Minireview
Gene expression profiling of gliomas: merging genomic and histopathological classification for personalised therapy

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The development of DNA microarray technologies over the past decade has revolutionised translational cancer research. These technologies were originally hailed as more objective, comprehensive replacements for traditional histopathological cancer classification systems, based on microscopic morphology. Although DNA microarray-based gene expression profiling (GEP) remains unlikely in the near term to completely replace morphological classification of primary brain tumours, specifically the diffuse gliomas, GEP has confirmed that significant molecular heterogeneity exists within the various morphologically defined gliomas, particularly glioblastoma (GBM). Herein, we provide a 10-year progress report on human glioma GEP, with focus on development of clinical diagnostic tests to identify molecular subtypes, uniquely responsive to adjuvant therapies. Such progress may lead to a more precise classification system that accurately reflects the cellular, genetic, and molecular basis of gliomagenesis, a prerequisite for identifying subsets uniquely responsive to specific adjuvant therapies, and ultimately in achieving individualised clinical care of glioma patients.

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Morphological evaluation of cancers by light microscopy has been the foundation for diagnosis, prognostication, and therapeutic stratification for well over a century. However, patients with morphologically identical tumours can have significantly different clinical outcomes. To address the pressing medical need for more accurate predictions, a variety of transformativ technologies have been developed over the last four decades – electron microscopy, molecular biology, immunohistochemistry, and quantitative RT–PCR – to refine traditional cancer classification or as outright replacements. The newest such technology, DNA microarrays, was introduced in 1995, and its potential clinical utility in oncology was quickly recognised. In fact, the Director of the US National Cancer Institute issued a challenge to the scientific community in 1999 (NCI, 1999) to ‘harness the power of comprehensive molecular analysis technologies to make the classification of tumours vastly more informative. This challenge is intended to lay the groundwork for changing the basis (emphasis added) of tumour classification from morphological to molecular characteristics.’ The response from the cancer research community has been intense: nearly 14 000 publications have utilised DNA microarrays for genome-wide gene expression profiling (GEP) in all aspects of cancer research, from basic to translational to clinical. GEP has unequivocally established that significant molecular heterogeneity exists within morphologically defined cancers and that potentially clinically relevant molecular subtypes can be identified. However, to date, only two molecular diagnostic tests, developed using DNA microarrays, have either been approved by the US Food and Drug Administration (MammaPrint) or incorporated into practice guidelines (Oncotype Dx) for clinical use in breast cancer (Weigelt et al, 2009).

This discordance between scientific productivity and clinical implementation over the course of a decade is not unexpected, given the stringent sample requirements, pace of technology development, data volume and complexity, continually evolving data analysis techniques, lack of defined best practices for analysis, and levels of evidence required for clinical use. A number of excellent review articles have discussed these and other impediments in implementing GEP clinically (Dupuy and Simon, 2007; Weigelt et al, 2009; Subramanian and Simon, 2010). Herein, we review a decade of DNA microarray-based GEP on the most common and biologically aggressive group of primary brain tumours, the diffuse gliomas (hereafter referred to as gliomas). The discussion will re-visit morphological classification and address the potential role of GEP in identifying clinically relevant molecular subtypes of gliomas. We will then primarily focus on studies that have examined the prognostic impact of multi-gene signatures for the most deadly glioma, glioblastoma (GBM).

MORPHOLOGICAL CLASSIFICATION OF GLIOMAS

Bailey and Cushing established the first diagnostic classification system for primary brain tumours in 1926, based on their
understanding of the histogenetic basis of brain development and the morphological resemblance of primary brain tumours to their presumed developmental counterparts by light microscopy. This system has been refined periodically, culminating in the presumed developmental counterparts by light microscopy. The morphological resemblance of primary brain tumours to their understanding of the histogenetic basis of brain development and (KPS), and therapy. This prognostic variability can be visualised prognostic factors, including age, Karnofsky performance status outcomes that are not otherwise accounted for by established patients within each diagnostic category can have vastly different has facilitated its widespread adoption for clinical patient manage- refinement into distinct prognostic groups is dictated by (Table 1). Further immunohistochemical evidence of differentiation along astro- pathological entities, each characterised by cytological and molecular and genetic features constitute an additional level of detail utilised not only to diagnostically differentiate among these entities but also increasingly to predict clinical outcomes and response to adjuvant therapies.

The prognostic power of the current WHO glioma classification has facilitated its widespread adoption for clinical patient management. However, it has long been recognised that individual patients within each diagnostic category can have vastly different outcomes that are not otherwise accounted for by established prognostic factors, including age, Karnofsky performance status (KPS), and therapy. This prognostic variability can be visualised using the 95% confidence intervals of Kaplan–Meier survival curves (Figure 1). The extent to which prognostic factors account for outcome variability in multivariate Cox proportional hazards models can be quantified with metrics such as Harrell’s C statistics (Table 1) (Miller et al, 2006). Using these two measurements, prognostic variability is least pronounced in astrocytic gliomas (Figure 1A), particularly GBM, and is substantially higher in mixed (Figure 1B) and pure oligodendrogial (Figure 1C) gliomas. Prognostic variability is most pronounced among the lower-grade gliomas (Figures 1D and E). For these gliomas in particular, accurate classification and prognostication have become increasingly dependent on molecular assays. The most notable test detects co-deletion of chromosomal arms 1p and 19q, a genetic signature and favourable prognostic factor, strongly associated with oligodendrogial differentiation (Miller et al, 2006). However, even with ancillary molecular testing, classification of a subset of morphologically ambiguous grade II and III gliomas remains challenging, even among experienced neuropathologists (Miller et al, 2006; Miller and Perry, 2007). Clearly, more objective, molecular methods for diagnostic discrimination among gliomas are needed.

The clinicopathological variables central to the WHO 2007 classification – patient age at diagnosis, differentiation (cytology), histological grade, and 1p19q co-deletion status – account for

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### Table 1 Prognostic utility of the WHO 2007 classification for diffuse gliomas

| WHO grade | Prognostic factor | HR | P-value | ΔC or overall C² |
|-----------|------------------|----|---------|------------------|
| II        | Grade            | 1.9| <0.001  | 0.61             |
|          | Age              | 1.9| <0.001  | 0.08             |
|          | All (N = 987)    | 0.69|         |                  |
| III       | Grade            | 2.2| 0.007   | 0.10             |
|          | Age              | 2.1| <0.001  | 0.15             |
|          | All (N = 559)    | 0.79|         |                  |
| IV        | 1p19q co-del     | 2.6| <0.001  | 0.54             |
|          | Age              | 2.1| <0.001  | 0.15             |
|          | All (N = 559)    | 0.79|         |                  |
|          | 1p19q co-del     | 2.1| 0.020   | 0.54             |
|          | Age              | 2.4| <0.001  | 0.17             |
|          | All (N = 539)    | 0.74|         |                  |
|          | 1p19q co-del     | 1.9| 0.002   | 0.61             |
|          | Age              | 1.8| <0.001  | 0.13             |
|          | Cytology         | 1.7| <0.001  | 0.04             |
|          | Grade            | 2.0| <0.001  | 0.03             |
|          | All (N = 1363)   | 0.83|         |                  |

**Abbreviations**: AA, A3 = anaplastic astrocytomas; AO, O3 = anaplastic oligodendroglioma; codel = co-deletion; CI = confidence interval; DA, A2 = diffuse astrocytoma; HR = hazard ratio; GBM, A4 = glioblastoma; GBM-O, MOA4 = glioblastoma with oligodendroglial features; OA, MOA2 = mixed oligoastrocytoma; ODG, O2 = oligodendroglioma; OS = overall survival; WHO = World Health Organization; y = years. aHarrell’s C statistic for the multivariable Cox proportional hazards model with all factors (C) or ΔC for each individual factor in the model Miller et al (2006). bAge at diagnosis trichotomized as follows: ≤40, 40–60, ≥60 y Miller et al (2006). cNote that GBM-O (MOA4) is not currently recognised as a distinct clinicopathological entity by the WHO; instead, it is considered a morphological pattern of GBM with a slightly more favourable prognosis Louis et al (2007). Data from adult patients (≥20 y) with newly diagnosed gliomas at Washington University School of Medicine (1977–2009 and Miller et al (2006)).
Molecular classification of gliomas

The earliest GEP studies utilised class comparison to identify differentially expressed genes among morphologically defined gliomas. Such genes were found in low-grade vs high-grade astrocytomas (Rickman et al, 2001), high-grade oligodendrogliomas vs GBM (Nutt et al, 2003; Shirahata et al, 2007), primary vs secondary GBM (Goddard et al, 2003; Shi et al, 2003; Tso et al, 2006), adult vs paediatric GBM (Faury et al, 2007), or a variety of morphologically defined glioma subtypes (Goddard et al, 2003; Shi et al, 2003; van den Boom et al, 2003). Using primarily hierarchical clustering on differentially expressed genes, transcriptional profiles of individual tumours were shown to be most similar to those from the same diagnostic category, that is, gliomas of similar differentiation and grade. These studies confirmed that morphological differences among gliomas are reflected at the mRNA transcript level and that differentially expressed genes could be utilised to distinguish among morphologically defined subtypes. However, discordance between morphological diagnosis and GEP-defined molecular subtype was frequent, likely due in part to inclusion of difficult to classify morphologically ambiguous gliomas.

Nutt, Louis, and colleagues provided a glimpse of the potential clinical utility of GEP as an ancillary diagnostic test for more accurate glioma classification (Nutt et al, 2003). These investigators identified genes significantly correlated with either morphologically classical GBM or anaplastic oligodendroglioma in a training set of 21 tumours, and built a class prediction model that showed 86% accuracy in assigning 29 diagnostically challenging gliomas.
GBM and anaplastic oligodendrogliomas to their respective diagnostic categories. More importantly, a statistically significant difference in overall survival for the GEP but not the morphologically defined groups was found, suggesting that GEP may provide more accurate classification and prognostication, particularly for morphologically ambiguous gliomas. These findings were confirmed by Shirahata et al. (2007), who identified 168 differentially expressed genes from PCR array data on 32 GBM and anaplastic oligodendrogliomas, and used a weighted voting algorithm to develop a 67-gene diagnostic assay with 96.6% accuracy in distinguishing between these two prognostically distinct high-grade gliomas from the published Nutt data set (Nutt et al., 2003). Li, Fine, and colleagues provided the first report of a comprehensive, molecular classification of all gliomas (Li et al., 2009). These authors utilised two unsupervised machine-learning methods on a large training set (N = 159) of WHO grade II–IV gliomas taken from all three histological categories. Guided only by molecular data, without influence of prior morphological diagnosis, they identified six hierarchically nested subtypes, divided into two main categories (O and G). The first category contained two subgroups (OA and OB) and the second had four nested subgroups (GA1, GA2, GB1, and GB2). These data confirm that morphological differences among gliomas are reflected at the mRNA transcript level. Survival analyses showed that the O and G main groups and the OA and OB subgroups of O-type tumours, but not the four G subgroups, were prognostically distinct. Importantly, the prognostic impact of the two main subgroups was confirmed in an independent data set from The Cancer Genome Atlas (TCGA), consisting entirely of GBM (Verhaak et al., 2010), whereas that of the six subgroups was confirmed in the REMBRANDT (Madhavan et al., 2009) and Phillips et al. (2006) data sets consisting of all seven gliomas. However, the concordance between GEP-defined subtypes and histopathological diagnoses was not assessed and multivariate survival analyses with known prognostic factors were not conducted.

In retrospect, the aforementioned studies utilised small (N < 100 per diagnostic category), ostensibly convenience cohorts of previously banked, frozen gliomas. As such, individual studies were statistically underpowered to assess the diagnostic discriminatory power of GEP vis-à-vis morphological classification. Moreover, the relatively small sample sizes and lack of data on known prognostic covariates precluded comprehensive, multivariable analyses. Particularly for the earlier studies, the prognostic impact of GEP signatures could not be validated in large, external data sets (Subramanian and Simon, 2010). Fortunately, most data have been deposited in publically available online repositories, including the Gene Expression Omnibus and REMBRANDT (Madhavan et al., 2009). These data have already been instrumental in both novel hypothesis-driven, mechanistic studies (Brennan et al., 2009) and subsequent GEP studies described below. Only through collection of GEP data on a sufficient number of all seven morphologically defined gliomas will it be possible to assess whether GEP will be diagnostically robust enough to replace morphology as the basis for glioma classification.

**GEP IDENTIFIES PROGNOSTICALLY DISTINCT MOLECULAR SUBTYPES OF GLIOMAS**

A number of GEP studies have identified prognostically distinct molecular subtypes of gliomas. In 2004, Freije, Nelson, and colleagues analysed 74 gliomas from four histological types and identified 595 differentially expressed genes that correlated with overall survival (Freije et al., 2004). Hierarchical clustering showed four molecular subtypes (labelled HC1A, HC1B, HC2A, and HC2B) that segregated into two distinct (P = 0.00011) survival clusters (SCs): SC1 (93% HC1A/B and 62% non-GBM) and SC2 (76% HC2A/B and 89% GBM) with 4.8 and 0.6 years (y) median overall survival, respectively. Prognostic significance was confirmed in the independent Nett data set (Nutt et al., 2003), and multivariate analysis showed that survival cluster was independent of patient age and histological grade. Functional annotation of the gene lists showed that HC1A subtype tumours were enriched for genes involved in neurogenesis (Kriegstein and Alvarez-Buylla, 2009), suggesting a more differentiated phenotype. In contrast, the poor survival subtypes were enriched for proliferation (HC2A) and extracellular matrix/invasion-related (HC2B) genes. A similar list of survival-related genes implicated in neurogenesis was identified by Liang et al. (2005), who also showed that GBM could be divided into two prognostically distinct molecular subtypes (median overall survival 2.1 vs 0.3 y).

In 2006, Phillips, Aldape, and colleagues analysed 76 high-grade astrocytomas and identified 108 differentially expressed genes significantly associated with overall survival (Phillips et al., 2006). Hierarchical and k-means clustering with these genes showed three distinct subtypes termed as proneural, proliferative, and mesenchymal, based on functional annotation of representative genes. Like Freije HC1A, the proneural subtype was defined by genes implicated in neurogenesis, composed predominantly (69%) of non-GBM, and associated with significantly more favourable median overall survival (3.6 vs ≤1.3 y), independent of histological grade. In contrast, the proliferative and mesenchymal gene signatures were enriched for proliferation- and extracellular matrix/invasion-related genes, similar to the Freije HC2A and HC2B subtypes, respectively. Prognostic significance of molecular subtype was validated in an independent cohort of 184 gliomas of various histological types. Taken together, these results suggest that (1) the molecular subtype of a majority of WHO grade II–III gliomas is HC1A/proneural, and (2) HC1A/proneural GBM may be more prognostically favourable.

Using published data sets and new GEP data on 86 GBM, a subsequent meta-analysis by Lee et al. (2008) utilised 377 differentially expressed genes that divided GBM into four distinct subtypes on hierarchical clustering: HC1A/proneural, HC2A/proliferative, HC2B/mesenchymal, and a fourth with hybrid HC2A/HC2B features termed ProMes. Survival analysis confirmed the more favourable prognosis of HC1A/proneural GBM vs the remaining three molecular subtypes (median 1.4 vs 0.9 y). With this larger data set of 267 GBM, the authors also confirmed an association first identified by Phillips et al. (2006), namely that the mean age at diagnosis of proneural glioma was significantly younger (51 vs 55 y, P = 0.02). Moreover, in multivariable analyses, only molecular subtype, but not age, was significantly associated with overall survival. These data suggest a molecular basis for the known association of younger age with improved overall survival in GBM patients.

However, it is of critical note that none of these prognostic studies distinguished among recognised morphological variants of GBM. As shown in Table 1, GBM with oligodendrogial features occur in younger patients and have a significantly prolonged overall survival compared with their GBM counterparts (P < 0.0001). Similarly, another morphological variant of GBM, small-cell GBM (Miller and Perry, 2007), characterised by frequent gains of chromosome 7 (PtEN), is morphologically similar to the prognostically more favourable anaplastic oligodendroglioma, but lacks 1p19q co-deletion. The recent recognition of these morphological patterns of GBM (Louis et al., 2007; Miller and Perry, 2007), prognostically distinct from anaplastic oligoastrocytoma and anaplastic oligoden-droglioma, respectively, raises the possibility that earlier studies were ‘contaminated’ with tumours known to have different prognoses. In addition, at least two significant design flaws were common in these studies (Dupuy and Simon, 2007; Subramanian and Simon, 2010): (1) subtype-specific signature genes were identified using heterogeneous training sets composed of various histological subtypes (e.g., anaplastic astrocytoma and GBM) with known differences in overall survival (Table 1) and (2) signature
genes were defined on the basis of their association with outcome in training sets, and their prognostic significance was re-analysed in independent test sets, raising the possibility that the correlation between GEP-defined subtypes and overall survival were a consequence of prior selection for outcome-related genes (Dupuy and Simon, 2007). To avoid the first problem, future studies should ideally define prognostic signatures in morphologically- and hence prognostically homogeneous cohorts of gliomas. Moreover, consensus diagnosis among multiple, experienced neuropathologists and/or utilisation of ancillary molecular testing such as 1p19q status for accurate assignment of morphologically ambiguous cases into established diagnostic categories will be important quality control measures.

The second problem is likely mitigated by two recently published studies that have identified the HC1A/proneural subset of GBM using gene signatures defined completely by unsupervised methods. In the largest single-institution study conducted to date, Gravendeel et al. (2009) defined molecular subtypes for 276 gliomas of all histological types. Using 5000 genes with highly variable expression, these authors identified six molecular subtypes with distinct prognostic. Glioblastoma largely (73–86%) fell into three clusters (18, 22, and 23) and these tumours showed inferior prognosis relative to GBM in other clusters (9, 16, and 17) (median overall survival 0.7 vs 2.1 y). Cluster 9 consisted primarily (86%) of oligodendroglial neoplasms and the vast majority (82%) appropriately harboured combined 1p19q loss-of-heterozygosity. Notably, the prognostically superior cluster 17 (median overall survival 3.3 and 2.1 y for all C17 gliomas and GBM, respectively) significantly (97%) overlapped with the Phillips proneural subtype, suggesting that detection of a subgroup of GBM with improved prognosis and transcriptional profiles similar to lower-grade gliomas was not a consequence of prior selection of outcome-related genes (Phillips et al., 2006). Notably, cluster 22 was enriched (38%) for secondary GBM, tumours that progress from lower-grade precursors, arise in younger patients (Miller and Perry, 2007), and feature IHD1 mutations (Yan et al., 2009), but lack EGFR amplification (Louis et al., 2007). These findings confirm those from a previous study that demonstrated distinct molecular profiles in primary vs secondary GBM (Tso et al., 2006). Clusters 18 and 23 contained predominantly GBM (78 and 86%, respectively) and showed significant overlap with Phillips proliferative (52%) and mesenchymal (93%) subtypes (Phillips et al., 2006). On analysis of data (Murat et al. 2008) from the definitive phase III clinical trial that established concomitant chemoradiotherapy and adjuvant temozolomide as the standard-of-care for newly diagnosed GBM patients (Stupp et al., 2005), these clusters were found to selectively benefit from combined chemoradiation vs radiation alone. Importantly, multivariate analysis included most known prognostic factors, including age, gender, histological type, grade, KPS, surgery, chemotherapy, EGFR amplification, 1p19q status, and IDH1 mutation (Yan et al., 2009). Only molecular subtype, KPS, and gender were significant, independent prognostic factors in this data set (P<0.02), suggesting that molecular subtyping may be more prognostically accurate than morphological classification alone. These authors validated the prognostic significance of their signatures in four independent data sets (Phillips et al., 2006; TCGA, 2008; Li et al., 2009; Madhavan et al., 2009).

The TCGA, established by the US National Cancer Institute and National Human Genome Research Institute in December 2005, with the mission of understanding ‘the molecular basis of cancer through the application of genome analysis technologies,’ selected GBM as its first cancer type for study, based on its uniformly poor prognosis and limited treatment options. As part of this multi-institutional project, we analysed 200 GBM on three different GEP platforms (Verhaak et al., 2010). Unsupervised hierarchical cluster analysis defined four subtypes, termed proneural, neural, classical, and mesenchymal, based on functional gene annotation and prior convention (Phillips et al., 2006). Significant overlap in molecular subtypes was found for TCGA mesenchymal/Phillips mesenchymal/ Freiße HC2B and TCGA proneural/Phillips proneural/Freiße HC1A (Freiße et al., 2004; Phillips et al., 2006). Unlike previous studies, the TCGA proneural subtype was not associated with improved prognosis in the TCGA data set consisting solely of GBM, but was in the validation of the data sets (Phillips et al., 2006; Madhavan et al., 2009) containing lower-grade gliomas. Conversely, re-analysis of the TCGA GBM data with Phillips molecular subtype designations confirmed a slightly more favourable prognosis of the Phillips proneural GBM (median overall survival 1.2 y) relative to Phillips mesenchymal/proliferative GBM subtypes (1.0 and 0.6 y, respectively; P=0.03). These findings suggest that subtyping based on outcome-defined, unsupervised gene expression signatures may identify a subset of GBM with more favourable prognosis. However, similar to previous findings (Gravendeel et al., 2009), the TCGA classical and mesenchymal subtypes showed significantly improved overall survival after conventional chemoradiation or ≥4 cycles of cytotoxic chemotherapy (P=0.02), suggesting that these subtypes may be particularly sensitive to DNA-damaging agents. These hypotheses will be tested further in two ongoing phase III clinical trials conducted by the Radiation Therapy Oncology Group (RTOG), as discussed below.

Capitalising on the unprecedented level of molecular data available for these tumours (TCGA, 2008), we identified recurrent genomic aberrations in each molecular subtype. The classical subtype was characterized by frequent EGFR amplification and EGFRvIII mutations, CDKN2A deletion, and a lack of TP53 mutations, whereas the mesenchymal subtype was characterised by NF1, TP53, and PTEN mutations. Consensus neuropathological review of a subset of TCGA cases has shown that the proneural, classical, and mesenchymal subtypes are enriched for GBM with oligodendroglial features, small-cell GBM, and gliosarcoma (a morphological variant of GBM with mesenchymal differentiation (Miller and Perry, 2007)), respectively (Cameron Brennan, personal communication). Moreover, pseudopalisading necrosis and to a lesser extent florid microvascular proliferation are frequent in mesenchymal GBM, but the proneural subtype typically lacks necrosis. These findings suggest that mesenchymal GBM may be uniquely susceptible to angiogenesis inhibitors, a hypothesis currently being tested in the RTOG 0825 trial discussed below. The proneural subtype, with increased expression of MGMT (Phillips et al., 2006; Lee et al., 2008) was found in younger patients, harboured frequent PDGFA and mutations in IDH1, TP53, and PIK3CA/PIK3R1, suggesting susceptibility to PDGFRA- and PIK3-targeted therapies. A recent proteomic analysis confirmed protein- and phosphorylation-level signalling abnormalities in the EGFR, PDGFR, and NF1 pathways in classical, proneural, and mesenchymal subtypes of GBM, respectively, further suggesting that these GBM subtypes may be uniquely susceptible to targeted agents (Brennan et al., 2009).

A recent TCGA effort utilised methylation profiling to identify a GBM CpG island methylator phenotype (G-CIMP) in a significant fraction (29%) of proneural GBM, particularly secondary, IDH1-mutant tumours, but not in oligodendroglial tumours (Nourshargh et al., 2010). This implies that G-CIMP might be common in lower-grade gliomas, the majority of which cluster with the proneural molecular subtype of GBM (Phillips et al., 2006; Gravendeel et al., 2009). To further investigate this hypothesis, Nourshargh and colleagues analysed eight G-CIMP gene regions in seven hypermethylated loci in an independent cohort of 152 WHO grade II and III gliomas by a MethyLight real-time PCR assay and found 46% of astrocytomas and 93% of oligodendrogliomas to be G-CIMP-positive. Furthermore, G-CIMP-positive GBM patients were younger (median 36 vs 59 y, P<0.0001) and survived longer than G-CIMP-negative GBM of both proneural and non-proneural subtypes (median overall survival 2.9 vs 0.8 and 1.0 y, P=7E–7). Importantly, G-CIMP positivity was independent of age and
Table 2  Summary of glioma microarray studies

| Reference                  | Data set | Histology* | Total (N) | Source | Findings                                                                 | Genes (N) | Biological process or molecular subtype |
|----------------------------|----------|------------|-----------|--------|---------------------------------------------------------------------------|-----------|----------------------------------------|
| Reitan et al (2001)        | T        | 19 PA, 21 GBM | 40        |        | Distinguishes PA and GBM.                                                 | 360       |                                         |
| Nutt et al (2003)          | V        | 15 non-classic GBM | 29        |        | 86% predictive accuracy for morphologically ambiguous cases. Improved prognostic stratification vs histological classification. | 19        |                                         |
| Shai et al (2003)          | T        | 5 A2, 3 O2, 18 priGBM, 9 secGBM | 35        |        | Distinguishes among histological subtypes.                                | 170       |                                         |
| van den Boom et al (2003)  | T        | 6 A2, two A3, two MOA2, four secGBM, two A3, two MOA3 | 16        |        | Correlates with malignant progression.                                   | 66        |                                         |
| Phillips et al (2003)      | V        | 9 A2, 10 A3, 17 priGBM, 7 secGBM | 43        |        | Progression-associated signature confirmed.                             | 9         |                                         |
| Godard et al (2003)        | T        | 12 A2, 14 priGBM, 5 secGBM | 31        |        | Confirmed findings of Shai et al (2003) and van den Boom et al (2003). Distinguishes A2/secGBM and priGBM. | 9         | Angiogenesis                            |
| Tso et al (2006)           | V        | 4 A2, 9 A3, 8 O2, 11 O3, 46 priGBM, 14 secGBM, 32 paediatric GBM | 92        |        | Distinguishes secGBM and non-GBM astrocytomas.                          | 21        | ECM                                     |
| Freije et al (2007)        | T        | 12 O3, 20 GBM | 32        |        | Distinguishes O3 and GBM.                                                | 108       | Phillips et al (2006) prolifeative      |
| Shirahata et al (2007)     | V        | 22 O3, 28 GBM | 50        | Nutt et al (2003) | 96.6% predictive accuracy. Improved prognostic stratification vs histological classification, confirming Nutt et al (2003). | 67        |                                         |
| Tso et al (2006)           | T        | 52 A2, 29 A3, 55 GBM, 11 O2, 11 O3, 1 MOA2 | 159       |        | Defined six hierarchically nested molecular subtypes with three distinct prognoses. 92% prediction accuracy. | 54        | G-O                                     |
| Li et al (2009)            | V        | 7 A2, 18 A3, 68 GBM, 12 O2, 9 O3, 7 MOA2, 68 gliomas | 187       |        | Reproduced six molecular subtypes.                                       | 69        | GA1/GA2/GB1/GB2                         |
| Phillips et al (2006)      | V        | 265 GBM | 265       | TCGA    | Prognostic significance confirmed.                                       |           |                                         |
| Freije et al (2004)        | T        | 8 A3, 7 O2, 9 O3, 50 GBM | 74        |        | Defined four molecular subtypes with two distinct prognoses. Prognostic independence from patient age and histological grade. | 595 → 44 | Survival                               |
| Li et al (2005)            | V        | 22 O3, 28 GBM | 50        | Nutt et al (2003) | Prognostic independence from patient age and histological grade. | 344       | Survival                               |
| Phillips et al (2006)      | V        | 31 A3, 1 O2, 13 O3, 7 MOA3, 132 GBM | 184       |        | Prognostic independence from patient age and histological grade. 89% of 73 WHO grade III gliomas are proneural. | 35        | Proneural – neurogenesis Proliferative – cell cycle |
| Murat et al (2008)         | V        | 35 A3, 9 O3, 102 GBM | 146       |        | Prognostic independence from patient age and histological grade. | 18        | HOX, self-renewal                      |
| Lee et al (2008)           | V        | 86 GBM from previous studies | 267       |        | Prognostic independence from patient age. | 595 → 377 | HOX2A-proliferative (Pro) HOX2B-Mesenchymal (mes) ProMes |

*Histology includes PA (preneural astrocytoma), GBM (glioblastoma multiforme), O (oligodendroglioma), MOA (mixed oligoastrocytoma), and A (astrocytoma).

- **Source** indicates the reference for each study.
- **Findings** describe the main outcomes or results of the study.
- **Genes (N)** refer to the number of genes identified in the study.
- **Biological process or molecular subtype** indicates the biological processes or molecular subtypes identified in the study.
histological grade on multivariable analysis. These findings suggest that G-CIMP defines a subset of proneural GBM and can be utilised to further refine expression-defined subtypes. The co-occurrence of G-CIMP/IDH1 mutation positivity in the proneural, neurogenesis-related subtype further suggests that IDH1 mutation and/or G-CIMP may confer neoplastic susceptibility to a common neuron/oligodendrocyte precursor cell of origin (Kriegstein and Alvarez-Buylla, 2009), a hypothesis supported by the comparative expression profiling data that showed enrichment of genes expressed in purified, cultured murine oligodendrocytes in proneural GBM (Verhaak et al, 2010).

**Table 2 (Continued)**

| Reference                        | Data set | Histologya | Total (N) | Source | Findings                                                                 | Genes (N) | Biological process or molecular subtype |
|----------------------------------|----------|------------|-----------|--------|--------------------------------------------------------------------------|-----------|----------------------------------------|
| Gravendeel et al (2009)          | T        | 80 GBM     | 80        | Murat et al (2008)            | Defined four ‘intrinsic’ molecular subtypes; not prognostic in TCGA data set. | 840       | Proneural – neurogenesis                 |
|                                  | V        | 76 gliomas | 76        | Phillips et al (2006)         | Prognostic significance confirmed.                                            | 1503      | Classic – EGFR                           |
|                                  | V        | 296 gliomas| 296       | Li et al (2007)               | Prognostic significance confirmed.                                            | 80        | Mesenchymal – immune response            |
|                                  | V        | 236 GBM    | 236       | Madhavan et al (2009)         | Prognostic significance confirmed.                                            | 276       | Survival                                |
| Verhaak et al (2010)             | T        | 200 GBM    | 200       | TCGA                             | Prognostic significance confirmed.                                            | 200       | Survival                                |
|                                  | V        | 173 GBM    | 173       | TCGA                             | Prognostic significance confirmed.                                            | 173       | Survival                                |
| Nsoumeh et al (2010)             | T        | 272 GBM    | 272       | TCGA                             | Prognostic significance confirmed.                                            | 272       | Survival                                |
|                                  | V        | 60 and 92 WHO grade II and III gliomas | 152    | TCGA                             | Prognostic significance confirmed.                                            | 60 and 92 WHO grade II and III gliomas | Survival |
| Colman et al (2010)              | V        | 68 GBM with FFPE tissues | 68        | TCGA                             | Prognostic significance confirmed.                                            | 68        | Survival                                |
|                                  | V        | 101 GBM from patients treated with standard-of-care TMZ/XRT → TMZ | 101    | TCGA                             | Prognostic significance confirmed.                                            | 101       | Survival                                |

Abbreviations: ECM = extracellular matrix; FFPE = formalin-fixed, paraffin-embedded; priGBM = primary GBM; secGBM = secondary GBM; T = training; TMZ/XRT → TMZ = concurrent temozolomide/radiation therapy and adjuvant temozolomide; V = validation. *See table 1 for histological subtype abbreviations. Studies listed in order of appearance in the text. Shading indicates the different datasets or gene signatures utilized in each individual study, along with the associated findings across each shaded row, where applicable.

**Clinical Implementation of GEP for Glioma Classification**

GEP-based diagnostic tests are currently being evaluated in prospective, randomised clinical trials in breast cancer (Weigelt et al, 2009). Similar progress in clinical neuro-oncology has
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