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Research Article

Glioblastoma Topography and Stem-Cell Markers Related with Survival

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Abstract

Introduction: Glioblastoma multiform is a malignant neoplasm of the central nervous system where preoperative variables, topography and cell lineage markers are associated with poor response to treatment and progression; however, no prospective studies have associated all these variables with survival.

Methods: 17 patients with newly glioblastoma who underwent to tumour resection were characterized. Pre and postoperative magnetic resonance image was performed to evaluate tumour topography, percentage of resection, and residual tumour. Percentage of medium fluorescence index for CD133+ and Glial Fibrilar Acid Protein (GFAP) was measured by flow cytometry. Nestin, tubulin, cathepsin B, GFAP and cystatin expressions were determined by real time RT-PCR.

Results: The most common tumor location was periventricular zone in 82%. The median Percentage of Resection (PR) and Residual Volume (RV) were 88.8% [78.4 - 99.2 (95%CI)] and 10 cm^3 [0.5 - 19.4 (95%CI)], respectively. From patients, who achieved Gross Total Resection (GTR) (PR >95% and RV <2cm^3) (12, patients, 70.5%), the median survival was 14 months in them who underwent adjuvant chemo- and radiotherapy. CD133, cathepsin B, GFAP and nestin were significantly associated with poor survival.

Conclusions: The overexpression of CD133, nestin, GFAP and enzyme cathepsin B located in or around of subventricular zones I and II are a prognostic factors to disease progression and poor clinical outcome.

Keywords: Glioma stem cells, Glioblastoma, Survival, Tumor topography.

Abbreviations

GBM	:	Glioblastoma
WHO	:	World Health Organization
CSCs	:	Cancer stem cells
GSCs	:	Glioma Stem-Like Cells
GFAP	:	Glial Fibrillary Protein
NG2	:	Neural/Glial 2
NINN	:	National Institute of Neurology and Neurosurgery
GTR	:	Gross Total Resection
TV	:	Tumor Volume
RV	:	Volume
PR	:	Percentage of Resection
EDTA	:	Trypsin-Ethylenediaminetetraacetic Acid
APC	:	Allophicocianin
FITC	:	Fluorescein Isothiocyanate
MFI	:	Mean Fluorescence Intensity
PCR	:	Quantitative Polymerase Chain Reaction
Ct	:	Cycle Threshold
RV	:	Residual Volume
PR	:	Percentage of Resection
SVZ	:	Sub-ventricular Zone

Introduction

Malignant brain tumors are among the most devastating forms of human cancer [1]. Glioblastoma (GBM), classified by the World Health Organization (WHO) as glioma grade IV [2], is the most aggressive and frequent primary brain tumor in adults, representing nearly 80% [1]. Despite radical treatment involving surgery, concomitant chemotherapy plus radiotherapy, in 90% of cases the median survival from GBM is 14.6 months [3-5] and less than 10% of patients survive more than 5 years [6].

GBM is characterized by an environment of non-differentiated astrocytic cells, necrosis, and vascular proliferation. In recent years, several studies described that within the environment of GBM there are small cell populations, named Cancer Stem Cells (CSCs), with tumorigenic propensity, which also share characteristics of stem cells such as self-renewal, multi lineage, unlimited proliferation potential and dedifferentiation [7-10]. Subpopulation of CSCs within the GBM have been named Glioma Stem-Like Cells (GSCs) and corresponds from 2 to 38% of cell population [7]. GSC can accumulate diverse mutations in their proto-oncogenes during lifespan, leading to resistant towards conventional treatment due to enhanced DNA repair. Besides, it is able to induce resistance to ionizing radiation, invasive growth, relapse and consequently in disease progression [11-13].

Some stem cell markers are used to isolate and characterize the GSCs subpopulation, and used as prognosis factors in patients [14,15]. The transmembrane protein CD133 is a stem cell marker that has been associated with aggressive biological behavior, poor prognosis and high rates of recurrence in various types of solid tumors located in the brain, prostate, liver, lung and colon [16-18]. Nestin, other stem cell marker, and Ki67+ (proliferation cell marker), has been associated also with low survival in GBM. This protein is up-regulated in cancer and promotes CSCs, tumor cell proliferation, migration and invasion [19]. Glial Fibrillary Protein (GFAP) is a major intermediate filament protein of mature astrocytes, its expression is increased following brain damage or during degeneration of the central nervous system, being directly downregulated during the differentiation of GSCs via the binding of Pax3 to the promoter region of GFAP [19-21]. Enzymes with proteolytic activity, called lysosomal cathepsins belong to a family with 3 subgroups (Cat B, Cat L and Cat S) are overexpressed in GBM [11]. The presence of Cat B and a chondroitin sulfate proteoglycan called neural/glial 2 (NG2), have been associated with both, local aggressiveness and tumor invasion [11,22].

GSCs show a co-expression of glial, neuronal and mesenchymal markers of multi lineage such as normal neural progenitors, the aim of this study was evaluate the clinical variables, tumor topography, and patient survival with glioma stem cells markers.

Material and Methods

Patient Selection

This study included 30 brain tumor samples obtained from adult patients (>18 years) with suspicion of high grade glioma whom underwent surgery at the National Institute of Neurology and Neurosurgery (NINN) in Mexico, during the study period from November 2012 to June 2013. All patients underwent surgical resection of the tumor and biopsies where collected for pathological assessment. In 17 of them the diagnosis of GBM was confirmed and the rest of them were lower-grade astrocytomas according to WHO classification criteria [2]. Patients with prior biopsy/surgery or previous resection of a lower-grade gliomas were excluded from the study. To determine and compare the percentage of stem cells, as well as markers associated with tumor´s invasiveness, we used as a control group, biopsies coming from the hippocampus,

a very well-known area rich in neural stem cells, obtained after surgery of 5 epileptic patients. The protocol was approved by the Bioethics Committee (CB/099/12) and the Institutional Review Board (Number 17/12) of the NINN.

Clinical Evaluation

Clinical information, location and tumor extent, surgical records, surgical complications, radiation and/or chemotherapy, hospital course, postoperative neurological function, Karnofsky Performance Status (KPS) and survival, were obtained prospectively from the clinical and radiological records. The use of fluorescent guide surgery, intraoperative mapping, motor and somatosensory evoked potentials, and surgical navigation were used according to neurosurgical guidelines. The use of postoperative radiation and/or chemotherapy was determined by a multidisciplinary team.

Volumetric Analysis

Pre- and postoperative MRIs were obtained for each patient. Patients without studies were excluded. All cases included were considered as amenable to Gross Total Resection (GTR) prior to surgery. Therefore, infra tentorial tumors, and multifocal or multi centric lesions as well as tumors with either deep-seated, located in eloquent regions, and/or spanned both hemispheres were all excluded. Preoperative tumor volume was measured by T1- weighted gadolinium-enhanced MRI, using OsiriX software (OsiriX, Los Angeles, California, USA).

The area of contrast enhancement was measured for each axial section. Tumor Volume (TV) was calculated by using the sum of all areas in axial sections. The Residual Volume (RV) was calculated similarly but evaluating postoperatively on MRIs obtained when possible within 48 hours of surgery. The Percentage of Resection (PR) was calculated using the following formula: (TV-RV)/TV. In this analysis, two clinicians blinded to each other's results and to the patient's outcomes measured the TV and RV of 17 mixed patients in this database. A comparison between T1-weighted gadolinium-enhanced and non-enhanced MRIs was done to exclude postoperative blood products from the RV calculations [23].

Flow Cytometry of CD133 and GFAP from Tumor Samples

Approximately, $1cm^3$ of brain tumor was washed with saline solution until blood was eliminated, after that; tumor samples were dissociated mechanically dissociated with a pipette using trypsin-Ethylene Diamine Tetra Acetic Acid (EDTA). Cell homogenates were maintained in MACS Neuro Medium (Miltenyi Biotech, Germany) supplemented with B27 supplement (Invitrogen), 20 ng/mL Fibroblast Growth Factor basic (FGF-b) and 20 ng/mL Epidermal Growth Factor (EGF) (Miltenyi Biotech, Germany) and antibiotic-antimycotic solution (Bio West, Nuaillé, France). Afterwards, we used monoclonal antibodies coupled to Allophicocianin (APC) or Fluorescein Isothiocyanate (FITC) in order to determine CD133+ cell percentage (Miltenyi Biotech, Germany) or GFAP expression (Sigma Aldrich, USA). Briefly, $1x10^6$ cells were incubated in the dark for 30 min with 10 µL of the corresponding monoclonal antibody and washed twice with 0.1 M PBS (pH 7.2), 0.1 % bovine serum albumin (BSA) and 0.1 % NaN_3. Cells were then fixed in 1 % of paraformaldehyde solution and stored at 4˚C until examination by flow cytometry (FACSCalibur, Becton Dickinson, USA), using the Cell Quest software, 10,000 total events were analyzed. The percentage of positive cells and Mean Fluorescence Intensity (MFI) from each sample were determined using Flowjo V10 (Oregon, USA).

Cell culture

Tumor cell line A172 was purchased from the ATCC (American Tissue Culture Collection, Rockville, MD, USA). Cells were cultured under sterile conditions at 37˚C in a humid environment with 5 % of CO_2 in Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (GIBCO BRL, Grand Island, NY, USA), 4 mM glutamine, 100 U/ml penicillin, and 100 mg/ ml streptomycin.

Quantitative Polymerase Chain Reaction

DNA from $10x10^6$ A172 cells was extracted with a mix of phenol, chloroform, and isoamyl alcohol, and precipitated with isopropanol; DNA was dried at room temperature and dissolved in 100µL of sterile water. Serial dilutions were made from this sample to obtain a standard curve to estimate the amount of mRNA for nestin, β3 tubulin, cathepsin B, and cystatin C from the different GBM samples. Relative quantifications from GBM samples against controls were made using ΔΔCt method. Total RNA from samples was extracted by phenol/chloroform method using trizol reagent (Invitrogen, USA). Quantitative Polymerase Chain Reaction (PCR) was performed using EXPRESS One-Step Superscript® qRT-PCR Kit, universal (Invitrogen, USA). The TaqMan probe for Nestin (Hs04187831-g1), β3-Tubulin (Hs00801390-s1), Cathepsin B (Hs00947433-m1), Cystatin C (Hs00264679-m1 CST3), and GADPH (Hs03929097-g1) were acquired from Applied Biosystem (USA). Each 20 µl of PCR mixture contained DNA from $1x10^6$ GBM cells in 5 µL of distilled water, 1.25 µL of Taqman probe, 0.04 µL of reference passive ROX, 10 µL EXPRESS SuperScript® qPCR SuperMix Universal, 2 µL EXPRESS SuperScript® Mix for One-Step qPCR and DEPC-treated water to 20 µL. Human endogenous control (GADPH) was used as internal control. PCR mixtures in 96-well plates were firstly incubated at 50˚C for 15 minutes (cDNA synthesis) followed by 95˚C for 20-second, and 40 cycles of 95˚C for 15 seconds and 60˚C for 1 minute using an ABI PRISM 7500 real-time PCR system (Applied Biosystem, USA). Each sample was analyzed by triplicate and the Cycle Threshold

(Ct) was calculated.

Statistical Analysis

For the descriptive analysis, continuous variables were summarized as means and standard deviations and discrete variables as frequencies and proportions. The independent samples Mann-Whitney U test was used to check the differences of the expression of the proteins when considering the presence or absence of the tumor. Cox Proportional Hazards Regression models and the Log Rank test (Mantel-Cox) were used to evaluate the impact of variables with the survival. A p-value less than or equal to $p \leq 0.05$ was considered for the significance. SPSS v20 (SPSS, Inc., Chicago, IL, USA) and R (R: A Language and Environment for Statistical Computing, R Core Team, Vienna, Austria) were employed for the data analysis.

Results

Clinical and Radiological Data

Between November 2012 to June 2013, 30 surgeries were performed in patients diagnosed as probable GBM according with pre-operative imaging assessment as safely capable of GTR; diagnosis of GBM was confirmed in 17 of them, which were included in the present study.

Pre and postoperative characteristics of patients are summarized in Table 1. Median age of patients was 57 years old (38-75) with 71 % of males. The major co-morbidity was hypertension in 24 % (4 patients) of them. Motor deficit was present in 71 % (12) of them preoperatively. No differences between pre and postoperative KPS were found [78 vs 68]. The most common tumor location was periventricular zone, being the zone l and lll the most common sites of location of the tumors (13 vs 3 cases respectively) (Table 1 and Figure 1).

Characteristics		Number	Percentage
Race	Hispanic	17	100%
Gender	Male	12	70.50%
	Female	5	29.50%
Age (years)	Mean*	56.7	(38-75)
Family history	Cardiopaties	2	11.70%
	Neoplasias	0	0%
Co-morbidities	HTN	4	23.50%
	DM	2	11.70%
	CAD	1	5.80%
	COPD	1	5.80%
Preoperative variables	Motor deficit	12	70.50%
	Oxford scale** 4/5	10	83.30%
	Oxford scale** 3/5	2	16.60%
	Headache	10	58.80%
	Confusion	10	58.80%
	Gait/balance	7	41.70%
	Sensory deficit	7	41.70%
	Seizures	6	35.20%
	Nausea	6	35.20%
	Language deficit	3	17.60%
	Visual deficit	2	11.70%
	Diplopia	1	5.80%
KPS*	Pre-operative	77.6	(70-90)
	Post-operative	67.6	(40-90)
Radiographic characteristic	Location***		
	Zone I	13	76.40%
	Zone II	1	5.80%
	Zone III	3	17.60%
	Zone IV	0	0%
Volumetric analysis (cm^3)	Tumor volume	81.6 cm^3	(59.2-104.1)
	Residual volume	10 cm^3	(0.5-19.4)
	Percentage of resection	88.8%	(78.4 -99.2)
Neurological complications	Stroke (MCAT)	1	5.80%
	Subdural hematoma	1	5.80%
	Delirium	1	5.80%
	Edema	1	5.80%
New neurological deficits	Motor deficit	1	5.80%
	Sensory deficit	1	5.80%
	Language deficit	1	5.80%
Length of hospital stay (days)	Patients without neurological complications (n=13)	4.5	(3-8)
Adjuvant therapy	Chemotherapy	9	52.90%
	Radiotherapy	9	52.90%

* Median (interquartile range).
** Oxford scale, muscle strength grading scale (0-5).
*** MRI-based classification of GBM according to Lim, 2007: Grupo I : SVZ+, Cortical+; Grupo II : SVZ+, Cortical–; Grupo III: SVZ–, Cortical+; Grupo IV: SVZ–, Cortical–.
^ Excluding four patients with the neurological complications mentioned above.
Afib, atrial fibrillation; CABG/stent, coronary artery bypass graft/stent; CAD, coronary artery disease; COPD, chronic obstructive pulmonary disease; DVT/PE, deep vein thrombosis/pulmonary embolism, DM, diabetes mellitus; KPS, Karnofsky performance scale; MCAT, middle cerebral artery territory; SVZ, subventricular zone.

Table 1: Pre-, peri-, and postoperative characteristics of patients undergoing surgery of a newly GBM capable to gross total resection from November 2012 to June of 2013 in the National Institute of Neurology and Neurosurgery from Mexico.

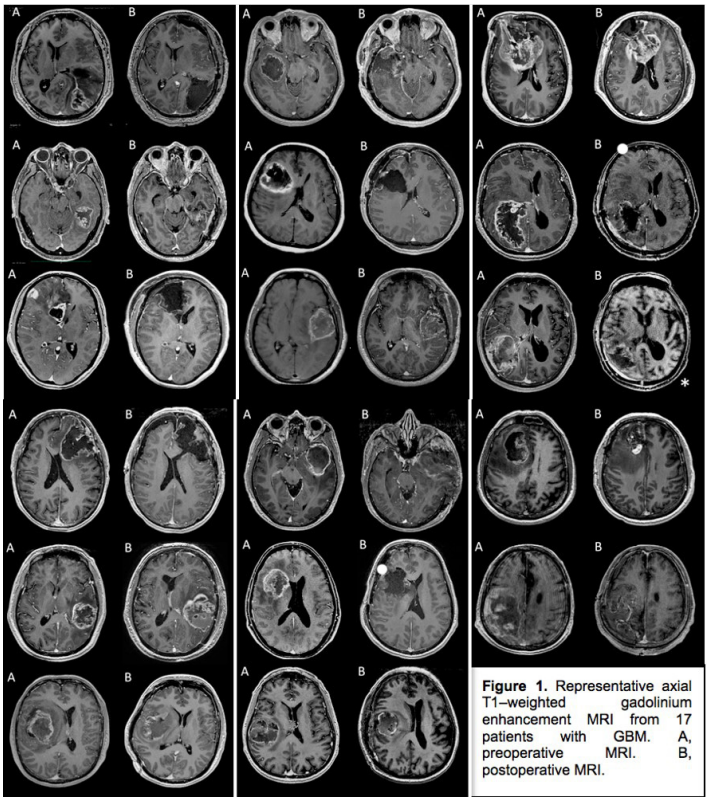


Figure 1: Representative axial T1-weighted gadolinium enhancement MRI from patients with newly GBM. A. preoperative MRI. B, Postoperative MRI.

The median preoperative contrast-enhancing TV was 81.6 cm^3 [59.2-104.1(95%CI)], and the range was 24.1-175.9 cm^3. After surgery, the median contrast-enhancing Residual Volume (RV) was 10 cm^3 [0.5-19.4 (95%CI)], and the range was 0.2-58.1 cm^3 (Figure 1). Median of percentage of resection (PR) was 88.8% [78.4 -99.2 (95%CI)]. From them, 12 patients (70.5%) achieved GTR (PR>95% and RV<2cm^3) with a median of percentage of resection of 98% and RV of 1.5cm^3. One patient had an irreversible neurological complication resulting in a deep hemiparesis associated with an ischemic event in the middle cerebral artery.

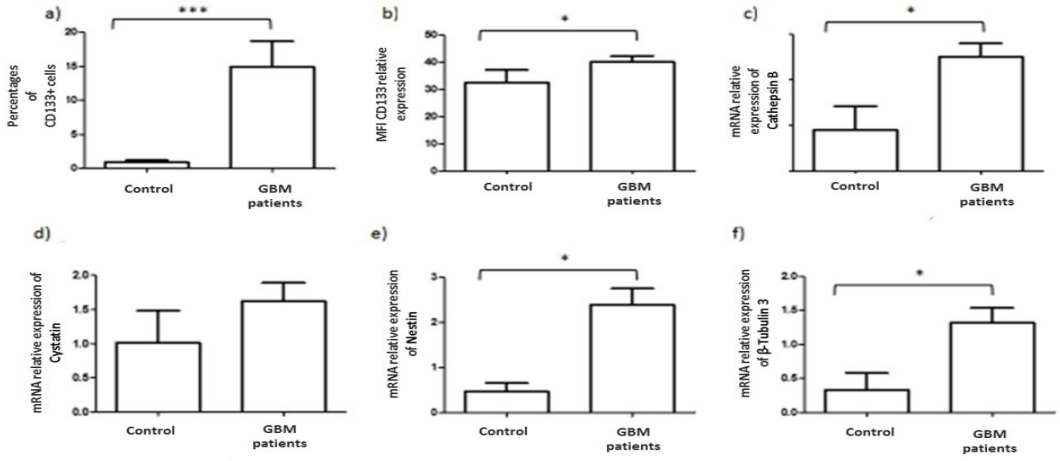
Overall survival of 8 months was observed in GBM patients; however, an improvement in survival of 9.4 months was observed in GBM patients with PR>95% and RV <2cm^3 (12 patients, 70.5%). From them, patients who also received adjuvant therapy, including chemotherapy with radiotherapy, had a median survival 14 months. Patients who did not undergo adjuvant therapy were because they were not considered candidates or because they refused additional therapy.

Inter-Observer Reliability

MRI was obtained within 48 hours after surgery in 12 patients. TV and RV were measured in all 17 where a interclass correlation coefficients of volumetric analysis were found in both TV=0.986 [0.903-0.996 (95%CI)] and RV=0.990 [0.973-0.996 (95%CI)].

Glioblastoma cell differentiation markers in non-tumor of tissue

The results showed that percentages of CD133, MFICD133, cathepsin B, cystatin, nestin and β3-tubulin expression varied from GBM patients and controls ($p < 0.05$), (Figure 2). Flow cytometry detection of CD133+ cells in tumoral tissue showed a high percentage (15±3.5%) compared with controls (1±0.6%) (Figure 2a), similar with prominin 1 expression in the MFI analysis (Figure 2b). Expression of cathepsin B showed two times more expression in tumoral tissue as compared with controls (p=0.03) (Figure 2c). No significant differences between controls and GBM were found in the mRNA expression of cystatin (Figure 2d). Three times more mRNA expression of nestin were found in GBM as compared with controls (p=0.01) (Figure 2e) and two times more mRNA expression of β3-tubulin in GBM against controls (p=0.01) (Figure 2f).



Figures 2(a-f): mRNA relative expression in GBM and controls. (a) Percentage of CD133+ cells in biopsies from GBM patients and controls (*p=0.002), (b) Mean Fluorescence Intensity (MFI) from CD133+ cells in GBM and controls, (c) Relative expression of cathepsin B from GBM and controls (*p=0.03), (d) Relative expression of cystatin from GBM as compared with controls, (e) Relative expression of nestin from GBM as compared with controls (*p= 0.01) and (f) Relative expression of β3-tubulin 3 from GBM as compared with controls (*p=0.01).

Correlation Between the Survival of Patients and the Percentages of CD133 cells and Nestin, GFAP, Cathepsin B, Cystatin and β3 Tubulin Expressions

The association of seven variables (CD133, MFICD133, cathepsin B, cystatin, nestin, GFAP and β3-tubulin) with the survival was analyzed in the group of 17 patients with the presence of GBM. We compared different cut-off of 10th and 90th percentiles of CD133, cathepsin B, cystatin, nestin and GFAP to predicted survival function (Figures 3 to 9). Overall survival was not associated with gender (p> 0.05), topography (p> 0.05) chemo-radio therapy (p> 0.05), age (p> .05) or KPS (p> 0.05) (data no shown).

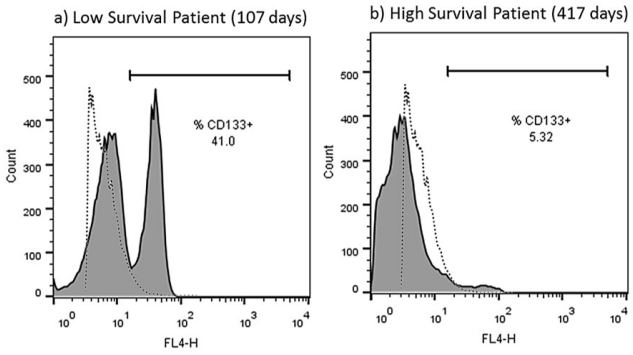


Figure 3: Percentage of CD133+ from GBM patients with low and high survival time. Representative histogram from two patients with GBM. Image shows (a) low survival patient (107 days) with 41% of CD133+ cells within the tumour and (b) a high survival (417 days) patient with 5% of CD133+ cells within the tumour. The dotted line show isotype control.

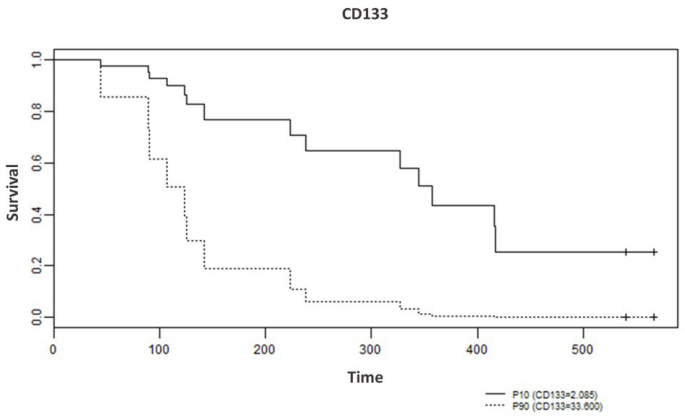


Figure 4: Figure shows the predicted survival function for the fitted Cox proportional hazards models when evaluating in the 10th and 90th percentiles, in the mean and in the median of the percent of CD133 within the tumor sample. Results show that CD133 was associated with survival time (p<0.05).

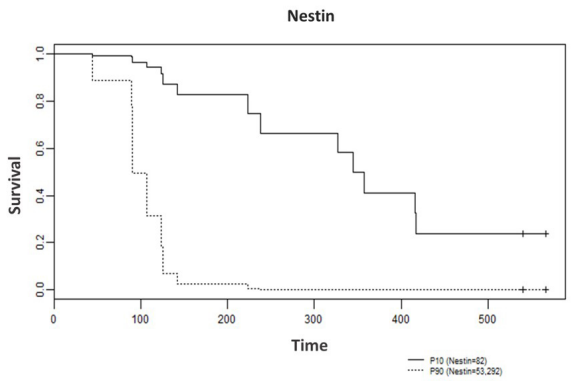


Figure 5: Predictive survival for the fitted Cox proportional hazards models when evaluating in the 10th and 90th percentiles, according to the mRNA expression of nestin. The results show that mRNA expression of nestin correlated negatively with survival time ($p < 0.05$).

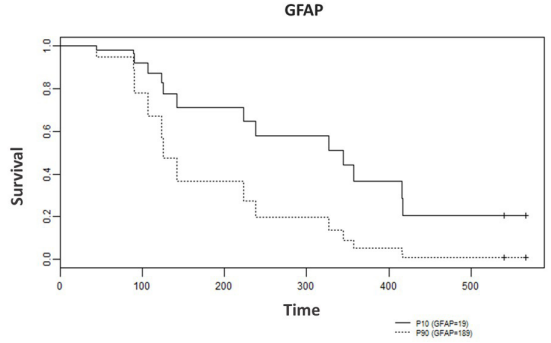


Figure 6: Predicted survivor for the fitted Cox proportional hazards models when evaluating in the 10th and 90th percentiles, of Glial Fibrilar Acid Protein (GFAP) contents in the tumor sample. The results show that high protein GFAP expression was associated with poor survival time ($p < 0.05$).

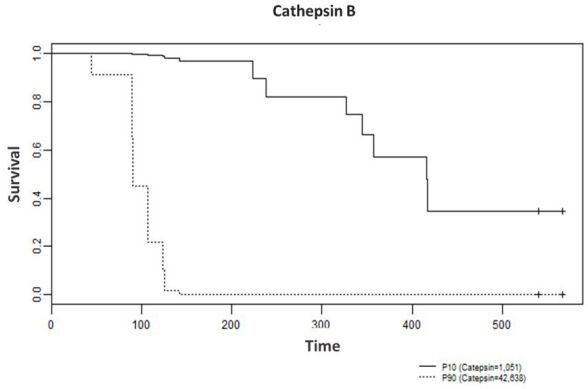


Figure 7: Predicted survival for the fitted Cox proportional hazards models when evaluating in the 10th and 90th percentiles, according to cathepsin B contents in the tumor sample. The results show that high mRNA cathepsin B expression was associated with poor survival time ($p < 0.05$).

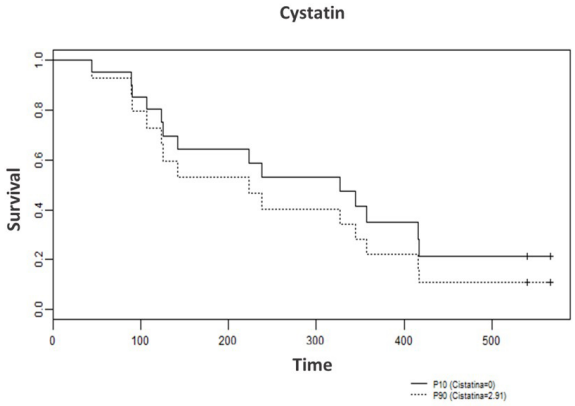


Figure 8: Predicted survival for the fitted Cox proportional hazards models when evaluating in the 10th and 90th percentiles, of mRNA expression of cystatin in the tumor sample. The results show that mRNA expression of cystatin was not associated with survival time ($p > 0.05$).

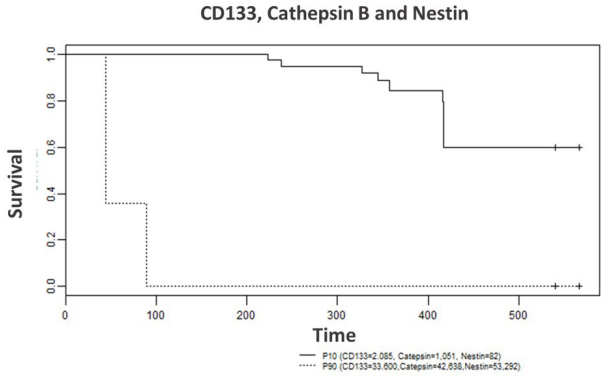


Figure 9: Cox Regression model considering the four variables: chemo-radio therapy, percentage of CD133+ cells, cathepsin B and nestin. Backward procedure was used to select a model. The final model includes three variables CD133, cathepsin B, and nestin when evaluating in the 10th and 90th percentiles, in the mean and in the median of the percentage of CD133+ cells, mRNA expression of cathepsin B and nestin in the tumor sample. These variables were significant when compared overall survival.

Percentage and MFI for prominin expression of CD133 positive cells were determinated by flow cytometry. The results showed a negative correlation between the percentage of CD133 positive cells and survival ($p=0.02$) (Figure 4), For example, percentage of CD133+ cells were higher (41%) in GBM patients with low survival (less than 200 days) as compared with high surviving patients with just 5% of CD 133+ (Figure 3). A percentage of CD133+ cells around 33% could predict low survival (less than 100 days with probability around 0.5) while a percentage around 2% predict until 3 times more survival in GBM patients. However, no correlation was observed in the expression of prominin protein ($p= 0.5$) (data no shown).

MFI for GFAP protein expression was determinate by flow cytometry. Patients with GFAP protein expression below than 19 MFI had around 3 times to increased survival (around 320 days with probability 0.5) than patients with expressions of 189 MFI (around 120 days). Those results showed a negative correlation between the GFAP protein expression and survival (p=0.01) (Figure 6)

On the other hand, lineage marker nestin mRNA expression showed a negative correlation with GBM patient survival (p=0.002). Patients with mRNA expression around 82 genomic equivalents showed 3.5 times more survival (around 350 days with probability around 0.5) than patients with expressions around 53000 genomic equivalents (100 days) (Figure 5). Patients who lived less than 150 days after surgery showed high levels of mRNA expression for cathepsin B as measured by quantitative real time PCR (Figure 7). Quantitatively, levels of 43 mRNA genome equivalents of cathepsin B correlated with a lower survival time (p=0.002). No correlation was found between the Cystatin (Figure 8) and β3-tubulin (data no shown) mRNA expression and GBM patient's survival (p=0.7 and p=0.6, respectively).

Discussion

Malignant tumor cells infiltrate from the primary tumour sites to the nearby healthy tissues. GBM has distinctive histopathological hallmarks including high cell density with poorly differentiation, with the frequent presence of pleomorphic astrocytic cells, marked nuclear atypia and brisk mitotic activity associated with intratumoral necrosis, angiogenesis and invasion surrounding brain parenchyma. This histology at microscopic level, frequently showing predominance of stem-like cells [24]. The intrinsic infiltration of glioma cells into brain parenchyma renders this cancer resistant to current treatments of surgical removal combined with radiation-, chemo- and immuno- therapies [25].

In this study, we found that the expression of CD133, cathepsin B, β3-tubulin and nestin are prognostic factors of disease progression and poor clinical outcome in GBM. These CD133-positive tumour cells may be a leading force for tumoral neo-genesis and progression. Patients with high CD133 expression had minor survival. Therefore, we found that in GBM the presence of a large proportion of CD133+ cells are a maker to anticipate poor prognosis. Some *in vitro* and *in vivo* studies have shown that CD133 induce the *novo* tumour and present strong capacity for self-renewal. The Sub Ventricular Zone (SVZ) has been proposed as the source of the most aggressive forms of GBM and possesses a large content of CD133+ which show a multifocality patterns of tumor recurrence, suggesting that the cell origin of this tumor might be migratory and invasive [11]. Transcriptional analysis of CD133+ GSCs have shown that these cells have an increased expression of anti-apoptotic genes, suggesting that GSCs also can develop intrinsic mechanisms for chemo-resistance [13]. Additionally, GSCs are resistant to radiotherapy, with the emergence of radiation-resistant clones that show increased expression of GSCs markers and upregulation of genes associated with DNA repair [12, 26]. Although CD133+ is a non-specific stem cell marker, recent studies have found that its high expression is associated with low survival it can be considered an independent risk factor for recurrence [19].

In addition to CD133, other biomarkers have been proposed as potential determinant of tumour aggressiveness and consequent poor prognosis for GBM. The expression of stem cell markers, such as podoplanin, CD15, CD44, CD133, and nestin, are correlated with malignant progression. Also, CD133 and nestin are commonly associated with poor PFS in patients with glioma grade II [27]. mRNA levels of nestin differ significantly between GBM and low-grade primary brain tumours [28,29] These biomarkers have been associated with resistance to treatment and recurrence. *In vitro* studies have shown that stem cells treated with temozolomide may acquire resistance to treatment, and this newly converted GSCs population express markers associated with pluripotency and stemless, such as CD133, SOX2, Oct4, and Nestin [30]. Nestin may also be expressed in astrocytes of the adult Central Nervous System (CNS) in response to cellular stress, such as neoplastic transformation [28,31]. Cathepsin B was strongly expressed in tumoral cells from half of the low-grade tumours, in contrast to intense expression for nestin in GBM.

In brain neoplasm, the down-regulation of the inhibitory activity of cystatins contributes to tumour malignancy [32]. The expression of cathepsin B, L and S increase with glioma malignancy; they have been implicated in glioma invasion. In contrast, the expression of cystatin C decrease with malignant progression towards GBM, this diminution of expression also correlates with shorter disease free survival in GBM patients [31, 33]. The process of glioma cell invasion into surrounding brain parenchyma is associated with increased degradation of the extracellular matrix, creating tracts within the white matter through which neoplastic cells can migrate [34, 35]. Proteolytic enzymes, cathepsins and the inhibitory proteins cystanins, have both a role in the infiltrative growth of GBM [36]. Interestingly, the localization of Cathepsin B in tumor-associated endothelial cells may participate in the process of tumor angiogenesis, possibly by increasing the invasive outgrowth of endothelial cells from normal existing vessels towards tumoral neovasculature [37]. The finding of high expression of cathepsin B in many tumors has led to the hypothesis that this enzyme plays a causal role in tumor invasiveness in experimental murine breast cancer. Antisense downregulation of cathepsin B reduce motility and invasion of osteosarcoma cells [38] and shRNA downregulation of cathepsin B reduce degradation of type I collagen *in vitro* and in bone metastasis *in vivo* carcinoma [39,40]. Downregulation of cathepsin B, in conjunction with downregulation of other proteases

and protease receptors has shown that cathepsin B is part of a proteolytic network that involves matrix metalloproteinase and the plasminogen activator cascade. For instance, in meningioma, the down-regulation of cathepsin B and MMP9 reduce tumor growth *in vivo* and decrease cell growth, invasion, angiogenesis, and regulation of downstream kinase signaling pathways *in vitro*; whereas the downregulation of cathepsin B and uPAR reduces contents of transforming growth factor β1 (TGF- β1) [40, 41].

Conclusions

There has been great interest in classifying molecular subtypes of GBM to offer personalized therapy and improve prognosis in patients suffering GBM. It is still debatable which subtype of GBM has a better or worse prognosis and is responsive or nonresponsive to any given therapies. This is the first prospective study aiming to analyze the correlation of stem cell markers and enzymatic expression in GBM with the topography and patient survival. Our results show that stem cell markers, CD133 and nestin, as well as GFAP and enzyme cathepsin B are overexpressed in cases where the GBM was located in or around SVZ (zones I and II) which presents the worst prognosis. Therefore, we propose that these biomarkers might be useful as predictive parameter of survival in patients with GBM. This study also indicates that therapeutic attempts could be directed against these molecules in GBM cells.

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