Progenitor-like Traits Contribute to Patient Survival and Prognosis in Oligodendroglial Tumors

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Abstract

Purpose: Patient-derived glioma-propagating cells (GPC) contain karyotypic and gene expression profiles that are found in the primary tumor. However, their clinical relevance is unclear. We ask whether GPCs contribute to disease progression and survival outcome in patients with glioma by analyzing gene expression profiles.

Experimental Design: We tapped into public sources of GPC gene expression data and derived a gene signature distinguishing oligodendroglial from glioblastoma multiforme (GBM) GPCs. By adapting a method in glioma biology, the Connectivity Map, we interrogated its strength of association in public clinical databases. We validated the top-ranking signaling pathways Wnt, Notch, and TGFB, in GPCs and primary tumor specimens.

Results: We observed that patients with better prognosis correlated with oligodendroglial GPC features and lower tumor grade, and this was independent of the current clinical indicator, 1p/19q status. Patients with better prognosis had proneural tumors whereas the poorly surviving cohort had mesenchymal tumors. In addition, oligodendroglial GPCs were more sensitive to Wnt and Notch inhibition whereas GBM GPCs responded to TGFB1 inhibition.

Conclusions: We provide evidence that GPCs are clinically relevant. In addition, the more favorable prognosis of oligodendroglial tumors over GBM could be recapitulated transcriptomically at the GPC level, underscoring the relevance of this cellular model. Our gene signature detects molecular heterogeneity in oligodendroglial tumors that cannot be accounted for by the 1p/19q status alone, indicating that stem-like traits contribute to clinical status. Collectively, these data highlight the limitation of morphology-based histologic analyses in tumor classification, consequently impacting on treatment decisions. Clin Cancer Res; 1–14. ©2012 AACR.

Introduction

Gliomas are among the most prevalent of adult malignant brain tumors and have a poor prognosis despite advanced surgical intervention and adjuvant radiotherapy and chemotherapy. In transgenic mouse models, the glioma cell-of-origin has been proposed to be the neural stem cell or oligodendroglial precursor (1–3). Such cells have been shown to be enriched in glioma-propagating cells (GPC) cultured in vitro (4); however, specific markers for the GPC have been controversial (5). This forces a reevaluation of the definition of the GPC to encompass the following characteristics (6): They must show long-term self-renewal, stemness and multipotentiality and the ability to form serially transplantable, orthotopic xenograft tumors that recapitulate the patient’s original histopathology. Laks and colleagues recently showed that the ability to form GPC spheres is correlated with clinical outcome (7). While the lengthy period of sphere culture precludes their use in clinical applications, these data nevertheless suggest that GPCs cultured in vitro contribute to patient survival. Here, we asked the critical question of whether a gene expression...
Translational Relevance

Gliomas predominate the spectrum of adult malignant brain tumors and can arise from stem-like glioma-propagating cells (GPC). Patient-derived GPCs are controversial because their cell-of-origin cannot be identified; however, they have been shown to contain karyotypic and gene expression profiles that mirror the primary tumor. We sought to determine the GPC contribution to patient survival and prognosis by analyzing gene expression profiles. By adapting the Connectivity Map, we tapped into public GPC data from multiple investigators and interrogated their contribution in public glioma clinical databases. We show that patients with better prognosis tend to exhibit oligodendroglial GPC progenitor characteristics independently of the 1p/19q status, the current clinical indicator. In addition, stem-like signaling pathways such as Wnt, Notch, and TGFβ distinguish oligodendroglial from glioblastoma multiforme (GBM) GPCs. Taken together, our data provide a direct link between GPCs and disease progression, highlighting the clinical relevance of these cells.

profile from GPCs is reflected in bulk tumor analysis and contributes to patient survival and prognosis. These data would be important because they: (i) provide evidence that in vitro cultured, tumor-propagating stem-like GPCs contain molecular information that contribute to patient survival outcome, thus directly linking these controversial GPCs to the primary tumor and (ii) validate that the transcriptomic changes driving the tumor phenotype reside in these GPCs, thus providing molecular evidence for their relevance as in vitro culture systems. The cancer stem cell (CSC) hypothesis has been studied by many in several tumor types, with evidence suggesting that CSCs are the most likely culprits of tumor recurrence and regrowth (8). Consequently, the general thought of the community is that effective treatments must target these CSC traits, often using survival as the endpoint to monitor in animal models. Such studies do not reveal the clinical impact of CSCs in databases of actual patient gliomas. As such, there is a need to show this direct correlation between GPC properties and survival outcome. Several studies have illuminated the clinical contribution of tumor-propagating acute myeloid leukemia stem cells, breast CSCs, and colorectal CSCs to disease outcome and relapse (9–12), thus highlighting that similar, important conclusions might be made from analyzing GPCs from major glioma variants currently available through public information.

Recent works have shown that major glioma variants glioblastoma multiforme (GBM) and oligodendroglial tumors are molecularly heterogeneous, with each subclass distinguished by unique gene expression, genetic aberrations, and clinical profile (13–16). These findings highlight that gene expression drives disease progression and survival outcome. We used gene expression analyses to explore the clinical relevance of GPCs derived from GBM and oligodendroglial tumors, by tapping into publicly available GPC gene expression data from our study and that of others (17–19) to enlarge the pool of cells and subsequently interrogating their contribution in 2 large, clinical databases, REMBRANDT (20) and “Gravendeel” (15). Specifically, we asked whether GPCs derived from these 2 major glioma variants show the uniqueness of each tumor subtype. Oligodendroglial tumors tend to have a favorable prognosis and chemosensitivity is predicted by the only known clinical indicator, the 1p/19q codeletion status (21). It would therefore be important to understand whether molecular heterogeneity exists that cannot be explained by current clinical indicators alone. We adapted the Connectivity Map method (22) to determine strengths of association between the GPC gene signature and patient gene expression profiles. The Connectivity Map was first used to define the strengths of association of a biologic state (represented by a gene signature) to the action of small-molecule therapeutics (a database of reference profiles). This method is advantageous in allowing us to make connections between different data platforms and biologic information through the common vocabulary of genome-wide expression profiling. Since then, the Connectivity Map has been successfully used to determine the degree of oncogenic pathway activation in gastric cancer (23). We also recently showed that such a method supported the mechanistic role of Parkin as a tumor suppressor in glioma (24). We show that this oligodendroglial gene signature is a positive prognostic factor in gliomas and detects heterogeneity in oligodendroglial tumor patient survival profiles that cannot be predicted by the 1p/19q codeletion status alone. Moreover, we could correlate our signature enrichment with tumor grade and the “Phillips” molecular classification of gliomas (25). Collectively, our data establishes the direct relation between GPCs and patient primary tumors, emphasizing the clinical relevance of these cultured cells.

Materials and Methods

Tissue collection and primary GPC culture

All GPCs except Pollard lines used in this study were cultured as spheroid structures in serum-free media supplemented with basic fibroblast growth factor and EGF (17–19). Although Pollard lines were cultured on laminin (19), a recent molecular classification study showed that both culture methods preserved the biologic and functional signaling pathways (26). This provides justification for our subsequent analyses.

Graded brain tumor specimens were obtained with informed consent, as part of a study protocol approved by the Institutional Review Board. NNI-1, 2, 3, 4, 5, 10, 11, and 12 were derived from patients with GBM as previously described and serially propagated in nonobese diabetic/severe combined immunodeficient (NOD/SCID) gamma mice (17). Only low passage GPCs were used for our studies (P1-10). We and others have shown previously that serial propagation as orthotopic xenografts maintains GPC traits.
Il2rgtm1Wjl also maintained for up to 3 passages (Supplementary Fig. S1B). GPC frequency and proliferation were assessed according to previous methods (Supplementary Figs. S1 and S2; ref. 17). NNI-8 GPCs showed presence of stemness marker expression such as Musashi-1 (Msi-1), octamer-binding transcription factor 4 (Oct4) and were actively dividing as indicated by approximately 30% of Ki-67–positive cells (Supplementary Fig. S1D). The GPCs also exhibited multi-lineage potential, showing presence of aberrant cells contaminating for neuron-specific class III β-tubulin (TuJ1) and glial fibrillary acidic protein (GFAP; Supplementary Fig. S1D). Finally, NNI-8 GPCs formed serially transplantable tumor xenografts in NOD/SCID gamma mice that recapitulated the patient’s original histopathology with maintenance of stemness expression in both primary tumors and low-passage GPCs (Supplementary Fig. S2). These data verify that serial tumor propagation maintains the GPC phenotype. 'Gunther’ lines: GS-1, 3, 4, 5, 6, 7, and 8 are GBM-propagating cells whereas GS-2 was derived from a high-grade tumor with oligodendroglial features as previously described (18). Cell lines were cultured for up to 14 passages in vitro with preservation of transcriptomic profiles. 'Pollard’ lines: G144, 144ED, 166, 179, and GliNS2 are GBM-propagating cells whereas G174 was derived from a patient with anaplastic oligoastrocytoma as previously described (19). Pollard lines could be cultured for 1 year (>20 passages) with preservation of key stemness/differentiation expression, karyotypic hallmarks, and tumor propagation.

**Sphere assays, immunofluorescence analysis, and intracranial glioma mouse model**

Sphere assays, immunofluorescence analysis, and the intracranial glioma mouse model were described in our earlier work (17). Approval for in vivo analysis was sought from the National Neuroscience Institute, Institutional Animal Care and Use Committee. Antibodies used for immunofluorescence included: (i) mouse monoclonal anti-Nestin (1:200, Chemicon, MAB3526); anti-Ki-67 (1:200, Chemicon, MAB4190); anti-Tuj1 (1:200, Chemicon, MAB1637), and anti-O4 (1:50, Chemicon, MAB345); (ii) rabbit polyclonal anti-Oct4 (1H-134, 1:100, Santa Cruz, SC9081); anti-GFAP (1:3000, DAKO, ZO334), and anti-Musashi (1:100, Chemicon, AB5977). Antibodies used for primary tumor or tumor xenograft paraffin sections included: (i) mouse monoclonal anti-Nestin (1:500, Chemicon, MAB5326); anti-ALDH (1:100, BD Biosciences, #611194), and anti-active β-catenin (1:300, Millipore, #05-665); and (ii) rabbit polyclonal anti-C133 (1:500, Abcam, ab19898); anti-activated Notch (1:500, Abcam, ab8925), and anti-phospho-Smad2 (Ser465/467; 1:50, Millipore, AB3849). NOD/SCID gamma mice (NOD.Cg-Prkd-acid il2rgtm1Wjl/SzJ; The Jackson Laboratory) were stereotactically injected with NNI-8 or lentivirally-transduced GPCs using pLKO.1-β-catenin knockdown constructs [clones: shβcat1 (TRCN0000003843) and shβcat2 (TRCN0000003844)] from Open Biosystems or a nontargeting (NT) control shRNA (SHC002, Sigma), packaged with pLenti-X packaging system according to the manufacturer’s instruction (Clontech). Animals were monitored for time to development of neurologic deficits. Kaplan–Meier survival analyses were carried out using the log-rank test in GraphPad Prism software.

**Small-molecule inhibitors and reagents**

The small-molecule inhibitor of Wnt signaling, cercosporin (28) was purchased from Sigma. CCT036477 (29) was manufactured and synthesized by Lavania Corporation according to the published chemical structure. The small-molecule inhibitors of Notch signaling, γ-secretase inhibitor (30) and DAPT [N-[3,5-difluorophenacetyl]-l-alanyl]-S-phenylglycine-t-butyler ester; ref. 31] were purchased from Sigma. TGFβRI inhibitor, SB525334 (32) was procured from TOCRIS bioscience. GPCs were treated at 10 μmol/L for γ-secretase inhibitor, DAPT, and SB525334, and at IC50 concentrations for cercosporin and CCT036477 (data not shown). TGFβ1, used at 200 pmol/L, was obtained from R&D.

**Immunoblot analysis**

GPC cells were pelleted and lysed in buffer containing 0.5% sodium deoxycholate, 1% NP-40 detergent, 0.1% SDS, 0.15 mol/L NaCl, 10 mmol/L Tris-HCl (pH 7.4), with protease and phosphatase inhibitors cocktail tablets (Roche). Equal amounts of protein lysate were resolved by SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes. Membranes were processed according to standard procedures and proteins detected using the imaging system, SYNGENE G:Box, iChemixXT. The following antibodies were used: anti-active β-catenin (BE7, 1:1,000; Millipore, #05-665), anti-β-catenin (1:1,000, BD Transduction Laboratories, #610153), anti-cleaved Notch 1 (NICD; 1:1,000; Cell Signaling, #2421), anti-phospho-Smad2 (Ser465/467; 1:1,000; Cell Signaling, #3108), anti-Smad2 (1:1,000; Cell Signaling, #3122), anti-phospho-Smad3 (Ser423/437; 1:1,000; Millipore, #07-1389), anti-Smad3 (1:1,000; Cell Signaling, #9523), and anti-β-actin (AC-15, 1:10,000; Sigma, A5441).

**Statistical analysis**

Data are expressed as means ± SEM of at least 3 independent experiments. Student t or Mann–Whitney U test was used where appropriate. P ≤ 0.05 was accepted as statistically significant.

**Processing of microarray data, gene signature generation, and pathway analysis**

Affymetrix U133 Plus 2.0 CEL files were mas5 processed and quantile normalized in the R statistical software using the affy packages (33, 34). Probes with “absent” call in all samples were removed. Microarray data were obtained from the Gunther and colleagues (18) and Pollard and colleagues.
Lamb and colleagues (22). With each activation score were calculated as described in nature and vice versa. The 2-tailed test expression profile is positively associated to the gene signature, and finally, (iv) The patients were sorted according to their pathway activation scores based on the strength of association to the GPC signature, and finally, (iv) The patients were sorted according to their pathway activation scores based on the strength of association to the GPC signature.

To further interrogate the oligodendroglial feature of glioma, we defined an "oligodendroglial GPC signature" using a log ratio cutoff value of 0.8. Similarly, the "NNI-8 GPC versus tumor" stemness signature was obtained by taking the top ranking differentially expressed probe sets using the log ratio cutoff value of 6. These signatures were used in the Connectivity Map analysis.

**Connectivity Map analysis**

We adapted the Connectivity Map method (22) to score glioma gene expression databases based on the extent of pathway activation associated with our GPC gene signature. (i) First, we defined an "oligodendroglial GPC signature"—a set of genes exhibiting altered expression between 2 cell states (oligodendroglial GPC vs. GBM GPC). (ii) Second, we generated databases of reference gene expression profiles from 2 glioma databases—REMBRANDT and "Gravendeel" (15, 20). (iii) Third, using a nonparametric, rank-based pattern matching procedure, we mapped the GPC signature onto each patient gene expression profile and calculated activation scores based on the strength of association to the GPC signature, and finally, (iv) The patients were sorted according to their pathway activation scores. Two patient classes were identified, (+) and (−), where a positive activation score indicates that the patient gene expression profile is positively associated to the gene signature and vice versa. The 2-tailed test $P$ values associated with each activation score were calculated as described in Lamb and colleagues (22). $P$ values $\leq 0.1$ were considered significant.

**Reference profile generation for Connectivity Map analysis**

Public GBM data sets with clinical data, in terms of survival length, histology, grade, and age were obtained from the REMBRANDT database (37) and the GEO database in the case of the Gravendeel data set (GSE16011). To generate the reference profiles, all raw files were processed separately using the mas5. Expression values less than the threshold value of 50 were replaced with the threshold value. Next, these data were quantile normalized and gene expression values were row-wise median centered. Median centering each probe set allows us to study the range of gene expression values in a large data set.

**Survival analysis**

Kaplan–Meier and Cox regression analysis of (+) and (−) groups were done in R using the survival package (38). For the REMBRANDT data set, only survival ranges were available. Hence, the lower limit of the range was used in this analysis.

**Prediction of Phillips Classification in REMBRANDT and Gravendeel data sets**

To classify the REMBRANDT and Gravendeel samples according to the Phillips and colleagues classification (25), Affymetrix U133A probes for the Phillips molecular subtypes were extracted from the publication. A shrunk centroid model was trained and tested on the Phillips data set (Supplementary Table S1; overall error rate 0.12) using the R package pamr (39). Next, classification of the REMBRANDT and Gravendeel data sets was conducted using the trained model.

**REMBRANDT SNP array processing and 1p19q LOH analysis**

CEL files from the Affymetrix 100K SNP Arrays of patients with oligodendroglioma and oligoastrocytoma were downloaded from the REMBRANDT database and all samples were normalized in dChip (40, 41). Genotyping calls were generated in the Affymetrix Genotyping Console (Affymetrix Inc.) software using the BRLMM algorithm. Chromosome 1p and 19q loss-of-heterozygosity inference was carried out using an HMM algorithm in dChip with default parameters.

**Gene set enrichment analysis**

The gene signature was further evaluated in molecular signature database using gene set enrichment approach. Gene set enrichment analysis (GSEA) tool was downloaded from Broad Institute portal (42). The significantly enriched gene sets in molecular signature database (MSigDB) were further analyzed for phenotypic correlation in the reference data sets.

**Results**

**An oligodendrogial GPC gene signature is defined**

For our purpose, we evaluated publicly available GPC gene expression data from 3 groups to enlarge our pool of cells. We first determined differentially regulated genes between oligodendrogial GPCs (NNI-8, GS-2, G174) and 17 GBM GPCs collectively obtained from our studies (this study and ref. 17), Gunther and colleagues (18), and Pollard and colleagues (ref. 19; Supplementary Fig. S3A, workflow). This differential gene list, "oligodendrogial GPC signature," is shown in Supplementary Table S2. An analysis of the associated pathway networks using the GeneGo program revealed that the signature was enriched in the Wnt, Notch, and TGFβ signaling pathways (Supplementary Fig. S3B). Interestingly, Notch (43, 44), TGFβ (45, 46), and the recently published Wnt (47) signaling pathways have been shown to be crucial in maintaining the growth of GBM GPCs.
Functional validation of the Wnt, Notch, and TGFβ pathways in GPCs

Although the Wnt, Notch, and TGFβ pathways regulate GBM GPC survival, their relation to the major glioma variants—oligodendrogial and GBM GPCs is unclear. Previously, the oligodendrogial gene signature enriched for the Wnt, Notch, and TGFβ signaling pathways (Supplementary Fig. S3B); however, their precise activation or downregulation remains to be tested. To assess pathway activation in GPCs (NNI-4, 7, 8, 10, 11, 12), we carried out 2 assays: (i) immunoblot analysis of key pathway components; and (ii) dependence on pathway by using well-established pharmacologic inhibitors.

NNI-7 and NNI-8 oligodendrogial GPCs showed increased sensitivity to cercosporin (28) and CCT036477 (29) compared with the other 2 of 3 GBM GPCs tested, consistent with the highest level of active β-catenin detected [nuclear-localized; Fig. 1A (i) and B (i)]. Sphere frequency was significantly reduced upon pathway inhibition, indicating that GPCs were targeted [Fig. 1B (i)]. Moreover, lentiviral-mediated β-catenin knockdown abrogated glioma formation in an orthotopic mouse model, consequently extending survival compared with the nontargeting vector control group (Fig. 2; P < 0.001).

Next, we assessed Notch pathway activation in our GPCs. Using γ-secretase inhibitor (30) and DAPT (31), we observed that NNI-7 and NNI-8 GPCs were most sensitive to pathway inhibition than NNI-11, 12, and 4 [Fig. 1B (ii)]. Again, these findings were consistent with the immunoblot analysis showing highest level of Notch intracellular domain (NICD) detected in NNI-8 GPCs [Fig. 1A (ii)].

Finally, we tested TGFβ signaling by using SB525334 (32). Interestingly, all 3 GBM GPCs showed sensitivity to SB525334 with up to 80% inhibition in NNI-4 [Fig. 1B (iii)]. A less clear pattern of phospho-Smad2 and phospho-Smad3 levels was observed upon TGFβ1 stimulation [Fig. 1A (iii)]. This may reflect the redundant roles of various Smad proteins in GPC regulation (46). Our data indicate that GPC frequency was preferentially targeted in GBM GPCs.

Collectively, our data indicate, albeit a limited panel of GPCs used, that Wnt and Notch pathways are upregulated in NNI-7 and NNI-8 oligodendrogial GPCs, whereas TGFβ pathway is active in all GBM GPCs tested.

The oligodendrogial GPC signature stratifies patient survival in gliomas

Next, we analyzed the strength of association of this gene signature with patient gene expression data from REMBRANDT and Gravendeel (15, 20). We assigned positive “(+)” and negative “(−)” activation scores with significant P values (Supplementary Table S3) and observed that the gene signature separated (+) and (−) patient cohorts that make up 30% to 50% of all patients in each database (Table 1). Important, the signature stratified patient survival (Fig. 3). Patients with better survival composed of (+) association (more oligodendrogial GPC association) whereas poorly surviving patients tended to be (−); i.e., more GBM GPC association; REMBRANDT P value, 1.93E-05; Gravendeel P value, 0.0082). The (+) activation score also contained more low-grade gliomas, especially enriched for oligodendrogliomas; whereas the (−) activation score enriched for high-grade gliomas with mainly GBMs. Cox regression analysis indicated that the GPC gene signature served as a significant prognostic indicator and the positive score patients (oligodendrogial GPC-like) in REMBRANDT had 54% lower risk of death; the HR (95% confidence interval, CI) was 0.462 (0.322–0.664) in a univariate model (P = 2.90E-05). Consistently, the positive score patients in Gravendeel were associated with 47% lower risk of death and the HR (95% CI) was 0.535 (0.334–0.856) in a univariate model (P = 0.009). This association remains significant in REMBRANDT after adjusting for other clinical factors such as age and tumor grade (P = 2.22E-05). Although we did not detect a significant multivariate analysis P value in the Gravendeel data set, this does not mean the absence of GPC transcriptome contribution to patient survival as shown in the REMBRANDT data set. First, most glioma databases are retrospectively generated and therefore, this limits our ability to assess the true predictive value of the gene signature. Second, a significant P value was observed in the univariate analysis, highlighting the relevance of the gene signature as an alternative prognostic tool. Collectively, these results suggest that distinct GPCs likely drive tumor formation and give rise to differences in response to therapy.

The oligodendrogial GPC signature correlates with "Phillips" molecular classification of gliomas

We next attempted to strengthen our findings based on the Connectivity Map (CMAP) by asking whether our GPC-derived gene signature could predict glioma survival outcome similar to other existing molecular-based classification schemes. This would be important to further validate the significance of the GPC-derived signature in relation to disease progression. We applied as an independent gene expression-based approach, the "Phillips" classification of gliomas (25) which molecularly categorizes the tumors into 3 subclasses: proneural, proliferative, and mesenchymal. The (+) activation score enriched for the proneural subclass, whereas the (−) activation score tended to be proliferative or mesenchymal (Fig. 3; Supplementary Table S4). Proneurals are typically lower grade gliomas with oligodendrogial features, frequently associated with better prognosis; in contrast, the mesenchymal subclass characterizes highly aggressive, recurrent gliomas such as GBM. Interestingly, recent work has suggested that oligodendrogliomas are more chemosensitive because their cells-of-origin are oligodendrocyte precursor cells (OPC), compared with the more resistant neural stem cells and astrocytes in GBM (48). Although this conclusion arose from transgenic mouse models, we find it intriguing that all cultured patient-derived GPCs from multiple studies are transcriptomically consistent with this hypothesis; however, we cannot definitively pinpoint the identity of GPCs due to its human origin. Furthermore, to eliminate the possibility that we
Figure 1. Functional validation of the Wnt, Notch, and TGFb signaling pathways in GPCs. Immunoblot analyses to evaluate key signaling components and use of pharmacologic agents to assess pathway dependence over 21 days (to detect slow-growing GPCs) were carried out for A (i), B (i), Wnt; A (ii), B (ii), Notch; and A (iii), B (iii), TGFb pathways. *, P < 0.05; **, P < 0.01; ***, P < 0.001. DMSO, dimethyl sulfoxide.
were biasing the gene signature selection toward better surviving oligodendroglial tumors by our filtering procedure, we in addition derived a "stemness" gene signature by comparing NNI-8 GPCs to its primary tumor (Supplementary Tables S5–S7). This, we rationalized, would allow an assessment of the GPC traits within the bulk tumor mass. This gene signature similarly stratified patient survival, with the (+) class enriched for lower grade tumors of proneural classification, whereas the (−) class enriched for higher grade tumors with mesenchymal features (Supplementary Fig. S4). Collectively, our data support that human oligodendroglial GPCs contribute to favorable prognosis, likely mediated by more chemosensitive OPC-like properties.

The oligodendroglial GPC signature is enriched in the Wnt, Notch, and TGFβ pathways in patient glioma databases

Our previous findings indicate that the oligodendroglial GPC signature is enriched in the Wnt, Notch, and TGFβ signaling pathways (Supplementary Fig. S3B); however, their activation or downregulation is unclear. On the basis of our in vitro data in a limited but unique GPC collection (Fig. 1), we suggested that oligodendroglial GPCs were more sensitive to Wnt and Notch inhibition, whereas GBM GPCs tended to be responsive to TGFβR1 inhibition. In recognizing the limitations posed by a small GPC panel, as with any such studies to-date, we sought to understand whether our GPC-derived conclusions bore similar significance in 2 of the largest patient glioma databases. We rationalized that our hypothesis would suggest the similar regulation of signaling pathways as predicted by our GPCs in Fig. 1 and the sheer number of patients in REMBRANDT (N = 298) and Gravendeel (N = 276) would provide firm evidence. In addition, we analyzed a panel of primary tumors by immunohistochemical staining and observed similar pathway regulation (Fig. 4); that is, GBM tumors exhibited elevated p-Smad2 expression (P = 0.0122) whereas oligodendroglial tumors displayed elevated NICD expression (P = 0.0331) and a trend toward elevated active β-catenin (3 of 4 tumors). Accordingly, using GSEA (49), we observed the following (Table 2): (i) The (−) activation score patients defined by our Connectivity Map, which correlate inversely with the oligodendroglial gene signature (i.e., more GBM GPC-like) in both databases, showed upregulated TGFβ1 response pathways upon closer analysis of the gene modules, further supported by downregulation of this pathway in Gravendeel (−) cohort. This is consistent with our in vitro data which suggest that GBM GPCs respond more strongly to TGFβR1 inhibition than oligodendroglial GPCs [Fig. 1A (iii) and B (iii)]. Furthermore, Gravendeel (−) patients showed upregulation of the Nutt_GBM versus AO (anaplastic oligodendroglioma) gene module, providing an independent verification that our GBM versus oligodendroglial GPCs mirror their primary tumor transcriptomic profile; (ii) The (+) patient cohort in Gravendeel showed upregulation of Wnt signaling pathway, again consistent with our in vitro data where NNI-7 and NNI-8 oligodendroglial GPCs were more sensitive to Wnt inhibition [Fig. 1A (i) and B (i)]; and (iii) The REMBRANDT (−) patients showed upregulation of Notch signaling. Upon

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<th>Table 1. Summary of results from Connectivity Maps, log-rank, and Cox regression analysis for all patient samples</th>
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NOTE: (+) represents patients with concordance to oligodendroglial GPC signature; (−) represents patients with inverse gene expression relationship to oligodendroglial GPC signature.
Closer analysis, this upregulation comprised the Notch inhibitor, Numbl homolog, which acts to inhibit Notch signaling. This is thus consistent with Fig. 1A (ii) and B (ii) findings where NNI-7 and NNI-8 oligodendroglial GPCs were more sensitive to Notch pathway inhibitors. In addition, we analyzed the enrichment of core stem cell programs (embryonic, hematopoietic, and neural stem cell) in the patient cohorts (12). CMAP\textsuperscript{+} patients display an enrichment of progenitor-like behavior with lower tumor grade, whereas CMAP\textsuperscript{-} patients resemble the CD34\textsuperscript{+} leukemia-initiating and -propagating cells (Table 2). These data suggest that core stem cell programs do contribute to the survival-correlated CMAP patient cohorts. Collectively, by interrogating independent, public glioma databases, we show that predictions made by our oligodendroglial GPC signature produced congruent data in GPCs, primary tumors, and patient databases. This thus supports our hypothesis that GPCs mirror their primary tumors and contribute to disease progression and survival outcome.

**Figure 3.** Oligodendroglial GPC gene signature stratifies patient survival. Patient survival is shown in all glioma patients in A, REMBRANDT; and B, Gravendeel databases. Tumor grade ("Grade") and molecular classification ("Phillips") distribution corresponding to (+) and (−) classes are shown below the activation score graphs.
The oligodendroglial GPC signature defines molecular heterogeneity within oligodendroglial tumors

Next, we interrogated this GPC gene signature in patients with oligodendroglial tumors. The (+) class enriched for lower tumor grades associated with the 1p/19q codeletion (Fig. 5). Interestingly, patients without loss-of-heterozygosity at 1p/19q (yellow) were spread throughout both classes, indicating that our stemness gene signature detected molecular heterogeneity and survival profiles that cannot be accounted for by the 1p/19q status alone. Although these retrospective data cannot determine whether the gene signature is an independent predictor of survival; furthermore, the 1p/19q status is specifically related to PCV chemotherapy (procarbazine, CCNU and vincristine; ref. 21); nevertheless, these data do suggest that the signature is a positive prognostic factor for human glioma patients.

Discussion

GPCs mirror the phenotypic and molecular fingerprint of their primary tumors (27). Consequently, they serve as a useful in vitro platform to carry out further investigations. However, much less is known about their direct contribution to disease progression and survival outcome. We attempted to address this gap in knowledge by: (i) tapping into publicly available GPC gene expression from several investigators and determining the differential gene list between 2 major variants, oligodendroglial GPCs versus GBM GPCs for which distinct patient survival patterns are seen in the primary tumors; (ii) using a rank-based, pattern-matching approach, the Connectivity Map (CMAP), to interrogate the strength of association between the oligodendroglial gene signature and individual patient gene expression profiles, as gene expression drives glioma disease outcome (16); (iii) drawing connections between (+) or (−) patients, tumor grade, and primary tumor molecular classification.

We found that oligodendroglial GPCs could be distinguished from GBM GPCs by Wnt, Notch, and TGFβ regulation. Although these findings are not entirely novel in that these pathways were previously implicated in GBM GPCs, their relation between the 2 major variants—oligodendroglial versus GBM GPCs is unclear. Our in vitro analysis showed that Wnt and Notch pathways were upregulated in NNI-7 and NNI-8 oligodendroglial GPCs, whereas TGFβ signaling was upregulated in GBM GPCs. Moreover, these pathways were similarly detected in primary tumors. Interestingly, Lottaz and colleagues showed that mesenchymal GPCs map into the mesenchymal class of primary tumors and exhibit upregulated TGFβ signaling pathway (26). In recognizing that a limited number of patient specimens were available for our in vitro and primary tumor analyses, we sought to tap into major patient glioma gene expression and molecular signature databases to substantiate our hypothesis that GPCs contribute to disease outcome. Indeed, using our oligodendroglial gene signature, our GSEA study indicated that patients with GBM (CMAP−) are enriched in the TGFβ signaling module, whereas patients with oligodendroglial tumors (CMAP+) are enriched in the Wnt and Notch pathways. Moreover, CMAP+ patients display a progenitor-like transcriptomic program that correlates with lower tumor grade, consistent with the idea previously established in a transgenic mouse model of oligodendroglioma that identified the more lineage-committed oligodendrocyte progenitor cell as the tumor cell-of-origin (48). Furthermore, these cells are more sensitive to standard chemotherapeutic drugs than neural stem cells. Our study is important because it provides clinical evidence, using large databases, that GPCs contain signaling pathways that dictate primary tumor progression, consequently impacting on survival outcome. These findings emphasize the relevance of in vitro cultured GPCs as investigative tools. Furthermore, our oligodendroglial gene signature stratified survival of even oligodendroglial tumor patients without 1p/19q LOH, suggesting that this
<table>
<thead>
<tr>
<th>Gene sets</th>
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<tbody>
<tr>
<td>ST_WNT_BETA_CATENIN_PATHWAY</td>
<td>Wnt/beta-catenin pathway (N = 31)</td>
<td>28</td>
<td>1.45</td>
<td>1</td>
<td>Wnt pathway is upregulated, whereas TGFβ1 signaling is downregulated in Gravendeel (+) patients</td>
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<td>JAZAG_TGFB1_SIGNALING_VIA_SMAD4_DN</td>
<td>Genes downregulated in PANC-1-S4KD cells (pancreatic cancer; SMAD4 knocked down by RNAi) after stimulation by TGFB1 for 2 hours (N = 64)</td>
<td>51</td>
<td>1.4</td>
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<td>VERRECCHIA_RESPONSE_TO_TGFB1_1</td>
<td>ECM-related genes upregulated in dermal fibroblasts within 30 minutes after TGFB1 addition and kept increasing with time (N = 18)</td>
<td>16</td>
<td>−1.58</td>
<td>0.704</td>
<td>TGFβ1 signaling and Nutt GBM vs. anaplastic oligodendroglioma (AO) are upregulated in Gravendeel (−) patients</td>
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<td>VERRECCHIA_EARLY_RESPONSE_TO_TGFB1</td>
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<td>47</td>
<td>−1.53</td>
<td>0.73</td>
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<tr>
<td>NUTT_GBM_VS_AO_GLIOMA_UP</td>
<td>Top 50 marker genes for GBM, a class of high-grade glioma (N = 47)</td>
<td>42</td>
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<td>0.746</td>
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<td>Notch signaling is downregulated (due to Numb) whereas TGFβ1 pathway is upregulated in REMBRANDT (−) patients</td>
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<td>−1.53</td>
<td>0.972</td>
<td>Stem cell core programs enriched in REMBRANDT (−) patients</td>
</tr>
<tr>
<td>VERRECCHIA_RESPONSE_TO_TGFB1_1</td>
<td>ECM-related genes upregulated in dermal fibroblasts within 30 minutes after TGFB1 addition and kept increasing with time (N = 22)</td>
<td>18</td>
<td>−1.47</td>
<td>0.63</td>
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<td>BENPORATH_ES_WITH_H3K27ME3</td>
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<td>0.16</td>
<td>Stem cell core programs enriched in REMBRANDT (−) patients</td>
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<td>0.13</td>
<td>Stem cell core programs enriched in REMBRANDT (−) patients</td>
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<tr>
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<td>Genes upregulated in CD34+ cells isolated from bone marrow of CML (chronic myelogenous leukemia) patients, compared with those from normal donors (N = 1,398)</td>
<td>5</td>
<td>−1.07</td>
<td>0.52</td>
<td>Stem cell core programs enriched in REMBRANDT (−) patients</td>
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(Continued on the following page)
previously "untreatable" class can now be further subdivided into drug-sensitive and -resistant patients. This indicates that our gene signature detects molecular heterogeneity in patients with oligodendroglial tumors that cannot be accounted for by the 1p/19q status alone and further highlights the limitation of morphology-based histologic analyses to diagnose and subsequently treat patients. Although oligodendroglial tumors are traditionally more chemosensitive than GBM tumors and would seemingly render our findings not unexpected, our study is important because we provide a direct clinical link between these controversial GPCs and their primary tumor. Essentially, we show that GPCs of different histologies not only mirror the phenotype and molecular fingerprint of their primary tumor, but also contain transcriptomic profiles that reflect the different survival outcomes. It is therefore of interest what signaling pathways are enriched in these transcriptomic profiles. The use of, for example, Wnt and Notch pathway inhibitors, should therefore be prioritized to treat not only oligodendroglial tumor patients but more importantly the sensitive group from 1p/19q LOH-negative patients. Our study provides strong evidence that long-term self-renewing GPCs are targeted by these pathway inhibitors and should thus prioritize their development for an effective cure, as opposed to inhibitors that abrogate bulk tumor and transient-amplifying cells.

In our study, we focused on defining the GPC by its long-term self-renewing potential, reflected in its ability at serial tumor propagation (6). We have avoided the use of markers as conflicting data have been obtained (5); furthermore, marker expression has been shown to be an artefactual consequence of experimental conditions (50). The importance of this trait was highlighted in several key studies linking CSCs of the breast and acute myeloid leukemia to survival outcome (9, 10), where only serially transplantable stem-like cells (and not marker-based) contributed to disease progression and patient survival, consequently emphasizing the importance of the self-renewing, tumor-initiating, and sustaining property. Indeed, recent works highlighted the contribution stem cell core programs to chemoresistance and survival outcome in other tumor systems (9, 10, 12).

Although our gene signature came from a limited number of GPC lines as with all studies to-date, we improved on its robustness and performance by 2 approaches: (i) We collated gene expression data from GPCs sourced from multiple investigators/laboratories that have published complete functional evidence of serial tumor-propagating

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<tr>
<td>RIGGI_EWING_SARCOMA_PROGENITOR_UP</td>
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<td>5</td>
<td>-0.71</td>
<td>0.89</td>
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<tr>
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<td>Genes possessing the trimethylated H3K27 (H3K27me3) mark in their promoters in human embryonic stem cells, associated with lower grade tumor expression and poor stemness nature (N = 1,117)</td>
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<td>1.12</td>
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<td>1.01</td>
<td>0.93</td>
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<tr>
<td>DIAZ_CHRONIC_MEYLOGENOUS_LEUKEMIA_UP</td>
<td>Genes upregulated in CD34⁺ cells isolated from bone marrows of CML (chronic myelogenous leukemia) patients, compared with those from normal donors (N = 1,398)</td>
<td>5</td>
<td>-1.09</td>
<td>0.76</td>
<td>Stem cell core programs enriched in Gravendeel (-) patients</td>
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<tr>
<td>BENPORATH_NANOG_TARGETS</td>
<td>Genes upregulated and identified by ChIP on chip as Nanog transcription factor targets in human embryonic stem cells (N = 988)</td>
<td>5</td>
<td>-1.01</td>
<td>0.48</td>
<td></td>
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</tbody>
</table>
activity; and (ii) We cross-compared the robustness of our signature performance in 2 large, independent glioma databases, REMBRANDT and Gravendeel, with gene expression generated from bulk tumor samples. In this manner, we leveraged the unavoidable small sample size with validation in much larger data sets. Thus, clinically amenable molecular tests may be developed by profiling unsorted bulk tumor cells because disease progression is in part, a manifest of the activation of stemness-related pathways. Taken together, we provide evidence that human-derived GPCs are clinically relevant.

Disclosure of Potential Conflicts of Interest

W.H. Ng is an employee for Duke-NUS GMS. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: F.S.L. Ng, C. Tang, B.T. Ang
Development of methodology: F.S.L. Ng, M. Phong, G. Tucker-Kellogg, C. Tang, B.T. Ang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.B. Toh, E. Ting, G. R.H. Koh, S.H. Leong, W.H. Ng, C. Tang, B.T. Ang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F.S.L. Ng, T.B. Toh, E. Ting, E. Sandanaraj, M. Phong, S.S. Wong, S.H. Leong, G. Tucker-Kellogg, I. Ng, C. Tang, B.T. Ang
Writing, review, and/or revision of the manuscript: F.S.L. Ng, T.B. Toh, M. Phong, S.S. Wong, O.L. Kon, I. Ng, C. Tang, B.T. Ang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F.S.L. Ng, C. Tang, B.T. Ang
Study supervision: M. Phong, C. Tang, B.T. Ang

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References


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