Differential Expression of 2',3'-Cyclic-Nucleotide 3'-Phosphodiesterase and Neural Lineage Markers Correlate with Glioblastoma Xenograft Infiltration and Patient Survival

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Abstract

Purpose: Glioblastoma multiforme (GBM) is a poorly treated human brain cancer with few established clinically useful molecular prognostic markers. We characterized glioblastoma stem–like cells (GSC) according to developmental neural lineage markers and correlated their expression with patient survival.

Experimental Design: Immunoblot array of neural lineage markers classified five independently isolated human GSC lines into three classes exhibiting differential expression of oligodendrocyte progenitor cells (OPC), astrocyte progenitor cells (APC), and neural progenitor cells (NPC) markers. Immunodeficient mice were orthotopically implanted with each cell line to evaluate tumor infiltration and recipient survival. 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNP) antigenic expression was used to evaluate a clinically annotated GBM tissue microarray with 115 specimens.

Results: We report that molecular classification of patient-derived GSCs using neural lineage markers show association with differential xenograft invasiveness, and also show significant correlation to survival in both the mouse model and human patients. Orthotopic implantation into immunodeficient mice showed Ki-67 proliferative index independent xenograft infiltration: class I GSCs (OPC and NPC positive) established focal lesions, class II GSCs (NPC positive) formed minimally invasive lesions, and class III GSCs (APC positive) established highly infiltrative lesions. The OPC marker, CNP also exhibited high expression in focal xenografts versus low expression in invasive xenografts. Differential CNP expression correlated with mouse model survival, and CNP immunoassay of a large GBM tissue microarray also showed significant differential patient survival.

Conclusions: GSC classification with developmental neural lineage markers revealed CNP as a novel and potentially useful clinical prognosis marker, and suggests clinical importance for patient-specific GSC analysis.

Introduction

Glioblastoma multiforme (GBM) is a deadly central nervous system malignancy with a median survival of 14.6 months after surgery followed by radiotherapy and temozolomide (1). Many studies show that GBM varies extensively in pathology and genotype features that likely determine differential therapeutic responses and patient outcomes (2); but unfortunately, very few molecular assays for classifying GBM are currently clinically validated and prognostically useful.

Currently, histopathologic classification of GBM is based on tumor architectural features, such as necrosis and/or endothelial proliferation, rather than on individual cellular morphology (3). Recent gene expression profiling of GBM tumor samples has provided better survival predictions than histology (4). Furthermore, mRNA and microRNA expression profiling technologies have identified genetically distinct GBM subclasses with differential survival and response to treatment (5–7). These studies categorize GBMs according to neural precursor cell markers, which are not revealed by current clinical pathology methods. In addition, GBM subtypes have not been well characterized among enriched stem-like populations that have tumor-initiating potential and contribute to therapeutic resistance. GBM stem-like cells (GSC) cultures share similar genotypes, gene expression patterns, and in vivo biology of human glioblastomas (8), suggesting GSC analysis is more applicable for clinically useful biomarker discovery.
In this study, we use a small set of neural lineage markers to molecularly subtype GSC lines via Western analysis. Our 5 GSC lines resemble either astrocyte progenitor cells (APC), oligodendrocyte progenitor cells (OPC), and/or neural progenitor cells (NPC). Each subtype initiates a distinct tumor phenotype in orthotopically implanted immunodeficient mouse xenografts, ranging from highly invasive to circumscribed focal lesions. Xenograft infiltration increases mortality in recipient mice and is correlated to differential expression of 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP), in patient survival was confirmed with a large tissue microarray of 115 human GBMs. These findings link different developmental neural lineage profiles with extent of tumor infiltration and patient survival, and reveal CNP as a novel GBM prognostic marker that can be clinically tested via current histopathologic assays, and new tumorigenesis mechanisms for study.

**Materials and Methods**

**Isolation of glioblastoma stem–like cancer cells**

All studies were conducted with approval from the University of Wisconsin-Madison Institutional Review Board (IRB) with informed consent obtained from patients, and with approval from the Animal Care and Use Committee. Glioblastoma stem–like cells (GSC) were isolated following protocols previously reported (8, 16–18), without the use of surface markers to capture the diversity of GBM tumors and enrich for their heterogeneous clones. Tumor tissue was collected directly from the operating room, weighed, coarsely minced with a scalpel blade, and subsequently chopped 2 times at 200 μm using a tissue chopper (Sorvall TC-2 Smith-Farquhar). Chopped tissue was directly plated in suspension or on laminin (19), at 10 mg/mL in NBE medium [Neurobasal™ medium with 0.5 × B27, 0.5 × N2, 2 mmol/L GlutaMAX, 5 μg/mL heparin, and penicillin/streptomycin/ampicillin (PSA); Invitrogen], supplemented with 50 ng/mL each of human recombinant EGF and bovine fibroblast growth factor (bFGF; PeproTech; ref. 8). Cultures were passaged approximately every 7 days by tissue chopping 2 × 100 μm or detached with Accutase (Millipore) before plating onto freshly coated laminin flasks. Using this method, we isolated 4 GSC neurosphere lines by suspension culture (recurrent 12.1, and primary 22, 33, 44) and 1 GSC line by laminin culture (primary 99) out of approximately 100 GBM tumors. Medium for later passages of GSCs was changed to “Passaging Medium” (PM: 70% DMEM-high glucose, 30% Ham’s F12, 1 × B27, 5 μg/mL heparin, 1% antibiotics, and 20 ng/mL each EGF and bFGF; ref. 17), as similar growth patterns were observed in both media formulations and media conditions near to normal human neural stem cells (hNSCs) were desired. Each GSC line was validated for self-renewal by neurosphere formation (Fig. 1, A1–C1), multipotency (20), and tumor initiation (below) before experiments were conducted. Standard serum conditions were used to maintain the U87 cell line (DMEM, 10% FBS, 1% antibiotics, and Invitrogen). In most studies, GSCs were compared with hNSCs. Human fetal cortical neural stem cells were a kind gift from Dr Clive Svendsen, and maintained as previously described (17). Establishing and cryopreservation of cell cultures ranged from passage 1 to 10. Cells used for experiments ranged from passage 20 to 25.

**GSC cancer cell orthotopic xenograft model**

Tumor initiation capacity of human GSCs was verified by orthotopic xenograft as previously described (8, 21, 22). Briefly, GSCs were enzymatically dissociated to single cells and varying cell numbers (10^2–10^6) were suspended in 5 μL of PBS. Using a Hamilton syringe, the cells were stereotactically implanted into the right striatum of anesthetized nonobese diabetic severe combined immunodeficient (NOD-SCID) mice at 0.33 μL/min at the after coordinates referenced from Bregma: 0 mm antero-posterior, +2.5 mm medio-lateral, and −3.5 mm dorso-ventral (22). At either 3 months or onset of neurologic symptoms, tumor formation was verified using MRI. Mice were anesthetized, contrast enhanced using 10 mmol/kg of intraperitoneal
Figure 1. GSCs exhibit diverse morphologies within each cell line and across patient samples. A1–C1, brightfield images of isolated neurosphere GSC cultures in stem cell media supplemented with 20 ng/mL each of EGF and bFGF. Numbers 12.1, 33, and 44 are named after 3 different patient-derived GSC samples. A2–C3, dissociated neurospheres plated on laminin (50 μg/mL) for 24 hours form morphologically heterogeneous colonies. Examples of 2 representative colonies from 12.1 (A2 and A3), 33 (B2 and B3), and 44 (C2 and C3) GSCs are shown. All images are ×100 magnification. Scale bar, A1–C1, 200 μm and A2–C3, 100 μm.

Western blot analysis

GSCs were lysed using cell extraction buffer (FNN0011; Invitrogen) containing protease inhibitor cocktail (P8340; Sigma-Aldrich). Total protein was quantified using a fluorescent-based total assay (EZQ Protein Quantitation, R33200; Invitrogen). Fifty micrograms of protein were resuspended in 2× reducing sample buffer (Novex, LC2676; Invitrogen), electrophoresed on 10% to 20% gradient Tris-glycine gels (Invitrogen), transferred using a semi-dry transfer system (Bio-Rad) to polyvinylidene difluoride membranes (Millipore), and probed with specific antibodies. Immunocomplex detection was accomplished using luminescence (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific) per manufacturer instructions. Supplementary Table S1 lists the primary antibodies used in alphabetical order according to the antigen name. Secondary antibodies used were HRP-conjugated goat-anti-rabbit-IgG (32460) and goat-anti-mouse-IgG (32430) from Thermo Scientific.

Immunohistochemistry

Immunohistochemistry was conducted on NOD-SCID mouse brains implanted with 200,000 GSCs from lines 12.1, 22, 33, 44, and 99 GSCs, as described (16). GSCs 12.1 and 22 were sacrificed when tumor was recognized by MRI and lines 33, 44, and 99 were sacrificed when moribund because tumor was not detected on T1/T2 MRI. General histology, including paraffin embedding and hematoxylin and eosin (H&E) staining, were conducted at the Experimental Pathology Shared Service of the University of Wisconsin Carbone Comprehensive Cancer Center. Briefly, formalin-fixed, paraffin-embedded tissue sections were mounted on positively charged microscope slides. Tissue sections were then deparaffinized and rehydrated to water, microwaved in antigen unmasking solution (Vector Laboratories) to retrieve epitopes, and blocked for endogenous peroxidase and biotin before the application of the primary antibody. Incubation of antibodies (Supplementary Table S2) was conducted overnight at 4°C. Subsequent immunodetection was conducted using the Elite Vector Stain ABC System (Vector Laboratories). Color visualization was conducted using 3′-diaminobenzidine (DAB) as the chromagen substrate (Sigma Chemical Company). Tissues were counterstained with hematoxylin to visualize cellular morphology. Images were acquired with a Nikon TE-2000 and EVOS XL Core (Advanced Microscopy Group) brightfield microscopes. Black boxes in whole-brain images indicate the corresponding location of acquisition for high magnification photomicrographs.

Ki-67 indexing

After Ki-67 immunolabeling, positive cells were defined as those with nuclei of any brown color, regardless of the intensity or pattern of staining. Human versus mouse nuclei were distinguished using 4′,6-diamidino-2-phenylindole (DAPI) labeling. Mouse nuclei were small, regular, and round displaying concentrated aggregate DAPI labeling, indicative of characteristic mouse chromocenters (23). Human GSC nuclei were comparatively large and atypically oblong with homogenous DAPI labeling. Human-specific nestin labeling did not colocalize with chromocentric DAPI labeling (data not shown). Counting of Ki-67–positive nuclei began at the site of implantation until 500 random human nuclei were identified with a 60× objective. Ki-67 percent positivity was derived by dividing the average Ki-67 positive cells by the average total number of human nuclei (i.e., ~500).

Mouse survival plot

Using a different cohort and strain of mice used for immunohistochemistry analysis, NOD.Cg-Prkdcaid Ifngm1WJf/SzJ (NOD-SCID Gamma; Jackson Laboratory) mice were stereotactically implanted with 2 × 10⁵ cells from GSC lines 12.1, 22, 33, 44, and 99 (n = 4 each). Tumor formation was verified by MRI in GSCs 12.1 and 22. Mice were sacrificed when moribund. Because some mice (i.e., 12.1 and 22 GSC xenografts) suffered extensive cerebral hemorrhaging before death, their brain tissue...
was not useful for histology and molecular characterization. Survival analysis was done using a log-rank test and presented as a Kaplan–Meier plot. \( P \) values of <0.05 were considered to be statistically significant. Plot was generated using MedCalc version 11.6.1.0 (Mariakerke).

GBM tissue microarray

A tissue microarray from 205 patients with GBM diagnosed between 1999 and 2009 was created from the UW Department of Pathology and Laboratory Medicine archives. One to 4 representative tissue punches/cores were obtained for each tumor sample depending on morphologic heterogeneity and tissue availability. Diagnosis and tissue punch location were defined by neuropathology (MSS) before incorporation into microarray. Each additional tissue punch contained classic features of GBM including nuclear pleomorphism, mitosis, vascular endothelial proliferation, and/or necrosis. Grades II and III astrocytoma, grade II oligodendroglioma, meningioma, hippocampus, and neocortex tissue punches were used as controls. Of 205 patients, 115 had a recorded value for overall survival and a preserved tissue punch. A summary of clinical characteristics is provided in Supplementary Materials (Supplementary Table S3). Rb-pAb-anti-CNP (Sigma-Aldrich) was used to label the tissue microarray at a concentration of 1:250 (Supplementary Table S2). Each punch was subjectively scored for presence or absence of CNP expression by light microscopic visualization of intensity of cytoplasmic (not extracellular matrix) DAB precipitate by 2 observers (MSS and MZ) blinded to clinical outcomes as described previously (24). Nuclear or fibrillary labeling was not scored as positive. In cases of multiple punches/cores for 1 tumor sample, at least 1 tissue punch required such labeling to obtain a positive score. Kaplan–Meier survival plot was generated as described earlier with an additional HR analysis.

Results

Inter- and intrapatient clonal diversity of GSCs

GSC lines were initially isolated as neurospheres in minimal stem-cell media. GSC spheres were morphologically identical under light microscopy (Fig. 1, A1–C1). When neurospheres are dissociated and plated on laminin for 24 hours, diverse colonies arise within each cell line and among different patient samples (Fig. 1, A2–C3). Some colonies from 12.1 and 44 GSCs resemble OPC (25), with small, phase-bright cell bodies and bipolar extensions (Fig. 1, A2, C2, and C3). Conversely, other colonies from 12.1 and 33 GSC appear more astrocytic, displaying triangular cell bodies with multiple short projections (Fig. 1, A3, B2, and B3). Each independent cell line showed heterogeneous clonal diversity. These morphologic observations prompted evaluation of the collective protein expression of developmental neural lineage markers in GSCs.

Neural lineage markers define three GSC subtypes

We categorized 5 GSC lines according to common progenitor and mature neural markers using immunoblot assays (Fig. 2). Three distinct classes were revealed via protein expression profiling of the GSC lines. Class I GSCs (lines 12.1 and 22) have low expression of APC markers (Fig. 2A) with upregulated NPC (Fig. 2B) markers and OPC markers (Fig. 2C). Class II GSCs (line 33) have low APC and OPC markers, with moderate expression of NPC proteins. Class III GSCs (lines 44 and 99) have high APC and low NPC and OPC markers. Mature neural markers for astrocytes (Fig. 2D), neurons (Fig. 2E), and oligodendrocytes (Fig. 2F) were not differentially expressed. In addition,

Figure 2. GSCs segregated according to neural lineage markers. A–F, immunoblots of common neurodevelopmental markers probed against normal hNSCs, 12.1, 22, 33, 44, and 99 GSCs; A, APC markers EGFR and glial fibrillary acidic protein (GFAP); B, NPC markers cluster of differentiation 133 (CD133), L1 cell adhesion molecule (L1CAM), nestin (NES), and SRY-box containing gene 2 (SOX2); C, OPC markers CNP, oligodendrocyte transcription factor 1 (OLIG1), oligodendrocyte transcription factor 2 (OLIG2), PDGFRA, and SRY-box containing gene 10 (SOX10); D, astrocyte (A) marker glutamate synthetase (GLUL); E, neuron (N) marker beta-3 tubulin (TUBB3); F, oligodendrocyte (OL) marker myelin basic protein (MBP). GSCs segregated into 3 distinct groups determined by expression of these neural lineage markers: class I, OPC and NPC marker positive (12.1, 22); class II, NPC marker positive (33); and class III, APC marker positive (44, 99). \( \beta \)-Actin (ACTB) was used for loading control. Blots were cropped to enhance clarity.
tumor xenografts initiated from distinct GSC subtypes exhibit molecular and phenotypic variation.

**GSC subtypes are differentially invasive and correlated with CNP expression**

After orthotopic GSC implantation into immunodeficient mice for tumor xenograft formation, GSC-derived human GBM xenografts showed marked histologic variation, although all contained hallmarks of human GBM (Supplementary Fig. S1). Class I GSC formed circumscribed and minimally invasive lesions (Supplementary Fig. S1, A1 and B1), with relatively mild nuclear atypia and mitotic features against a fibrillary astrocytic background (Supplementary Fig. S1, A5 and B5). These tumors integrate into normal mouse brain parenchyma, as opposed to traditional nodular U87 xenografts that develop a pushing border at the margin of the tumor. Neoplastic class I GSCs were also found in the mouse corpus callosum (data not shown). Class II GSC formed moderately invasive lesions spanning both cerebral hemispheres (Supplementary Fig. S1, C1), with some irregular and elongated nuclei (Supplementary Fig. S1, C5). A human-specific nestin antibody was used to confirm location of human GSC-derived tumor cells (Fig. 3, C1; Supplementary Fig. S1, C6). Microscopic evaluation of Class III GSC-derived xenografts (Supplementary Fig. S1, D1 and E1) with human-specific nestin labeling revealed remarkable neoplastic cellular infiltration throughout mouse brains (Fig. 3, D1 and E1; Supplementary Fig. S1, D6 and E6). Class III GSC-derived xenografts exhibited the most atypical nuclei with elongated and irregular morphologies arranged in bundles (Supplementary Fig. S1, D5 and E5). Furthermore, we tested whether the observed larger size of Class III xenografts was because of tumor infiltration or enhanced proliferation via Ki67 staining, and found no proliferation differences (equivalent Ki67 positivity) among the xenografts derived from all independent GSC lines (Supplementary Fig. S1). GFAP expression was high in class III GSCs (Fig. 3, D2 and E2; Supplementary Fig. S1, D2 and E2). Conversely, GFAP expression was absent in classes I and II GSC xenografts, only labeling reactive mouse astrocytes (Fig. 3, A2–C2; Supplementary Fig. S1, A2–C2). Interestingly, EGFR receptor (EGFR) expression was absent in vitro for the 12.1 GSC line but reactivated in vivo for 12.1 GSC xenografts (Supplementary Fig. S1, A4 and A7), yet remained consistent in vitro for all other GSC lines and in vivo xenografts (Fig. 2A; Supplementary Fig. S1, B4–E4 and B7–E7). Most consistent was the finding that the OPC-like classes I and II GSC-derived xenografts showed CNP expression (Fig. 3, A3–C3; Supplementary Fig. S1, A3–C3), whereas CNP was absent in APC-like class III xenografts (Fig. 3, D3 and E3; Supplementary Fig. S1, D3 and E3). Therefore, high CNP expression was correlated with reduced tumor cell infiltration of classes I and II xenografts, in which the Rb-pAb-anti-CNP (Sigma-Aldrich) had precise cellular labeling and specificity. In addition, differential survival of implanted mice harboring GSC-xenografts showed encouraging associations with GSC classes (Supplementary Fig. S2), therefore independent validation of survival correlation with a large array of clinically annotated human GBM specimens was conducted.

**CNP expression is prognostically favorable in GBM**

We extended our GSC-derived xenograft observations to GBM patient outcomes by creating and analyzing a large human GBM tissue microarray (clinically annotated data from 115 patients). For this analysis, we immunolabeled GBM tissue microarray slides for CNP expression, which reliably discriminated between the different GSC classes in mouse xenografts (Fig. 3). For human patients with CNP-negative tumors (Fig. 4) showed a significantly lower survival probability ($P = 0.0154$) with a 0.6443 HR [95% confidence interval (CI), 0.4307–0.9639; Fig. 4A]. Median survival was 14 months for patients with CNP-positive ($n = 70$) versus 10 months for patients with CNP-negative tumors ($n = 45$), which is similar to the median survival of patients receiving the current standard of care with temozolomide (1). Furthermore, 38% of the CNP-positive patients (35% for CNP-negative patients) received temozolomide in the first round of
chemotherapy in our tissue microarray (Supplementary Table S3). Given this sample size, about 61% of patients with GBM potentially have CNP-positive tumors accounting for 1.95 of 100,000 persons per year according to the Central Brain Tumor Registry of the United States (CBTRUS) with a reported GBM incidence rate of 3.20 of 100,000 persons per year (95% CI, 3.17–3.23; ref. 26). The median age between cohorts of patients with CNP-positive (57 years) versus CNP-negative (53 years) tumors was equivalent. Patients included in the tissue microarray received variable treatment regiments as is reflected in precedence of GBM therapy since 1999 (Supplementary Table S3). Among CNP-positive and -negative samples, patients were comparable according to sex, ECOG/KPS performance status, tobacco usage, alcohol use, multiple surgeries, radiation, multiple rounds of chemotherapy, and enrollment status in a clinical trial (Supplementary Table S3). Consistent with mouse xenograft observations, CNP-negative gross GBM samples had elongated cells arranged in bundles with atypical nuclei (Fig. 4C) and CNP-positive samples contained minimally atypical nuclear morphologies (Fig. 4D). Analysis of gene expression data available through the National Cancer Institute Repository of Molecular Brain Neoplasia Data (NCI REMBRANDT) did not show a survival advantage for CNP upregulated GBM tumors \((n = 6)\) versus intermediate \((n = 91)\) and downregulated tissues \((n = 84; \text{ref. 27})\). In addition, similar analysis with the NCI The Cancer Genome Atlas (TCGA) shows no statistical difference between CNP altered \((n = 14)\) and nonaltered \((n = 413)\) GBM tumors \((28)\).

### Discussion

Through protein expression analysis of developmental neural lineage markers, we have identified GSC classes resembling: (1) OPCs and NPC, (2) NPC, and (3) APC (Fig. 5). Each of these GSC types exhibited distinct and particular hallmarks found in GBM, including varied cellular and nuclear morphologies, invasive potential, and survival (Fig. 5). Because only 5 cell lines were used for sampling different GSC classes, other GSC categories potentially exist with similar phenotypic features but different molecular markers. These neural lineage markers may not be specific to the stem-like cells and may be present in the other cells of the tumor. Further analysis of 1 OPC-specific marker showed that CNP expression correlated with longer
survival in mice harboring GSC-derived xenografts (Figs. 3 and 4) and in human patients via assaying a clinically-annotated tissue microarray containing 115 GBM samples (Fig. 4), suggesting the predictive power of our classification scheme in GSCs. Use of CNP as a prognostic marker will need to be validated on an additional clinically annotated GBM tissue microarray. Taken together, these data reveal molecular variation of patient-derived GSCs with the clinically applicable strategy of immunoassaying with neural lineage markers.

Because of GBM's phenotypic diversity between patients and the lack of efficacious treatment regimens, many groups previously report molecular classification of gross GBM specimens by gene expression analysis (5–7, 29–31). Each of these groups uncovered specific gene signatures to subclassify GBM that predict molecular phenotype or survival better than histologic analysis alone. Some of these GBM subclasses such as "proneural" (7), "oligoneural" (5), and other classifications (31) suggest neurodevelopmental links in gliomagenesis (6, 7, 31). In relationship to Verhaak and colleagues (6), class I GSCs resemble the "proneural" subtype according to Olig2 expression, class II GSCs may resemble the "neural" subtype with a preponderance of NPC markers, and class III GSCs resemble the "classical" GBM subtype with elevated EGFR expression. Genome-wide expression profiling is ongoing to cluster our GSC classes into these previously identified GBM subtypes. Instead of previous subtyping methodologies (5–7, 29, 31–35), our approach was to evaluate patient-derived GSCs, which are hypothesized to drive gliomagenesis, without contamination from non-GSCs and other cell types present in clinical resection samples. A few groups have also reported gene expression analyses of GSCs, isolated either through sphere-forming capacity (8, 35) or AC/CD133 expression (36), and these GSCs were found to segregate into distinct subgroups with some phenotypic correlation. Our study aimed to discover clinically applicable biomarkers using protein expression assays with a developmental array of neural lineage markers. We show that different GSC subtypes correlate with tumor invasiveness and likely has clinically relevant prognosis for survival in human patients. This study is also unique in that GSC protein marker expression rather than transcriptional analysis was conducted. As previously posited by Brennan and colleagues (37), total mRNA or microRNA levels do not always correlate with protein levels, therefore reducing the predictive ability of gene expression arrays to identify pathologically useful biomarkers. With their identification of EGFR- and platelet-derived growth factor alpha (PDGFRα)-specific subclasses, GSC line 44 showed an abundance of both EGFR and PDGFRα expression noting the potential differences in GSC cultures and probable mosaic molecular features characteristic of GBM (Fig. 2A and C). Additional evidence for the advantage of protein expression analysis is reflected in comparing our tissue microarray results with an assay of the NCI REMBRANDT and TCGA databases. No significant survival difference was observed in CNP-expressing versus nonexpressing GBMs with REMBRANDT or TCGA data despite a rather robust difference revealed with tissue microarray analysis. CNP expression may be a suitable classifier within the proneural or oligoneural group because of its link to oligodendrocyte-related genes (5, 6), but the lack of adequate CNP upregulated tumors (n = 1) in this GBM subset with 56 cases discourages conjecture (28). CNPs potential activity in RNA editing (12, 13) may explain differences in mRNA transcript and protein levels, yet these speculations remain to be resolved. The selective neural lineage marker approach is also advantageous to rapid clinical translation because many of the proteins studied are already familiar to neuropathologists and highly validated detection tools (i.e., antibodies and immunoassay protocols) are already available.

Not surprisingly, extensive cellular and molecular GBM heterogeneity was found between and within our patient GSC samples (Fig. 1). Furthermore, when GSCs were implanted into immunodeficient mice, GSC lines that expressed and exhibited a preponderance of oligodendrocytic markers and features in vitro shifted to an astrocytic morphology in vivo (Fig. 2, A2 and B2). This in vivo data diminishes the likelihood that we isolated a lower-grade or even "oligodendroglioma stem cell" from a GBM sample, either because of the existence of multiple stem-like cell clones within a single GSC line (38) or cell culture conditions (39). Xenografts from one class I GSC line (12.1) even showed new expression of EGFR (Supplementary Fig. S1, A4 and A7), an APC marker that correlated with the invasiveness of class III GSCs. It is possible that although 12.1 GSC xenografts expressed EGFR in vivo, EGFR is not critical for in vitro propagation of the GSC line. Activation of EGFR expression in xenografts may explain why mice with 12.1 GSC-derived xenografts had markedly worse survival probability compared with another class I GSC line (12.1 versus 22; Supplementary Fig. S2). In addition, EGFRVIII which is found mutated in 40% of EGFR amplified GBMs has not been detected in any of our GSC lines to date (40). Interestingly, 22 GSC xenografts (least infiltrative) had the highest survival probability along with the highest proliferative index at 83% Ki67 positivity, suggesting that invasiveness is more important for GBM mortality. Class III 99 GSC xenografts are more invasive but had the second best survival (Supplementary Fig. S2). Altogether, it seems that studies to identify anti-GBM therapies will need to factor in GSC heterogeneity showed in this and other studies (41).

Because interpreting survival data in GSC xenografts is limited to a small sample size, we used a large clinically annotated GBM tissue microarray to link GSC neural lineage marker protein expression findings with clinical patient outcomes, and found significantly improved survival in patients with CNP-positive GBM (Fig. 4). CNP expression in GBM and GSCs has been previously showed in vitro and in vivo (31, 35, 42, 43). We extend these studies by linking CNP expression to reduced invasiveness and survival with in vivo mouse xenograft studies, and in human
GBM patient specimens. Although protein or genetic expression in enriched GSCs does not necessarily correlate with fully developed GBM as represented by tissue microarray, it seems that in some cases, such as for CNP, the GSCs follow a predetermined "differentiation" program not unlike normal stem cells. Because CNP is associated with OPC development and myelin formation (44), CNP-positive tumors in GBM may be considered to contain an "oligodendroglialoma-like" component, currently not taken into account by the World Health Organization (WHO) classification scheme (45). In addition, CNP expression might be hypothesized to indicate an OPC cell-origin in as a GBM subset (43, 46). CNP may not be a GSC-specific marker, but its expression in mouse GSC xenografts and human GBM tumor specimens reliably correlates with decreased infiltration and improved patient survival.

This contrasts with the continuing controversy of using CD133 expression as a GSC-specific marker. Although nuclear elongation correlates with increased invasion in this study, little to no CD133 expression was found in our GSC lines—all were carefully validated for GSC properties of growth in minimal stem cell media, self-renewal, multi-potential differentiation, and highly efficient tumor initiation (Figs. 2 and 3). It is interesting that tumor cells with elongated nuclei were frequently observed and correlated with the class III APC, highly invasive GSC-derived xenografts. In contrast, Chen and colleagues (34) observed similar elongated nuclei in invasive mouse xenografts from CD133+ GSC clones.

The phenotypic and histologic aberrations found in the different GSC and GSC-derived xenograft classes also have much potential for clinical application. GBM cell invasion into normal brain parenchyma limits neurosurgeons' ability to remove tumors, often leading to recurrence immediately adjacent to the resection cavity (47). Better understanding and elucidating mechanisms of GSC invasion would aid in clinical treatment planning and defining prognosis.

In conclusion, with the emergence of molecular therapeutics and personalized medicine, understanding and identifying the various molecular pathologies of GBM and its intrinsic GSCs will be increasingly important in developing novel diagnostic and therapeutic strategies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interests were disclosed.

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