki67⁺ cells in tumor lesions were independent prognostic factors of tumor recurrence and short survival.

Generation of *in vitro* growing CSCs capable of self-renewal, multipotency, and longevity was achievable from 32% of glioblastomas. The analysis of multiple tissue sampling suggests that the pathologic heterogeneity of the glioblastoma does not affect CSC generation, which can be regarded as an intrinsic feature of a subset of tumors, possibly related to the low requirements for survival and expansion of their tumorigenic cells. We found that patients whose tumors generated CSCs *in vitro* had an unfavorable outcome with significantly shorter overall and progression-free survival in comparison with patients with tumors not generating CSCs. Generation of CSCs was an independent prognostic factor. With the exception of CD133 expression and the presence of CD133⁺/Ki67⁺ cells, none of the clinical prognostic factors and biological markers of glioblastoma was significantly related to CSC generation.

In a recent retrospective study on brain gliomas of different WHO grades, Zeppernick et al. (15) showed that both the proportion of CD133⁺ cells and their topological organization in clusters were significant prognostic factors. More specifically, tumors containing $> 1\%$ CD133⁺ cells associated with significantly shorter progression-free and overall survival than tumors containing $\leq 1\%$ CD133⁺ cells (15). This report was the first

Table 2. Results of multivariate analyses with the Cox proportional hazard models

Variable	Overall survival		Progression-free survival	
	HR (95% CI)	P	HR (95% CI)	P
A				
CSC generation (vs no CSC generation)	2.92 (1.37-6.20)	0.005	3.60 (1.69-7.68)	0.0009
Symptom duration ≤ 3 mo (vs > 3 mo)	2.39 (1.01-5.66)	0.05	1.70 (0.80-3.64)	0.17
Partial surgery (vs complete surgery)	2.92 (1.35-6.33)	0.007	3.36 (1.56-7.23)	0.002
Age	1.05 (1.01-1.09)	0.02	1.04 (1.00-1.07)	0.05
MGMT status negative (vs positive)	1.04 (0.51-2.13)	0.92	1.00 (0.50-1.96)	0.99
p53 status positive (vs negative)	1.53 (0.60-3.90)	0.37	1.03 (0.44-2.43)	0.95
B				
CD133/Ki67 positive (vs negative)	4.48 (1.68-11.9)	0.003	6.33 (2.48-16.2)	0.0001
Symptom duration ≤ 3 mo (vs > 3 mo)	4.18 (1.67-10.4)	0.002	3.14 (1.38-7.16)	0.007
Partial surgery (vs complete surgery)	2.99 (1.34-6.65)	0.007	3.67 (1.67-8.05)	0.001
Age	1.03 (0.98-1.07)	0.23	1.02 (0.98-1.06)	0.30
MGMT status negative (vs positive)	1.04 (0.50-2.14)	0.92	0.94 (0.48-1.87)	0.87
p53 status positive (vs negative)	1.20 (0.48-3.02)	0.70	0.97 (0.43-2.18)	0.93
C				
CD133 $\geq 2\%$ (vs $< 2\%$)	2.22 (1.09-4.52)	0.03	2.10 (1.05-4.21)	0.04
Ki67 expression	1.05 (1.00-1.10)	0.03	1.05 (1.00-1.09)	0.03
Symptom duration ≤ 3 mo (vs > 3 mo)	2.57 (1.04-6.33)	0.04	1.61 (0.72-3.60)	0.25
Partial surgery (vs complete surgery)	3.07 (1.41-6.67)	0.005	2.73 (1.29-5.81)	0.009
Age	1.07 (1.02-1.12)	0.003	1.05 (1.01-1.09)	0.02
MGMT status negative (vs positive)	1.40 (0.66-2.97)	0.39	1.36 (0.67-2.73)	0.39
p53 status positive (vs negative)	1.65 (0.67-4.10)	0.28	1.25 (0.52-3.00)	0.62

demonstration in neural tumors that the analysis of a putative CSC marker could be prognostically relevant. However, because the vast majority of glioblastomas evaluated (45 of 47) contained >1% of CD133⁺ cells, the prognostic potential of CD133 analysis in grade IV tumors cannot be clearly established from this study.

In our cohort of glioblastoma patients, the presence of $\geq 2\%$ CD133⁺ cells was associated with a significant poorer prognosis, suggesting that an increased number of cancer stem and progenitor cells may negatively affect the disease progression. On the other hand, the absence of a direct correlation in univariate analysis between the number of CD133⁺ cells and the clinical outcome of the patients suggests that the relative presence of glioblastoma stem and progenitor cells is prognostically relevant only if these cells have a detectable proliferation activity. This hypothesis is in line with recent microarray data indicating that CD133 mRNA is increased in glioma patients with poor prognosis and whose tumors express genes associated to cell proliferation (26). Our results suggest that the ability to grow *in vitro* characterized the most aggressive CSCs, which may be responsible for the faster disease progression observed in such group of glioblastoma patients. Among those patients whose tumors generated CSCs *in vitro*, we observed a dramatic correlation between the time required for CSC expansion and patient survival. The strict correlation between CSC expansion and tumor growth suggests that fast-growing CSCs maintain *in vitro* the aggressive features responsible for disease recurrence or progression. A further confirmation of these data comes from the particularly negative prognosis of patients whose tumors carried proliferating CD133⁺ cells. The presence of CD133⁺/Ki67⁺ cells in 22.7% of tumors was a considerable negative prognostic factor for patient survival, extremely significant to identify patients at risk of disease progression, even within the group of tumors generating CSCs. Thus, although the *in vitro* generation of glioma neurospheres might not be easily transferred to clinical laboratories, the detection of CD133⁺/Ki67⁺ cells by immunohistochemistry or immunofluorescence may be of particular interest in the clinical setting. The high prognostic potential of the detection of CD133⁺/Ki67⁺ cells is particularly relevant because in our study Ki67 expression does not directly correlate with poor survival. As pointed out in Results, the lack of prognostic value of Ki67 depends on the values obtained in long survival patients. Although the relationship between Ki67 and overall survival in glioblastoma has been shown by several groups, such a relationship becomes less straightforward for the long-term glioblastoma survivors (27).

The DNA repair protein MGMT seems to play an important role in the resistance of glioblastoma cells to alkylating agents, such as nitrosoureas and temozolomide (22, 28). Transcriptional silencing of the MGMT gene by promoter hypermethylation is seen in $\sim 50\%$ of glioblastomas and has been

linked to prolonged progression-free and overall survival in glioblastoma patients treated with alkylating agents (22, 28). It has been hypothesized that resistance to temozolomide may be related to the CD133-expressing cell fraction of glioblastoma (12). This assumption stems from *in vitro* data showing that CD133⁺ cells, which were collected by fluorescence-activated cell sorting from primary glioblastoma cultures, exhibit levels of MGMT mRNA 32.4 times higher than autologous CD133⁻ cells (12). In the *in vivo* condition, however, where the CD133⁺ cells represent a relatively small population and where several other factors affect the progression of the disease, a direct relationship between CD133 expression in the tumor tissue and resistance to temozolomide may be more difficult to show.

In our study, all patients were treated with temozolomide during and after radiotherapy. Nevertheless, MGMT promoter methylation did not associate with a favorable outcome as recently described (28). Although the recursive partitioning analysis classification of our patients (14% class III, 61% class IV, and 25% class V) is very similar to that of the entire patient population of the European Organization for Research and Treatment of Cancer/National Cancer Institute of Canada trial, the subgroup of patients analyzed for MGMT promoter methylation and prognosis by Hegi and colleagues differs from our series both for performance status and for extent of surgical resection (29). In our study, the patient population is closer to those included in other reports where a significant benefit of MGMT methylation in glioblastoma patients treated with alkylating agents was not found (30, 31).

In conclusion, the ability to generate CSCs *in vitro* may distinguish a severe prognostic subtype of glioblastoma, which can be further dissected based on the growth potential of these cells *in vitro*. Moreover, the detection of CD133⁺/Ki67⁺ cells in tumor lesions may represent a valuable and informative analysis on the disease outcome. In addition to its unfavorable prognostic value, generation of CSCs from this very aggressive subtype of tumors may be crucial for the development of new and effective therapies. Although glioblastoma stem cells seem to be resistant to chemotherapy (16) and radiotherapy (13), the availability of considerable numbers of these tumorigenic cells may allow high-throughput analyses of new compounds for the development of stem cell-targeted therapeutic strategies to improve the clinical outcome of these patients (32).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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