Running title: Stem-like cells contribute to glioma disease outcome

PROGENITOR-LIKE TRAITS CONTRIBUTE TO PATIENT SURVIVAL AND PROGNOSIS IN OLIGODENDROGLIAL TUMORS

Felicia Soo-Lee Ng1,2*, Tan Boon Toh3†, Esther Hui-Ling Ting3, Geraldene Rong-Hui Koh4, Edwin Sandanara1, Mark Phong2, Swee Seong Wong2, Siew Hong Leong5, Oi Lian Kon5, Greg Tucker-Kellogg2,6, Wai Hoe Ng7,9, Ivan Ng7,9, Carol Tang4,5,6*, Beng Ti Ang1,3,7,8*

1 Singapore Institute for Clinical Sciences, Agency for Science, Technology and Research (A*STAR), Singapore; 2 Lilly Singapore Centre for Drug Discovery, Singapore (Eli Lilly and Company); 3 Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore; 4 Department of Research, National Neuroscience Institute, Singapore; 5 Division of Medical Sciences, Humphrey Oei Institute of Cancer Research, National Cancer Centre, Singapore; 6 Department of Biological Sciences, Faculty of Science, National University of Singapore; 7 Department of Neurosurgery, National Neuroscience Institute, Singapore; 8 Duke-National University of Singapore Graduate Medical School, Singapore

†Authors contributed equally. *Address correspondence to: Carol Tang, Department of Research, National Neuroscience Institute, 11 Jalan Tan Tock Seng, Singapore 308433. Phone: 65-6357-7634; Fax: 65-6256-9178; Email: Carol_Tang@nni.com.sg; Beng Ti Ang, Department of Neurosurgery, National Neuroscience Institute, 11 Jalan Tan Tock Seng, Singapore 308433. Phone: 65-6357-7627; Fax: 65-6357-7137; Email: Beng_Ti_Ang@nni.com.sg or Felicia Soo-Lee Ng, Singapore Institute for Clinical Sciences, A*STAR, Brenner Centre for Molecular Medicine, 30 Medical Drive, Singapore 117609. Phone: 65-6407-0100; Fax: 65-6776-8840; Email: felicia.sl.ng@gmail.com.

Key words: Glioma, cancer stem cell, REMBRANDT, Gravendeel, Connectivity Map
STATEMENT OF TRANSLATIONAL RELEVANCE

Gliomas predominate the spectrum of adult malignant brain tumors and can arise from stem-like glioma-propagating cells (GPCs). 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 oligodendrogial 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 oligodendrogial from GBM GPCs. Taken together, our data provide a direct link between GPCs and disease progression, highlighting the clinical relevance of these cells.

ABSTRACT

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

Experimental Design: We tapped into public sources of GPC gene expression data and derived a gene signature distinguishing oligodendrogial from 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 TGFβ, in GPCs and primary tumor specimens.
Results: We observed that patients with better prognosis correlated with oligodendrogial 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 while the poorly surviving cohort had Mesenchymal tumors. Additionally, oligodendrogial GPCs were more sensitive to Wnt and Notch inhibition while GBM GPCs responded to TGFβR1 inhibition.

Conclusions: We provide evidence that GPCs are clinically relevant. In addition, the more favorable prognosis of oligodendrogial 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 oligodendrogial 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 histological analyses in tumor classification, consequently impacting on treatment decisions.
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 oligodendrogial precursor (1-3). Such cells have been shown to be enriched in glioma-propagating cells (GPCs) cultured in vitro (4); however, specific markers for the GPC have been controversial (5). This forces a re-evaluation of the definition of the GPC to encompass the following characteristics (6): They must demonstrate long-term self-renewal, stemness and multipotentiality, and the ability to form serially transplantable, orthotopic xenograft tumors that recapitulate the patient’s original histopathology. Kornblum and colleagues recently demonstrated 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, the data nevertheless suggest that GPCs cultured in vitro contribute to patient survival. Here, we asked the critical question of whether a gene expression 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 re-growth (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 cancer stem cells and colorectal cancer stem cells 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, GBM and oligodendrogial tumors are molecularly heterogeneous, with each sub-class 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 utilized gene expression analyses to explore the clinical relevance of GPCs derived from GBM and oligodendrogial 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 if GPCs derived from these 2 major glioma variants demonstrate the uniqueness of each tumor subtype. Oligodendrogial tumors tend to have a favorable prognosis and chemosensitivity is predicted by the only known clinical indicator, the 1p/19q co-deletion status (21). It would therefore be important to understand if 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 biological 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 biological 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 demonstrated that such a method supported the mechanistic role of Parkin as a tumor suppressor in glioma (24). We show that this oligodendrogial gene signature is a positive prognostic factor in gliomas and detects heterogeneity in oligodendrogial tumor patient survival profiles that cannot be predicted by the 1p/19q co-deletion 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 glioma-propagating cell 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 epidermal growth factor (17-19). Although Pollard lines were cultured on laminin (19), a recent molecular classification study demonstrated that both culture methods preserved the biological 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 NOD-SCID gamma mice (17). Only low passage GPCs were utilized for our studies (P1-10). We and others have shown previously that serial propagation as orthotopic xenografts maintain GPC traits (17, 27). NNI-7 and NNI-8 are new GPC lines derived from patients with anaplastic oligoastrocytoma, and characterized according to previous methods (Supplementary Figs. S1-2) (17). Accordingly, NNI-8 GPCs exhibited positivity for CD133 and Nestin markers, with low levels of SSEA-1/CD15 and aldehyde dehydrogenase (ALDH) (Supplementary Fig. S1B). GPC frequency and proliferation were also maintained for up to 3 passages in vitro (Supplementary Fig. S1C). NNI-8 GPCs demonstrated presence of stemness marker expression such as Musashi-1 (Msi-1), octamer-binding transcription factor 4 (Oct4) and were actively dividing as indicated by ~30% of Ki-67-positive cells (Supplementary Fig. S1D). The GPCs also exhibited multi-lineage potential, showing presence of aberrant cells co-staining for neuron-specific class III beta-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 while GS-2 was derived from a high grade tumor with oligodendrogial 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 while 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: (1) Mouse monoclonal anti-Nestin (1:200, Chemicon, MAB5326); anti-Ki-67 (1:200, Chemicon, MAB4190); anti-TuJ1 (1:200, Chemicon, MAB1637) and anti-O4 (1:50, Chemicon, MAB345); (2) Rabbit polyclonal anti-Oct4 (H-134, 1:100, Santa Cruz, SC9081); anti-GFAP (1:3000, DAKO, Z0334) and anti-Musashi (1:100, Chemicon, AB5977). Antibodies used for primary tumor or tumor xenograft paraffin sections included: (1) 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 (2) Rabbit polyclonal anti-CD133 (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-Prkdc<sup>scid</sup> Iloc<sup>m1Wvs</sup>/SzJ, The Jackson Laboratory) were stereotaxically injected with NNI-8 or lentivirally-transduced GPCs utilizing pLKO.1-β-catenin knockdown constructs (Clones: shβcat1 (TRCN0000003843) and shβcat2 (TRCN0000003844)) from OpenBiosystems or a non-targeting (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 neurological 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 Laviania Corporation (CA, USA) according to the
published chemical structure. The small molecule inhibitors of Notch signaling, γ-secretase inhibitor (30) and DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) (31) were purchased from Sigma. TGFβR1 inhibitor, SB525334 (32) was procured from TOCRIS bioscience. GPCs were treated at 10 μM for γ-secretase inhibitor, DAPT, and SB525334, and at IC₅₀ concentrations for Cercosporin and CCT036477 (data not shown). TGFβ1, used at 200 pM, 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% sodium dodecyl sulphate, 0.15 M NaCl, 10 mM 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 PVDF membranes. Membranes were processed according to standard procedures and proteins detected using the imaging system, SYNGENE G:Box, iChemXiXT. The following antibodies were used: Anti-active β-catenin (8E7, 1:1000; Millipore, #05-665), anti-β-catenin (1:1000, BD Transduction Laboratories, #610153), anti-cleaved Notch 1 (NICD) (1:1000; Cell Signaling, #2421), anti-phospho-Smad2 (Ser465/467) (1:1000; Cell Signaling, #3108), anti-Smad2 (1:1000; Cell Signaling, #3122), anti-phospho-Smad3 (Ser423/425) (1:1000; Millipore, #07-1389), anti-Smad3 (1:1000; Cell Signaling, #9523) and anti-β-actin (AC-15, 1:10000; Sigma, A5441).

Statistical analysis

Data are expressed as means ± standard error of the mean (SEM) of at least 3 independent experiments. Student’s 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 (18) and Pollard (19) publications and were processed similarly. To understand the transcriptomic differences between oligodendroglial gliomas and
GBM, a linear model was fitted with batch correction using the limma package (35). Additionally, a linear model was applied to gene expression data of NNI-8 GPC cells and its primary tumor. For both analyses, probesets with adjusted p-value ≤ 0.05 were considered significant and used as inputs for pathway analysis in MetaCore from GeneGo, Inc. Significantly enriched process networks and canonical pathways were analyzed and top ranking results were reported. All array platform gene annotation was derived from Rinmart (36). Raw and processed data are available on the GEO public database website.


To further interrogate the oligodendroglial feature of glioma, we defined an "oligodendroglial GPC signature" using a log ratio cut-off of 0.8. Similarly, the "NNI-8 GPC versus tumor" stemness signature was obtained by taking the top ranking differentially expressed probesets using the log ratio cut-off 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 versus 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 non-parametric, 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 two-tailed test p-values associated with each activation score were calculated as described in Lamb et al. (22). P-values ≤ 0.1 were considered significant.
Reference profile generation for Connectivity Map analysis

Public GBM datasets 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 dataset (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, the data was quantile normalized and gene expression values were row-wise median centered. Median centering each probeset allows us to study the range of gene expression values in a large dataset.

Survival analysis

Kaplan-Meier and Cox regression analysis of (+) and (-) groups were done in R using the survival package (38). For the REMBRANDT dataset, 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 datasets

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

REMBRANDT SNP array processing and 1p19q LOH analysis

CEL files from the Affymetrix 100K SNP Arrays of oligodendroglioma and oligoastrocytoma patients 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 performed 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. GSEA tool was downloaded from Broad Institute portal (42). The significantly enriched genesets in molecular signature database (MSigDB) were further analyzed for phenotypic correlation in the reference datasets.
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 (17)), Gunther (18) and Pollard (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 demonstrated 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 oligodencrogial 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: (1) Immunoblot analysis of key pathway components; and (2) Dependence on pathway by utilizing well-established pharmacological inhibitors.

NNI-7 and NNI-8 oligodendrogial GPCs demonstrated increased sensitivity to Cercosporin (28) and CCT036477 (29) compared to the other 2 of 3 GBM GPCs tested, consistent with the highest level of active β-catenin detected (nuclear-localized) (Fig. 1Ai, Bi). Sphere frequency was significantly reduced upon pathway inhibition, indicating that GPCs were targeted (Fig. 1Bi). Moreover, lentiviral-mediated β-catenin knockdown abrogated glioma formation in an orthotopic mouse model, consequently extending survival compared to the non-targeting 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 compared
to NNI-11, 12 and 4 (Fig. 1Bii). Again, these findings were consistent with the immunoblot analysis demonstrating highest level of Notch intracellular domain (NICD) detected in NNI-8 GPCs (Fig. 1Aii).

Finally, we tested TGFβ signaling by utilizing SB525334 (32). Interestingly, all 3 GBM GPCs demonstrated sensitivity to SB525334 with up to 80% inhibition in NNI-4 (Fig. 1Biii). A less clear pattern of phospho-Smad2 and phospho-Smad3 levels was observed upon TGFβ1 stimulation (Fig. 1Aiii). 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, while 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 30-50% of all patients in each database (Table 1). Importantly, the signature stratified patient survival (Fig. 3). Patients with better survival comprised 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; while 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) 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% confidence interval) 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 dataset, this does not mean the absence of GPC transcriptome contribution to patient survival as demonstrated in the REMBRANDT dataset. Firstly, most glioma databases are retrospectively generated and therefore, this limits our ability to assess the true predictive value of the gene signature. Secondly, 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 oligodendroglial GPC signature correlates with “Phillips” molecular classification of gliomas. We next attempted to strengthen our findings based on the Connectivity Map (CMAP) by asking if 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 sub-classes: Proneural, Proliferative and Mesenchymal. The (+) activation score enriched for the Proneural sub-class, while the (-) activation score tended to be Proliferative or Mesenchymal (Fig. 3; Supplementary Table S4). Proneurals are typically lower grade gliomas with oligodendroglial features, frequently associated with better prognosis; in contrast, the Mesenchymal sub-class 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 (OPCs), compared to 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 were biasing the gene signature selection towards better surviving oligodendroglial tumors by our filtering procedure, we additionally derived a “stemness” gene signature by comparing NNI-8 GPCs to its primary tumor (Supplementary Tables S5-7). 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, while 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. Based on 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, while 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 if 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); i.e. GBM tumors exhibited elevated pSmad2 expression (p=0.0122) while oligodendroglial tumors displayed elevated Notch intracellular domain expression (p=0.0331) and a trend towards elevated active β-catenin (3 of 4 tumors). Accordingly, using Gene Set Enrichment Analysis (GSEA) (49), we observed the following (Table 2): (1) 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 module, further supported by downregulation of this pathway in Gravendeel (+) cohort. This is consistent with our in vitro data that suggests GBM GPCs respond more strongly to TGFβR1 inhibition than oligodendroglial GPCs (Fig. 1Aii, Biii). Furthermore, Gravendeel (-) patients showed upregulation of the Nutl_GBM vs AO (anaplastic oligodendroglioma) gene module, providing an independent verification that our GBM versus oligodendroglial GPCs mirror their primary tumor transcriptomic profile; (2) 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. 1Ai, Bi); and (3) The REMBRANDT (-) patients showed upregulation of Notch signaling. Upon closer analysis, this upregulation comprised the Notch inhibitor, Numb homolog, which acts to inhibit Notch signaling.

This is thus consistent with Fig. 1Aii, Bii 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+ patients display an enrichment of progenitor-like behavior with lower tumor grade, while CMAP- patients resemble the CD34+ 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.

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 co-deletion (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) (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: (1) 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; (2) 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, since gene expression drives glioma disease outcome (16); (3) 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 demonstrated 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 et al. 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 GBM patients (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 investigational tools. Furthermore, our oligodendrogial gene signature stratified survival of even oligodendrogial tumor patients without 1p/19q LOH, suggesting that this 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 oligodendrogial tumors that cannot be accounted for by the 1p/19q status alone, and further highlights the limitation of morphology-based histological analyses to diagnose and subsequently treat patients. Although oligodendrogial 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 oligodendrogial 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 cancer stem cells 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 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 datasets. 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.
ACKNOWLEDGEMENTS

The authors acknowledge financial support from competitive grants awarded by the Biomedical Research Council, A*STAR to C. Tang (07/1/33/19/530, 09/1/33/19/611) and institutional grant funding to B.T. Ang at the Singapore Institute for Clinical Sciences, A*STAR. We are grateful to Dr. Khoon Leong Chuah of Tan Tock Seng Hospital (Department of Pathology) for pathological analyses and Selamat Wata for excellent technical assistance. We thank lab members and Jonathon D. Sedgwick of Lilly Singapore Centre for Drug Discovery for critical review of the manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The other authors indicate no potential conflicts of interest.
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TABLES

Table 1. Summary of results from Connectivity Maps, Logrank and Cox Regression Analysis for all patient samples.

Table 2. Summary of Gene Set Enrichment Analysis.
Table 1. Summary of results from Connectivity Maps, Logrank and Cox Regression Analysis for all patient samples. (+) represents patients with concordance to oligodendrogial GPC signature; (-) represents patients with inverse gene expression relationship to oligodendrogial GPC signature.

<table>
<thead>
<tr>
<th>Dataset</th>
<th># of probes</th>
<th># of samples</th>
<th>(+)</th>
<th>(-)</th>
<th>total (+)(-)</th>
<th>%(+)(-)</th>
<th>Log Rank p-value</th>
<th>Hazard Ratio</th>
<th>p-value</th>
<th>Multivariate Cox</th>
<th>Hazard Ratio</th>
<th>p-value</th>
<th>Univariate Cox</th>
<th>Hazard Ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>REMBRANDT</td>
<td>95</td>
<td>298</td>
<td>86</td>
<td>81</td>
<td>147</td>
<td>49.33</td>
<td>1.93E-05</td>
<td>0.440</td>
<td>0.001-0.643</td>
<td>2.22E-05</td>
<td>0.462</td>
<td>0.322-0.664</td>
<td>2.90E-05</td>
<td>0.536</td>
<td>0.334-0.856</td>
</tr>
<tr>
<td>Gravendeel</td>
<td>95</td>
<td>276</td>
<td>58</td>
<td>34</td>
<td>92</td>
<td>33.33</td>
<td>0.0082</td>
<td>0.851</td>
<td>0.504-1.436</td>
<td>0.546</td>
<td>0.536</td>
<td>0.334-0.856</td>
<td>0.009</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 2. Summary of Gene Set Enrichment Analysis.

<table>
<thead>
<tr>
<th>Genesets</th>
<th>Description</th>
<th>Size</th>
<th>Normalized enrichment Score</th>
<th>FDR q-value</th>
<th>CMAP Class Association</th>
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<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, while TGFβ1 signaling is downregulated in Gravendeel (+) patients</td>
</tr>
<tr>
<td>JAZAG_TGFβ1_SIGNALING_VIA_SMAD4_ON</td>
<td>Genes down-regulated in PANC-1-S4KD cells (pancreatic cancer, SMAD4 knocked down by RNAi) after stimulation by TGFβ1 for 2 h (N=64)</td>
<td>51</td>
<td>1.4</td>
<td>0.978</td>
<td></td>
</tr>
<tr>
<td>VERRECHIA_RESPONSE_TO_TGFβ1_C1</td>
<td>ECM related genes up-regulated in dermal fibroblasts within 30 min after TGFβ1 addition, and which kept increasing with time (N=18)</td>
<td>16</td>
<td>-1.58</td>
<td>0.704</td>
<td></td>
</tr>
<tr>
<td>VERRECHIA_Response_TO_TGFβ1</td>
<td>ECM related genes up-regulated early (within 30 min) in dermal fibroblasts after addition of TGFβ1 (N=51)</td>
<td>47</td>
<td>-1.53</td>
<td>0.73</td>
<td>TGFB1 signaling and Numb GBM versus Anaplastic Oligodendroglioma (AO) are upregulated in Gravendeel (-) patients</td>
</tr>
<tr>
<td>NUTT_O6MSA_Q_GLIOMA_UP</td>
<td>Top 50 marker genes for glioblastoma multiforme (GBM), a class of high grade glioma (N=47)</td>
<td>42</td>
<td>-1.52</td>
<td>0.746</td>
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<tr>
<td>KG1_BUL_H_S15_NALING_PATHWAY</td>
<td>Notch signaling pathway (N=47)</td>
<td>40</td>
<td>-1.86</td>
<td>1</td>
<td></td>
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<tr>
<td>VERRECHIA_RESPONSE_TO_TGFβ1</td>
<td>ECM related genes up-regulated early (within 30 min) in dermal fibroblasts after addition of TGFβ1 (N=51)</td>
<td>47</td>
<td>-1.53</td>
<td>0.972</td>
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<tr>
<td>VERRECHIA_RESPONSE_TO_TGFβ1_C5</td>
<td>ECM related genes up-regulated in dermal fibroblasts within 30 min after TGFβ1 addition, and which kept increasing with time (N=22)</td>
<td>18</td>
<td>-1.47</td>
<td>0.63</td>
<td>Notch signaling is downregulated (due to Numb) while TGFβ1 pathway is upregulated in REMBRANDT (-) patients</td>
</tr>
<tr>
<td>VERRECHIA_RESPONSE_TO_TGFβ1_C1</td>
<td>ECM related genes up-regulated in dermal fibroblasts within 30 min after TGFβ1 addition, and which kept increasing with time (N=18)</td>
<td>18</td>
<td>-1.43</td>
<td>0.559</td>
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<tr>
<td>BENPORATH_ES_WITH_H3K27ME3</td>
<td>Genes possessing the trimethylated H3K27 (H3K27me3) mark in their promoters in human embryonic stem cells, associated with lower grade tumour expression and poor stemness nature (N=1117)</td>
<td>8</td>
<td>1.36</td>
<td>0.16</td>
<td>Stem cell core programs enriched in REMBRANDT (+) patients</td>
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<tr>
<td>BENPORATH_EED_TARGETS</td>
<td>Eed targets genes identified by ChIP on chip as targets of the Polycomb protein EED in human embryonic stem cell, associated with lower grade tumour expression and poor stemness nature (N=1062)</td>
<td>6</td>
<td>1.34</td>
<td>0.13</td>
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<td>Author Manuscript Published OnlineFirst on June 6, 2012; DOI: 10.1158/1078-0432.CCR-11-3064</td>
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<tr>
<td>Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.</td>
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<td></td>
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</table>

<table>
<thead>
<tr>
<th>Gene Set</th>
<th>Description</th>
<th>Cell Line</th>
<th>q-value</th>
<th>FDR</th>
<th>Enrichment Status</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diaz Chronic Myelogenous Leukemia</strong> Up</td>
<td>Genes up-regulated in CD34+ cells isolated from bone marrow of CML (chronic myelogenous leukemia) patients, compared to those from normal donors (N=1368)</td>
<td>5</td>
<td>-1.07</td>
<td>0.52</td>
<td>Stem cell core programs enriched in REMBRANDT (-) patients</td>
</tr>
<tr>
<td><strong>Rigli Ewing Sarcoma Progenitor</strong> Up</td>
<td>Genes up-regulated in mesenchymal stem cells (MSC) engineered to express EWS-FL1 fusion protein (N=42)</td>
<td>5</td>
<td>-0.71</td>
<td>0.89</td>
<td>Stem cell core programs enriched in REMBRANDT (-) patients</td>
</tr>
<tr>
<td><strong>Benforath ES With H3K27Me3</strong></td>
<td>Genes possessing the trimethylated H3K27 (H3K27me3) mark in their promoters in human embryonic stem cells, associated with lower grade tumour expression and poor stemness nature (N=117)</td>
<td>8</td>
<td>1.12</td>
<td>1</td>
<td>Stem cell core programs enriched in Gravendeel (+) patients</td>
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<tr>
<td><strong>Benforath EED Targets</strong></td>
<td>Eed targets genes identified by ChIP on chip as targets of the Polycomb protein EED in human embryonic stem cell, associated with lower grade tumour expression and poor stemness nature (N=1062)</td>
<td>5</td>
<td>1.01</td>
<td>0.93</td>
<td>Stem cell core programs enriched in Gravendeel (+) patients</td>
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<tr>
<td><strong>Diaz Chronic Myelogenous Leukemia</strong> Up</td>
<td>Genes up-regulated in CD34+ cells isolated from bone marrow of CML (chronic myelogenous leukemia) patients, compared to those from normal donors (N=1368)</td>
<td>5</td>
<td>-1.09</td>
<td>0.76</td>
<td>Stem cell core programs enriched in Gravendeel (+) patients</td>
</tr>
<tr>
<td><strong>Benforath Nanog Targets</strong></td>
<td>Genes upregulated and identified by ChIP on chip as Nanog transcription factor targets in human embryonic stem cells (N=938)</td>
<td>5</td>
<td>-1.01</td>
<td>0.48</td>
<td>Stem cell core programs enriched in Gravendeel (+) patients</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Functional validation of the Wnt, Notch and TGFβ signaling pathways in GPCs. Immunoblot analyses to evaluate key signaling components, and use of pharmacological agents to assess pathway dependence over 21 days (to detect slow-growing GPCs) were carried out for Ai, Bi, Wnt; Aii, Bii, Notch and Aiii, Biii, TGFβ pathways. *, p<0.05; **, p<0.01; ***, p<0.001.

Figure 2. Lentiviral-mediated β-catenin knockdown abrogates GPC tumorigenicity. NOD-SCID gamma mice were implanted with GPCs lentivirally transduced with non-targeting control (NT), shβcat1 or shβcat2 vectors according to (A). The time to development of neurological deficits was monitored (B). ***, p<0.001. Representative images of mouse brains are shown in (C). Scale bar denotes 0.2 cm.

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.

Figure 4. Analysis of Wnt, Notch and TGFβ signaling pathways in primary tumors. A, active β-catenin; B, Notch intracellular domain (NICD); and C, phospho-Smad2 were immunohistochemically detected in patient tumors of GBM and oligodendroglial (Oligo) features.

Figure 5. Oligodendroglial GPC gene signature is associated with lower tumor grade and 1p/19q co-deletion. Tumor grade (“Grade”) and 1p/19q co-deletion distribution corresponding to (+) and (-) classes are shown below the activation score graphs in A, REMBRANDT; and B, Gravendeel databases.
Figure 2, Ng et al.

(A) Neurological sign-free survival (%)

(B) Mice:
- NT shRNA: 8/8
- shJcat1: 0/8
- shJcat2: 0/8
Figure 3, Ng et al.

A

Activation Score

Survival Outcome

p = 1.93E-05

Grade Phillips

B

Gravendeel

p = 0.0082

Grade Phillips

Legend:

Activation Score

Normalized Activation Score

Probability of Survival

Survival Outcome

Samples

REMBRANDT:

Months in study

Gravendeel:

Years in study

- Grade I
- Grade II
- Grade II/III
- Grade III
- Grade IV
- Proneural
- Proliferative
- Mesenchymal

Red: Oligo. GPC (+)
Blue: Oligo. GPC (-)
Figure 4, Ng et al.

A

\[ p = 0.2829 \]

% Active \( \beta \)-catenin expression

GBM (n=9)  Oligo (n=4)

GBM 1  GBM 2  GBM 19  GBM 25  GBM 26  GBM 34  NNI-4_PT  NNI-10_PT  NNI-11_PT  Oligo 24  Oligo 40  NNI-7_PT  NNI-8_PT

B

\[ p = 0.0331 \]

% NICD expression

GBM (n=9)  Oligo (n=4)

GBM 1  GBM 2  GBM 19  GBM 25  GBM 26  GBM 34  NNI-4_PT  NNI-10_PT  NNI-11_PT  Oligo 24  Oligo 40  NNI-7_PT  NNI-8_PT

C

\[ p = 0.0122 \]

% p-SMAD2 expression

GBM (n=9)  Oligo (n=4)

GBM 1  GBM 2  GBM 19  GBM 25  GBM 26  GBM 34  NNI-4_PT  NNI-10_PT  NNI-11_PT  Oligo 24  Oligo 40  NNI-7_PT  NNI-8_PT
Figure 5. Ng et al.

A

Activation Score

Samples

REMBRANDT

Grade

1p/19q

B

Activation Score

Samples

Gravendeel

Grade

1p/19q

Legend:

Activation Score

Normalized Activation Score

Samples

Grade II

Grade II/III

Grade III

LOH

Partial LOH

No LOH

NA
PROGENITOR-LIKE TRAITS CONTRIBUTE TO PATIENT SURVIVAL AND PROGNOSIS IN OLIGODENDROGLIAL TUMORS

Felicia S.L. Ng, Tan Boon Toh, Esther Ting, et al.

Clin Cancer Res  Published OnlineFirst June 6, 2012.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-11-3064

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