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

Michael Zorniak¹,², Paul A. Clark², Heather E. Leeper³, Matthew D. Tipping², David M. Francis², Kevin R. Kozak⁴, M. Shahriar Salamat⁵, John S. Kuo¹,²,⁴,⁶

¹Neuroscience Training Program, ²Department of Neurological Surgery, ³Department of Neurology, ⁴Department of Human Oncology, ⁵Department of Pathology and Laboratory Medicine, ⁶Carbone Cancer Center, University of Wisconsin School of Medicine and Public Health, Madison, WI, 53792-8660, USA

Running Title

CNP Linked to GBM Patient Survival

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Author Contribution Summary

Michael Zorniak: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript

Paul A. Clark: Conception and design, administrative support, collection and assembly of data, data analysis and interpretation, final approval of manuscript

Heather Leaper: Collection and assembly of data

Matthew Tipping: Collection and assembly of data
David Francis: Collection and assembly of data

Kevin Kozak: Administrative support, financial support, collection and assembly of data, final approval of manuscript

M. Shahriar Salamat: Conception and design, provision of study material or patients, collection and assembly of data, data analysis and interpretation, final approval of manuscript

John S. Kuo: Conception and design, financial support, administrative support, provision of study materials, data analysis and interpretation, manuscript writing, final approval of manuscript

Correspondence

John S. Kuo, University of Wisconsin School of Medicine and Public Health, Department of Neurological Surgery, CSC K3/803 Mail Code 8660, 600 Highland Ave, Madison, WI 53792-8660, Phone: 608-262-7303, Fax: 608-263-1728, E-mail: j.kuo@neurosurgery.wisc.edu

Key Words

2',3'-Cyclic-Nucleotide 3'-Phosphodiesterase; Prognostic Marker; Glioblastoma; Stem-Like Cancer Cells; Infiltration

Conflicts of Interest

We report no conflicts of interest.
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Abbreviations

3-3’-diaminobenzidine (DAB) • 4’,6-diamidino-2-phenylindole (DAPI) • Astrocyte (A) • Beta 3 tubulin (TUBB3) • Beta actin (ACTB) • Bovine fibroblast growth factor (bFGF) • Central Brain Tumor Registry of the United States (CBTRUS) • Cluster of differentiation 133 (CD133) • 2’,3’-Cyclic-nucleotide 3’-phosphodiesterase (CNP) • Epidermal growth factor (EGF) • Epidermal growth factor receptor (EGFR) • Glial fibrillary acidic acid (GFAP) • Glioblastoma multiforme (GBM) • Glioblastoma stem-like cell (GSC) • Glutamine synthetase (GLUL) • Human neural stem cell (hNSC) • Immunohistochemistry (IHC) • Institutional Review Board (IRB) • Kilodaltons (kDA) • L1 cell adhesion molecule (L1CAM) • Magnetic resonance imaging (MRI) • Myelin basic protein (MBP) • Nestin (NES) • Neuron (N) • Non-obese diabetic severe combined immunodeficiency (NOD-SCID) • Oligodendrocyte (OL) • Oligodendrocyte transcription factor 1 (OLIG1) • Oligodendrocyte transcription factor 2 (OLIG2) • Penicillin/streptomycin/ampicillin (PSA) • Platelet-derived growth factor alpha (PDGFRA) • SRY-box containing gene 10 (SOX10) • SRY-box containing gene 2 (SOX2) • The Cancer Genome Atlas (TCGA) • World Health Organization (WHO)
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 demonstrate significant correlation to survival in both the mouse model and human patients. Orthotopic implantation into immunodeficient mice demonstrated 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.

Word Count: 250/250
Translational Relevance

Median survival for glioblastoma patients is less than two years despite aggressive therapy. Glioblastoma stem-like cells (GSC) can grow in stem cell media, exhibit multipotent differentiation and therapeutic resistance, and are highly efficient at tumor initiation. We hypothesized that GSC lines isolated from different patients will exhibit distinct expression profiles of neural development proteins that possess prognostic value. We discovered that classifying GSC lines, via differential expression of neural cell lineage proteins, correlated with extent of tumor infiltration and survival in GSC-initiated mouse tumor models. Validation of prognostic utility for the oligodendroglial progenitor marker, 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 histopathology assays, and new tumorigenesis mechanisms for study.

Word Count: 150
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, histopathological 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. Additionally, 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 five 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), found
membrane-bound (9) in myelin-rich regions of the central nervous system (10).
Furthermore, CNP expression in human GBMs (assayed in a large clinically-annotated
GBM tissue microarray), is a favorable prognostic indicator of overall patient survival.
After 50 years since CNPs initial description as a phosphodiesterase (11), substrate or
product complexes have still not been found. Recently, some groups have indicated a role
in RNA metabolism, trafficking, or splicing of the exposed N-terminal domain (12, 13) and
potentially involving the maturation of oligodendrocytes through the regulation of myelin-
associated genes (14, 15). Our data show that differential expression of developmental
neural lineage markers is correlated with differential survival in both mouse model and
patients based on CNP expression and tumor invasiveness.

Materials and Methods

Isolation of Glioblastoma Stem-Like Cancer Cells

All studies were performed 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-Farquahar). Chopped tissue was directly plated in suspension or on laminin (19), at 10 mg/ml in NBE medium (Neurobasal™ medium with 0.5X B27, 0.5X N2, 2 mM Glutamax, 5 µg/ml heparin, and penicillin/streptomycin/ampicillin (PSA)) (Invitrogen, Grand Island, NY), supplemented with 50 ng/ml each of human recombinant epidermal growth factor (EGF) and bovine fibroblast growth factor (bFGF) (Peprotech, Rocky Hill, NJ) (8). Cultures were passaged approximately every 7 days by tissue chopping $2 \times$ at 100 µm or detached with Accutase (Millipore, Billerica, MA) 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, 1X B27, 5 µg/ml heparin, 1% antibiotics, and 20 ng/ml each EGF and bFGF) (17), as similar growth patterns were observed in both media formulations and media conditions near to normal human neural stem cells (hNSC) were desired. Each GSC line was validated for self-renewal by neurosphere formation (Fig. 1A1-C1), multipotency (20), and tumor initiation (below) before experiments were performed. Standard serum conditions were used to maintain the U87 cell line (DMEM, 10% fetal bovine serum, 1% antibiotics) (Invitrogen, Grand Island, NY). In most studies, GSCs were compared to 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 1-10. Cells used for experiments ranged from passage 20 to 25.

**Glioblastoma Stem-Like 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 non-obese diabetic severe combined immunodeficient (NOD-SCID) mice at 0.33 μl/min at the following 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 neurological symptoms, tumor formation was verified using magnetic resonance imaging (MRI). Mice were anesthetized, contrast enhanced using 10 mmol/kg of intra-peritoneal gadodiamide (Omniscan, GE Healthcare, Piscataway, NJ), placed onto a small animal MRI scanner (Varian 4.7T horizontal bore imaging/spectroscopy system, Palo Alto, CA), and T1- and T2-weighted images were obtained. After MRI showed tumor xenograft growth or when neurological symptoms were observed, implanted NOD-SCID mice were euthanized by perfusion fixation with 4% paraformaldehyde. Brains were then excised, embedded in paraffin, and processed for general histology. Human-specific nestin immunohistochemistry was used to discriminate between mouse and human cells.

**Western Analysis**
GSCs were lysed using cell extraction buffer (FNN0011, Invitrogen, Grand Island, NY) containing protease inhibitor cocktail (P8340, Sigma Aldrich, St. Louis, MO). Total protein was quantified using a fluorescent-based total assay (EZQ Protein Quantitation, R33200, Invitrogen). 50 µg of protein were resuspended in 2× reducing sample buffer (Novex, LC2676, Invitrogen), electrophoresed on 10-20% gradient tris-glycine gels (Invitrogen), transferred using a semi-dry transfer system (Bio-Rad, Hercules, CA) 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. Supplemental Table 1 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 performed on NOD-SCID mouse brains implanted with 200,000 GSCs from lines 12.1, 22, 33, 44, and 99 GSCs, as described (16). GSC 12.1 and 22 were sacrificed when tumor was recognized by MRI and lines 33, 44, and 99 were sacrificed when moribund since tumor was not detected on T1/T2 MRI. General histology, including paraffin embedding and hematoxylin and eosin (H&E) staining, were performed 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, Burlingham, CA) to retrieve epitopes, and blocked for endogenous peroxidase and biotin before the application of the primary antibody. Incubation of antibodies (Supp. Table 2) was performed overnight at 4°C. Subsequent immunodetection was performed using the Elite Vector Stain ABC System (Vector Laboratories, Burlingham, CA). Color visualization was performed using 3-3′-diaminobenzidine (DAB) as the chromagen substrate (Sigma Chemical Company, St. Louis, MO). Tissues were counterstained with hematoxylin to visualize cellular morphology. Images were acquired with a Nikon TE-2000 (Tokyo, Japan) and EVOS XL Core (Advanced Microscopy Group, Bothell, WA) 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-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ (NOD-SCID Gamma) (Jackson Laboratory, Bar Harbor, ME) mice were stereotactically implanted with 2x10<sup>5</sup> 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. Since some mice (i.e. 12.1 and 22 GSC xenografts) suffered extensive cerebral hemorrhaging prior to 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, Belgium).

GBM Tissue Microarray

A tissue microarray from 205 GBM patients diagnosed between 1999 and 2009 was created from the UW Department of Pathology and Laboratory Medicine archives. One to four representative tissue punches/cores were obtained for each tumor sample depending on morphological heterogeneity and tissue availability. Diagnosis and tissue punch location were defined by neuropathology [MSS] prior to incorporation into microarray. Each additional tissue punch contained classic features of GBM including nuclear pleomorphism, mitosis, vascular endothelial proliferation, and/or necrosis. Grade II and III astrocytoma, grade II oligodendroglioma, meningioma, hippocampus, and neocortex tissue punches were used as controls. Out of the 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 (Supp. Table 3). Rb-pAb-anti-CNP (Sigma Aldrich, St. Louis) was
used to label the tissue microarray at a concentration of 1:250 (Supp. Table 2). 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 two 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 one tumor sample, at least one tissue punch required such labeling to obtain a positive score. Kaplan-Meier survival plot was generated as described above with an additional hazard ratio analysis.

Results

Inter- and intrapatient clonal diversity of GSCs

Glioblastoma stem-like cancer cell (GSC) lines were initially isolated as neurospheres in minimal stem-cell media. GSC spheres were morphologically identical under light microscopy (Fig. 1A1-1C1). 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 oligodendrocyte progenitor cells (OPC) (25), with small, phase-bright cell bodies and bipolar extensions (Fig. 1, A2, C2, & 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, & B3). Each independent cell line demonstrated heterogeneous clonal diversity. These morphological observations prompted evaluation of the collective protein expression of developmental neural lineage markers in GSCs.
Neural lineage markers define three GSC subtypes

We categorized five 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 astrocyte progenitor cell (APC) markers (Fig. 2A) with upregulated neural progenitor cell (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, 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 histological variation, although all contained hallmarks of human GBM (Supp. Fig. 1). Class I GSC formed circumscribed and minimally invasive lesions (Supp. Fig. 1, A1 & B1), with relatively mild nuclear atypia and mitotic features against a fibrillary astrocytic background (Supp. Fig. 1, A5 & 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 (Supp. Fig. 1, C1), with some irregular and elongated nuclei (Supp. Fig. 1, C5). A human-specific nestin antibody was used to confirm location of human GSC-derived tumor cells (Fig. 3, C1; Supp. Fig. 1, C6). Microscopic evaluation of Class III GSC-derived xenografts (Supp. Fig. 1, D1 & E1) with human-specific nestin labeling revealed remarkable neoplastic cellular infiltration throughout mouse brains (Fig. 3, D1 & E1; Supp. Fig. 1, D6 & E6). Class III GSC-derived xenografts exhibited the most atypical nuclei with elongated and irregular morphologies arranged in bundles (Supp. Fig. 1, D5 & E5). Furthermore, we tested whether the observed larger size of Class III xenografts was due to 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 (Supp. Fig. 1). GFAP expression was high in Class III GSCs (Fig. 3, D2 & E2; Supp. Fig. 1, D2 & E2). Conversely, GFAP expression was absent in Class I and II GSC xenografts, only labeling reactive mouse astrocytes (Fig. 3, A2-C2; Supp. Fig. 1, A2-C2). Interestingly, epidermal growth factor receptor (EGFR) expression was absent \textit{in vitro} for the 12.1 GSC line but reactivated \textit{in vivo} for 12.1 GSC xenografts (Supp. Fig. 1, A4 & A7), yet remained consistent \textit{in vitro} for all other GSC lines and \textit{in vivo} xenografts (Fig. 2A; Supp. Fig. 1, B4-E4 & B7-E7). Most consistent was the finding that the OPC-like Class I and II GSC-derived xenografts showed CNP expression (Fig. 3, A3-C3; Supp. Fig. 1, A3-C3), whereas CNP was absent in APC-like Class III xenografts (Fig. 3, D3 & E3; Supp. Fig. 1, D3 & E3). Therefore, high CNP expression was correlated with reduced tumor cell infiltration of Class I and II xenografts, in which the Rb-pAb-anti-CNP (Sigma Aldrich, St. Louis) had precise cellular labeling and specificity. In addition,
survival of implanted mice harboring GSC-xenografts showed encouraging associations with GSC classes (Supp. Fig. 2), therefore independent validation of survival correlation with a large array of clinically annotated human GBM specimens was performed.

_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) demonstrated a significantly lower survival probability (p=0.0154) with a 0.6443 hazard ratio (95% 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 (Supp. Table 3). Given this sample size, about 61% of GBM patients potentially have CNP-positive tumors accounting for 1.95 of 100,00 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% C.I., 3.17-3.23) (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 (Supp. Table 3).
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 (Supp. Table 3). 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 up-regulated GBM tumors (n=6) versus intermediate (n=91) and down-regulated tissues (n=84) (27). Additionally, similar analysis with the NCI The Cancer Genome Atlas (TCGA) shows no statistical difference between CNP altered (n=14) and non-altered (n=413) GBM tumors (28).

Discussion

Through protein expression analysis of developmental neural lineage markers, we have identified GSC classes resembling: I) oligodendrocyte progenitor cells (OPC) and neural progenitor cells (NPC), II) neural progenitor cells (NPC), and III) astrocyte progenitor cells (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). Since only five 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 one OPC-
specific marker demonstrated that CNP expression correlated with longer survival in mice harboring GSC-derived xenografts (Fig. 3 & 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. Utility 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, 29-33). Each of these groups uncovered specific gene signatures to subclassify GBM that predict molecular phenotype or survival better than histological analysis alone. Some of these GBM subclasses, such as “proneural” (29), “oligoneural” (5), and other classifications (33), suggest neurodevelopmental links in gliomagenesis (29, 32, 33). In relationship to Verhaak and colleagues (32), 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, 29, 30, 32-37), 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, 38) or AC/CD133 expression (39), 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 demonstrate 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 performed. As previously posited by Brennan and colleagues (40), 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 PDGFRA specific subclasses, GSC line 44 demonstrated an abundance of both EGFR and PDGFRA expression noting the potential differences in GSC cultures and probable mosaic molecular features characteristic of GBM (Fig. 2A & 2C). 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 non-expressing 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 due to its link to oligodendrocyte related genes (5, 32), but the lack of adequate CNP up-regulated 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, since 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 & 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 (41) or cell culture conditions (42). Xenografts from one Class I GSC line (12.1) even showed new expression of EGFR (Supp. Fig. 1, A4 & 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 to another class I GSC line (12.1 versus 22) (Supp. Fig. 2). Additionally, EGFRvIII which is found mutated in 40% of EGFR amplified GBMs has not been detected in any of our GSC lines to date (43). 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 (Supp. Fig. 2). Altogether, it seems that studies to identify anti-GBM
therapies will need to factor in GSC heterogeneity demonstrated in this and other studies (44).

Since 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 demonstrated in vitro and in vivo (33, 37, 45, 46). 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. Since CNP is associated with oligodendrocyte progenitor cell development and myelin formation (47), CNP-positive tumors in GBM may be considered to contain an ‘oligodendroglioma-like’ component, currently not taken into account by the World Health Organization (WHO) classification scheme (48). Additionally, CNP expression might be hypothesized to indicate an OPC cell-of-origin in as a GBM subset (46, 49). 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, multipotent differentiation and highly efficient tumor initiation (Fig. 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 (36) observed similar elongated nuclei in invasive mouse xenografts from CD133+ GSC clones.

The phenotypic and histological 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 (50). 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.

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References


Figure Legends

Figure 1. Glioblastoma stem-like cells (GSC) 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 epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Numbers 12.1, 33, and 44 are named after three different patient-derived GSC samples. (A2-C3): Dissociated neurospheres plated on laminin (50 µg/mL) for 24 hours form morphologically heterogeneous colonies. Examples of two representative colonies from 12.1 (A2 & A3), 33 (B2 & B3), and 44 (C2 & C3) GSCs are shown. All images are 100X magnification. Scale bar (A1-C1), 200 µm. Scale bar (A2-C3), 100 µm.

Figure 2. GSCs segregated according to neural lineage markers. (A-F): Immunoblots of common neurodevelopmental markers probed against normal human neural stem cells (hNSCs), 12.1, 22, 33, 44, and 99 GSCs. (A): Astrocyte progenitor cell (APC) markers epidermal growth factor receptor (EGFR) and glial fibrillary acidic protein (GFAP). (B): Neural progenitor cell (NPC) markers cluster of differentiation 133 (CD133), L1 cell adhesion molecule (L1CAM), nestin (NES), and SRY-box containing gene 2 (SOX2). (C): Oligodendrocyte progenitor cell (OPC) markers 2’,3’-cyclic-nucleotide 3’-phosphodiesterase (CNP), Oligodendrocyte transcription factor 1 (OLIG1), Oligodendrocyte transcription factor 2 (OLIG2), Platelet-derived growth factor alpha (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.

**Figure 3.** Neural lineage subclasses are maintained in GSC-derived tumors and predict invasiveness. (A1-E1): Whole-brain human-specific nestin immunohistochemical (IHC) labeling. (A2-E3): IHC for GFAP and CNP were visualized at the site of stereotactic implantation. Black boxes indicate magnified sections in subsequent panels near the site of tumor implantation. Scale bar, 200 µm.

**Figure 4.** 2’,3’-Cyclic-nucleotide 3’-phosphodiesterase (CNP) expression in a GBM tissue microarray is a favorable prognostic indicator. (A): Kaplan-Meier survival probability curve for patients with CNP positive versus negative GBM tumors (n=115). CNP positive tumors showed a statistically significant higher survival probability (Log Rank Test, p=0.015). Hazard ratio is 0.6443 (95% CI 0.4307-0.9639). Median survival 10 months (range of 0.75 – 156 months) for CNP negative (n=45) and 14 months (range of 0-54 months) for CNP positive (n=70) tumors. (B): Examples of negative and positive CNP labeling from the GBM tissue microarray. Black boxes indicate magnified sections in subsequent panels. Counter-stained with hematoxylin. (C-D): CNP negative and positive labeling. Scale bar, 40 µm.
Figure 5. GSCs categorized into various classes based on differential expression of neural lineage markers, nuclear irregularities, xenograft invasiveness in vivo, and patient survival via human GBM microarray analysis.
Figure 2

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### Figure 3

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### Figure 5

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

Michael Zorniak, Paul A. Clark, Heather E. Leeper, et al.

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