BMJ Neurology Open

Potential biomarkers and challenges in glioma diagnosis, therapy and prognosis

Liyen Katrina Kan,[1,2] Kate Drummond,[3] Martin Hunn,[4] David Williams,[2] Terence J O'Brien,[1,5] Mastura Monif[1,2,5,6]

To cite: Kan LK, Drummond K, Hunn M, *et al*. Potential biomarkers and challenges in glioma diagnosis, therapy and prognosis. *BMJ Neurology Open* 2020;**2**:e000069. doi:10.1136/bmjno-2020-000069

Received 18 May 2020
Revised 29 June 2020
Accepted 08 July 2020

Check for updates

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[1]Department of Neuroscience, Monash University Faculty of Medicine, Nursing and Health Sciences, Melbourne, Victoria, Australia
[2]Department of Physiology, The University of Melbourne, Melbourne, Victoria, Australia
[3]Department of Neurosurgery, Royal Melbourne Hospital, Melbourne, Victoria, Australia
[4]Department of Neurosurgery, Alfred Health, Melbourne, Victoria, Australia
[5]Department of Neurology, Alfred Health, Melbourne, Victoria, Australia
[6]Department of Neurology, Royal Melbourne Hospital, Melbourne, Victoria, Australia

Correspondence to
Dr Mastura Monif;
mastura.monif@monash.edu

ABSTRACT

Gliomas are the most common central nervous system malignancies and present with significant morbidity and mortality. Treatment modalities are currently limited to surgical resection, chemotherapy and radiotherapy. Increases in survival rate over the previous decades are negligible, further pinpointing an unmet clinical need in this field. There is a continual struggle with the development of effective glioma diagnostics and therapeutics, largely due to a multitude of factors, including the presence of the blood–brain barrier and significant intertumoural and intratumoural heterogeneity. Importantly, there is a lack of reliable biomarkers for glioma, particularly in aiding tumour subtyping and measuring response to therapy. There is a need for biomarkers that would both overcome the complexity of the disease and allow for a minimally invasive means of detection and analysis. This is a comprehensive review evaluating the potential of current cellular, proteomic and molecular biomarker candidates for glioma. Significant hurdles faced in glioma diagnostics and therapy are also discussed here.

INTRODUCTION

Gliomas are heterogeneous glial cell malignancies which primarily develop as diffuse tumours that extensively infiltrate the brain parenchyma. These neoplasms are the most common central nervous system (CNS) cancers, comprising approximately 80% of all brain malignancies.[1] Among the glioma subtypes, glioblastomas are the most predominant and aggressive, killing 225 000 people annually.[1] Glioblastomas account for over 60% of all brain tumours in adults.[1] The current median survival time for patients with glioblastoma is approximately 14–15 months postdiagnosis with treatment and 3–4 months without treatment. This is a review summarising and evaluating the current genetic, proteomic and cellular biomarkers of glioma, discussing major hurdles to overcome in glioma diagnostics and treatment.

Classification of gliomas

The WHO 2016 classification of CNS tumours has fundamentally shifted the focus of glioma classification from a histological perspective to one that is largely based on genetic and molecular alterations.[2] Importantly, the classification of diffuse gliomas (WHO grade II–IV) now involves the detection of mutations in isocitrate dehydrogenase (IDH) 1 and 2. IDH 1/2 mutations are largely associated with WHO grade II and III gliomas and secondary glioblastomas. With the updated WHO 2016 glioma classification, glioblastomas have been reclassified into IDH-wildtype glioblastoma and IDH-mutant glioblastoma. Interestingly, clinical outcome also differs between histologically identical diffuse gliomas with IDH-wildype and IDH-mutant genotypes. There is an improved prognosis in patients with secondary IDH-mutant glioblastomas, compared with those with IDH-wildtype glioblastomas.[3] Among the diffuse gliomas with IDH mutations, there further exist two molecular groups: (1) IDH-mutant diffuse gliomas with 1p/19q co-deletion and mutated *TERT* promoter and (2) IDH-mutant diffuse gliomas with mutations in *ATRX* and *TP53*. The combined genetic and molecular expression profiles also supersede histological diagnosis. For example, the presence of a complete 1p/19q co-deletion differentiates between an astrocytoma and an oligodendroglioma despite histological appearance.[4] Deletions in the 1p and 19q chromosomes have been observed in 40%–90% of biopsies and are the most common genetic alteration of oligodendrogliomas.[5] This deletion further favours an improved survival time and response to conventional therapy.[6] Hence, IDH genotyping and its associated molecular subgroups has since been termed as a decisive marker for glioma classification.[2 4] A comprehensive review of the WHO 2016 glioma classification is available.[4]

Molecular diagnosis

Conventional diagnosis of glioblastoma is made based on clinical presentation, MRI and histopathological analysis. Whereas histopathological imaging can provide a generic classification of glioma grade, an advantage of molecular diagnosis is the potential for

developing patient-specific therapies that target various oncogenic pathways. For example, the molecular aberrations most commonly associated with glioma development include IDH mutations, epidermal growth factor receptor (EGFR) amplification, mutations in P53 and retinoblastoma protein, as well as abnormalities in the pathways involving receptor tyrosine kinase, protein kinase B (Akt), phosphoinositide 3-kinase and Ras.[7–9] In this regard, a large area of research has been focused on developing therapeutic agents that target EGFR and its mutant EGFR variant III (EGFRvIII) in glioblastoma.[10] This is a notable example of using molecular diagnosis to develop more targeted therapies for glioma. With the aid of RNA sequencing, studies have also identified 20 genes associated with reduced survival in glioblastoma.[9]

Additionally, molecular profiling by The Cancer Genome Atlas (TCGA) has enabled the classification of glioblastoma into four further subtypes—classic, mesenchymal, neural and proneural—and the subsequent identification of aberrantly expressed genes in each subtype.[11] While this classification does not reflect primary intratumour heterogeneity and clonal tumour evolution in disease progression and treatment response, it provides a valuable means of patient stratification. In classic glioblastomas, chromosome 7 amplification (coupled with chromosome 10 loss) and EGFR amplification were present in 100% and 97% of cases, respectively.[11] Mesenchymal glioblastomas commonly contained deletions and mutations in neurofibromatosis type 1 (*NF1*), a tumour suppressor gene associated with increased tumourigenesis and malignancy in glioblastoma.[12] In most mesenchymal cases, there was an occurrence of *NF1*-containing focal hemizygous deletions at 17q11.2 and an increased expression of genes associated with necrosis and inflammation.[11] Neuronal markers, including neurofilament light (*NEFL*), gamma-aminobutyric acid receptor subunit alpha-1 (*GABRA1*), chloride-potassium symporter 5 (*SLC12A5*) and synaptotagmin-1 (*SYT1*), were characteristic of neural glioblastoma, whereas *IDH1* point mutations and platelet derived growth factor receptor alpha (*PDGFRA*) aberrations predominantly marked the proneural subtype.[11] Importantly, these subtypes have also been correlated to treatment efficacy.[11] Aggressive treatment involving either concurrent chemotherapy and radiotherapy or over three successive cycles of chemotherapy reduced mortality in classic, mesenchymal and neural glioblastomas, but did not affect survival in the proneural subtype.[11] Moreover, chemosensitivity has also been reported to vary depending on the progenitor origin of each tumour.[13] These molecular signatures will serve a pivotal role in improving diagnosis and the generation of more targeted and personalised therapeutics.

CURRENT DIAGNOSTIC AND THERAPEUTIC CHALLENGES

Current therapeutics for gliomas are limited and only offer a modest prognostic benefit. Moreover, treatments are coupled to an array of limitations and adverse side effects. The standard of care involves maximal safe surgical resection and radiotherapy with concomitant chemotherapy. Complete elimination of tumour mass by surgery is not possible because of the highly complex and infiltrative nature of gliomas. As a result, surgical resection only increases survival time by several months.[14] Radiotherapy and the conventional chemotherapeutic drug, temozolomide, also provide a modest survival benefit and can be associated with debilitating side effects. Despite treatment, the 5-year survival rate is 5% for glioblastoma and 30% for patients with anaplastic astrocytoma.[15 16] Lower grade gliomas also commonly progress into high-grade tumours; 10% of all glioblastomas fall within this category.[17 18] It is evident that gliomas present a critically high, unmet clinical need; hence, the development of more effective therapeutics is pivotal.

To date, there are no targeted therapies approved for glioblastoma.[9] Furthermore, almost all glioblastoma tumours that initially respond to first-line treatment recur and minimally respond to second-line therapy.[9 19] There is a desperate need for improvement in both glioma diagnosis and treatment. An innumerable combination of factors are attributed to the challenges faced in this field, but an overarching contributor is the lack of reliable glioma biomarkers. Ideally, biomarker candidates should be able to surpass limitations associated with (1) imaging and pathology, (2) intertumoural and intratumoural heterogeneity and (3) the blood–brain barrier (BBB). A visual summary of the current diagnostic and therapeutic challenges is shown in figure 1.

MRI is the current diagnostic and monitoring standard for newly diagnosed and recurrent gliomas. Results obtained from MRI play an important role in pretreatment characterisation and measuring response to therapy. However, challenges exist in the ability for conventional MRI to distinguish between primary tumours versus metastases and CNS masses, and true progression versus pseudoprogression, as it is not uncommon for the radiological features of these to overlap.[19 20] Gliomas, metastases and primary CNS lymphomas are known to appear as contrast-enhancing tumours bordered by a T2-hyperintense oedema.[19] Moreover, glioblastomas also have reported similarities with metabolite ratios, rendering more advanced imaging modalities, including dynamic susceptibility contrast-enhanced MRI, inadequate.[20] Pseudoprogression occurs in 20%–30% of patients who undergo continual radiotherapy and treatment with temozolomide.[21 22] It can often be mistaken for true tumour recurrence, as it is, too, characterised by new or enlarging regions of contrast enhancement.[21] The fundamental differences are that pseudoprogression occurs in the absence of tumour growth and stabilises without changes in treatment.[22] The recovery period, however, takes an approximate 7 months.[21] The challenge here is overcoming the consequential uncertainty in outcome evaluation prior to stabilisation: to alter the course of therapy or to do nothing at all? It would be useful to establish biomarkers unique to gliomas that would aid in these differentiations.

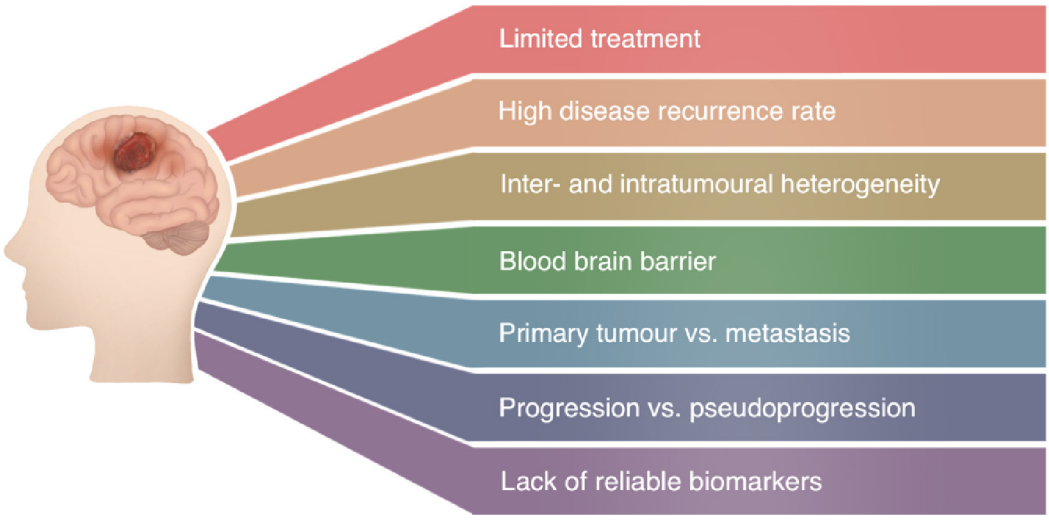

Figure 1 Summary of current challenges in glioma diagnosis, therapy and prognosis.

Another hurdle faced in glioma diagnostics and therapy is the high heterogeneity within and between tumours. It is said that the cellular complexity associated with glioblastomas argues against the notion that gliomagenesis is induced by a single cell of origin.[9] Rather, the cause of disease is multifactorial and a range of cell types (not necessarily the originating cells) may contain the transforming mutation. In fact, the expression patterns of copy number alteration (CNA) have been demonstrated to vary between different stages of tumour development.[23] CNAs in the early phases of gliomagenesis were typically orientated in chromosomes 7 and 10, while mutations in the late phases were more dispersed across the genome.[23] Further studies focused on identifying any fundamental differences in genomic profiles across various stages of tumour development would aid in determining whether such observations can potentially serve as reliable biomarkers of disease. Specific biomarkers released across various stages of tumour progression might be useful in determining tumour stage and serve as a stage-specific target for treatment. Interestingly, single-cell RNA sequencing of 430 cells from five primary glioblastomas revealed significant variability in genes modulating oncogenic signalling, the immune response and cell proliferation.[24] There were also evident intratumour variations in markers used to subtype glioblastoma by the TCGA.[24] This was also demonstrated in an earlier study which conducted microarray gene profiling on 51 tumour fragments from 10 patients with glioblastoma.[23] The expression of molecular signatures corresponding to multiple glioblastoma subtypes within a single tumour adds another layer of complexity, not detected by population-level analyses, to diagnosis. As a consequence of substantial intertumour and intratumour heterogeneity, many difficulties exist in therapeutic target identification, diagnostic stratification and treatment optimisation. A single, reliable diagnostic biomarker would be abundantly expressed within the tumour across all stages and in the majority of patients with glioma.

BBB integrity is known to be compromised at the tumour site, but a challenge arises in the surrounding nutrient-providing vasculature that largely remains intact. Hence, a barrier remains in the transportation of molecules from the peripheral blood to the parenchymal brain and vice versa. Therapeutic agents must therefore be of appropriate size and lipophilicity for effective delivery into the tumour site via the bloodstream. The BBB also restricts the biomarker candidate pool—there may be reliable glioma-specific molecules released within the tumour microenvironment that are unable to cross the barrier and enter the peripheral bloodstream for detection. This is a major hurdle, as it is important for biomarkers to be measured and detected via techniques that are as minimally invasive as possible. There is a need for biomarkers unique to patients with glioma that are either expressed abundantly in the bloodstream or able to surpass the BBB for adequate detection.

POTENTIAL GLIOMA BIOMARKERS

A summary of all major biomarkers is presented in table 1.

Circulating tumour cells

Circulating tumour cells (CTCs) are ample reflections of homogeneous tumour characteristics in situ, but it is unlikely that these cells accurately mimic the milieu of complex, heterogeneous malignancies, such as gliomas. Despite this, CTCs likely contain the genome of the primary tumour and may serve as a valuable biomarker for glioma diagnostics or treatment. There is evidence supporting the presence of CTCs in the peripheral blood of patients with glioblastoma.[25–28] In a study conducted by Müller and colleagues,[25] immunocytochemical analyses using glial fibrillar acidic protein (GFAP) as a marker for glioblastoma cells detected CTCs in 20.6% (29 of 141) of participating patients with glioblastoma. The effect of surgical resection on the presence of CTCs in the peripheral blood was additionally assessed. Out of 67 patients

Table 1 Summary of current glioma biomarker candidates and their associations with diagnosis, treatment and prognosis

	Associations		
Biomarker	**Diagnosis**	**Treatment**	**Prognosis**
Circulating tumour cells	↑ with disease progression (Sullivan *et al* 2014).[26] ↑ in IDH wildtype high-grade gliomas (Li *et al*, 2019).[27]	↓ postradiotherapy (MacArthur *et al*, 2014).[30]	
Extracellular vesicles	↑ in glioblastoma (Osti *et al*, 2019).[34] Exosomal protein levels correlated with WHO glioma grade (Muller *et al*, 2015).[25] EGFR-positive serum EVs correlated with glioma malignancy (Wang *et al*, 2019).[42]	↓ with temozolomide in T103 mice (Shao *et al*, 2012).[32] TPI from T103 mouse microvesicle measurements revealed response to temozolomide before observable changes in tumour size (Shao *et al*, 2012).[32] TPI from microvesicle measurements predicted response to temozolomide and radiotherapy (Shao *et al*, 2012).[32] ↑ release after radiation (Arscott *et al*, 2013).[38] ↑ release of EV adhesion molecules after temozolomide treatment (Andre-Gregoire *et al*, 2018).[39]	
Circulating tumour DNA	ctDNA sequencing facilitated diagnosis of diffuse gliomas (Martínez-Ricarte *et al*, 2018).[47]		ctDNA in CSF of patients with glioma contributed to fourfold increased risk of mortality (Miller *et al*, 2019).[46]
MicroRNAs	↑ exosomal miR-21, miR-222 and miR-124–3p in patients with high-grade glioma (Santangelo *et al*, 2018).[53] ↑ miR-21 in human glioblastoma cell lines, serum and tissue (Yang *et al*, 2014[55]; Skog *et al*, 2008; Chan *et al*, 2005).[56] ↑ miR-21 in high-grade gliomas (Chan *et al*, 2005).[56]	Serum miR-181 level correlated with response to temozolomide in patients with glioblastoma (Zhang *et al*, 2012).[54] ↓ miR-21, miR-222 and miR-124–3p levels in postsurgery blood samples of patients with glioma (Santangelo *et al*, 2018).[53] miR-21 downregulation boosted the proapoptotic effect of temozolomide in glioblastoma cells (Lan *et al*, 2015).[58] miR-21 overexpression in U87MG cells inhibited temozolomide-mediated apoptosis (Shi *et al*, 2010).[59]	Patients with glioblastoma with lower risk scores from miRNA expression profiles had longer survival (Li *et al*, 2014).[52] miR-21 levels inversely correlated with patient survival (Yang *et al*, 2014).[55]
Epidermal growth factor receptor variant III	↑ microvesicle EGFRvIII in glioblastoma cells (Shao *et al*, 2012).[32]	↓ microvesicle EGFRvIII with temozolomide and geldanamycin (Shao *et al*, 2012).[32]	
YKL-40	↑ in glioblastoma (Shostak *et al* 2003[64] ; Qin *et al*, 2017).[65] ↓ in patients with radiographically absent disease (Hormigo *et al*, 2006[66] ; Iwamoto *et al*, 2011).[67]	↑ in patients who had undergone partial resection, compared with total resection (Bernardi *et al*, 2012).[68]	↑ YKL-40 associated with worse survival (Qin *et al*, 2017).[65]
Matrix metalloproteinase-9	↑ in patients with glioblastoma with radiographically evident disease (Hormigo *et al*, 2006).[66]	↑ MMP-9 in the CSF of patients with recurrent glioma treated with doxycycline suggested a failed response to treatment (Wong *et al*, 2008).[73] ↑ serum MMP-9 in high-grade glioma after surgical resection (Hormigo *et al*, 2006).[66]	
Haptoglobin	Expression levels proportional to increasing tumour grade (Kumar *et al*, 2010).[75]		Haptoglobin overexpression in tumours implanted in mice resulted in a worse prognosis (Kumar *et al*, 2010).[75]
A2-Heremans-Schmid glycoprotein			↓ AHSG expression linked to lower median survival time (Petrik *et al*, 2008).[76]
S100A8/A9	↑ in glioblastoma sera (Arora *et al*, 2019).[78]		↑ transcript levels linked to lower median survival time (Arora *et al*, 2019).[78]

AHSG, A2-Heremans-Schmid glycoprotein; CSF, cerebrospinal fluid; ctDNA, circulating tumour DNA; EGFR, epidermal growth factor receptor; EGFRvIII, EGFR variant III; EVs, extracellular vesicles; IDH, isocitrate dehydrogenase; miRNA, microRNA; MMP-9, matrix metalloproteinase-9; TPI, tumour progression index.

with glioblastoma, 13.4% had CTCs in both presurgical and postsurgical blood samples, 6% had CTCs only in presurgical blood, and 7.5% had CTCs only in postsurgical blood.[25] Although there was no evidence of CTC release due to surgery,[25] the study did not quantify the amount of CTCs within peripheral blood samples obtained at the presurgical and postsurgical time points—this should be an aim for future research. In this study, blood collected during dural opening at the initial phase of surgery represented the 'pre-surgical' time point, whereas blood collected during dural closure at the later phase of surgery represented the 'post-surgical' time point. A further criticism in this aspect is that blood extracted during surgery may not be accurately illustrative of physiological conditions prior to and after surgery. Rather, samples should be drawn before any surgical intervention takes place and several weeks following surgery. Hence, it cannot be concluded that surgery has no effect on CTC levels in the peripheral blood. From these data, there might be differences in peripheral blood CTC levels before and after tumour resection. Indeed, anaesthesia has been shown to augment BBB permeability,[29] which may increase transport of CTCs into the peripheral blood.

Recent research by Li *et al*[27] identified CTCs in 90.9% of 88 patients with glioma via the capture of heteroploid chromosome cells. However, in this study, GFAP was not expressed on 96.1% of CTCs detected,[27] indicating that GFAP may not be a reliable marker of CTCs. A study conducted by Macarthur *et al*[30] identified CTCs in 72% of 11 participating preradiotherapy patients with glioblastoma, with CTC detection decreasing (8%) in postradiotherapy patients. Hence, from this study, CTCs may also be a useful biomarker of response to treatment. Using a negative-depletion system to remove leucocytes and subsequently enrich for CTCs, another study identified CTCs in 39% of participating patients with glioblastoma.[26] These data reflect the presence of CTCs in the peripheral blood of patients with glioma, but as CTCs are estimated to be present at a rate of one CTC per 1×10^9 blood cells,[31] the scarcity of these cells remains a major limitation. On a positive note and despite the variation in detection rates between studies, the presence of CTCs in the peripheral bloodstream is reflective of their ability to surpass the BBB. Limited studies have focused on the relationship between CTCs and glioma malignancy. Interestingly, the frequency of CTCs was increased in patients with progressing disease.[26] In support of this, CTC frequency was also significantly increased in patients with high-grade gliomas without IDH mutations.[27] However, there was no significant difference in CTC level among different glioma grades in a study conducted by Gao and colleagues.[28] Collectively, data from these few studies highlight the potential for CTCs to be used as a biomarker for gliomas to aid in grading and measuring response to surgery/treatment, but this area of study requires further elucidation. Additional studies are required to optimise CTC measurement techniques and clarify the effect of routine surgery/treatment on CTCs. This would help gain a deeper understanding of the roles that CTCs could serve as biomarkers of disease severity and treatment response.

Extracellular vesicles

Extracellular vesicles (EVs) are small lipid bilayer compartments comprising a combination of microRNAs (miRNAs), circulating tumour DNA (ctDNA) and proteins. These vesicles are released by both non-malignant cells and tumour cells into the extracellular space either as exosomes that form intracellularly and are released after fusion with the plasma membrane, or as microvesicles that are released via plasma membrane budding. An advantage is that molecules within EVs are safeguarded from rapid degradation that often occurs for molecules freely circulating in the plasma. Furthermore, unlike CTCs that are sparse in population, large quantities of EVs have been detected in the peripheral blood of patients with glioblastoma.[32] The concentration of EVs per 1 mL of plasma has been estimated to total 10 billion.[33] Recent research has also demonstrated increased levels of EVs in patients with glioblastoma compared with healthy controls and patients with other CNS malignancies.[34]

In gliomas, EVs primarily serve a role in intercellular signalling, specifically to mediate the horizontal transmission of tumourigenic species to neighbouring cells. The differentiation of microvesicles released by glioblastoma cells from that of normal cells has previously been demonstrated.[32 35 36] The genetic and proteomic phenotype of glioblastoma-derived EVs has been shown to provide an accurate snapshot of tumour cells in situ.[36 37] Using a highly sensitive combination of nanoparticle protein typing and miniaturised nuclear magnetic resonance (NMR) detection, research by Shao and associates[32] further confirmed the upregulation of protein markers (EGFR, EGFRvIII, PDPN, IDH1 and R132H), distinctively reflective of parental glioblastoma cells, within microvesicles.[35] However, microvesicle profiling of blood samples of 24 patients with glioblastoma demonstrated significant heterogeneity between the expression levels of each protein marker between patients.[32] While microvesicles may provide an accurate proteomic representation of original tumour mass, heterogeneity remains a major hurdle in the search for an ideal glioma biomarker candidate.

Nonetheless, EVs have been shown to indicate treatment efficacy both in vitro and in vivo. A recent study demonstrated a reduction in the number of plasma EVs following tumour resection in patients with glioblastoma.[34] Treatment with temozolomide induced a dose-dependent reduction in both T103 mouse glioblastoma cells and microvesicles, indicating that microvesicles, alone, were able to signal therapeutic efficacy.[32] In vivo experiments on T103 tumour-bearing mice demonstrated that tumour progression index (TPI) values obtained via NMR microvesicle measurements revealed response to temozolomide treatment before changes in tumour size could be observed.[32] In a subsequent longitudinal study conducted by Shao *et al*,[32] TPI from blood microvesicle NMR measurements of

patients with glioblastoma prior to and after routine temozolomide and radiation therapy adequately scoped and predicted treatment response. Alternatively, radiation has been shown to stimulate the release of EVs by glioblastoma cells, promoting the transition of surrounding tumour cells into a migratory phenotype on uptake.[38] Treatment of glioblastoma stem-like cells with temozolomide similarly promoted the release of EV-associated molecules involved in cellular adhesion.[39] Hence, in this aspect, EVs were able to reveal the upregulation of adhesion and migratory molecules in response to conventional therapy. An association has been established between post-therapy increases in tumour size and increased levels of exosomal transforming growth factor-beta, interleukin-8, TIMP1 (TIMP metallopeptidase inhibitor 1), ZAP70 (zeta-chain associated protien kinase 70).[40] From these data, a possible mode of indicating response to treatment by EVs is by measuring post-treatment immune responses. There needs to be further research on the potential roles of EVs as indicators of treatment response.

EVs additionally fuel glioma angiogenesis via interaction with endothelial cells and promote glioblastoma growth in an autocrine manner.[37] Interestingly, EVs released by glioblastoma cells have been demonstrated to be taken up by microglia and monocytes/macrophages within the brain in vitro and in a mouse model of glioblastoma.[41] The resulting effect was increased microglial proliferation and polarisation towards an immunosuppressive activation state.[41] Exosomal protein levels have been shown to positively correlate with WHO glioma grade.[40] EGFR-positive serum EVs have also recently been shown to correlate with glioma malignancy.[42] Flow cytometry analysis of sera from 4 patients with low-grade (WHO grade II) and 13 patients with high-grade (WHO grade III and IV) glioma by Wang *et al*[42] revealed that the expression levels of EGFR in EVs were substantially higher in patients with high-grade gliomas, compared with those with low-grade tumours. EVs may therefore aid in glioma subtyping, although further studies with larger sample sizes are needed to clarify this.

While EVs show vast potential in aiding glioma diagnosis and indicating therapeutic efficacy, a limitation is that EV isolation and measurement techniques, including ultracentrifugation, high-resolution flow cytometry, nanoparticle tracking analysis and tunable resistive pulse sensing, are not often employed in basic laboratories. Techniques differentiating between microvesicles, exosomes and endosomal components are also limited. This is in part due to the possibility of overlap between various types of EVs, as size ranges are not well defined.[43] Hence, further research is required to further characterise EV subtypes and optimise procedures for EV isolation and purification.

Nucleic acids

Circulating tumour DNA

ctDNA contains mutations present in the parental tumour and exists in higher abundance than CTCs.[44 45] Moreover, the sensitivity of ctDNA detection is comparatively higher than that of CTCs alone,[44] making it a good candidate as a diagnostic biomarker for gliomas. Importantly, the genomic landscape encoded in ctDNA has been shown to closely resemble that of the primary glioma.[46] Indeed, ctDNA sequencing has been demonstrated to successfully facilitate the diagnosis of diffuse gliomas, allowing for the genomic analysis of *IDH1*, *IDH2*, *TP53*, *TERT*, *ATRX*, *H3F3A* and *HIST1H3B* mutations.[47]

Furthermore, ctDNA was detected in the cerebrospinal fluid (CSF) of 42 out of 85 patients with glioma and contributed to prognosis and burden of disease.[46] Notably, the presence of ctDNA in the CSF of patients with glioma was associated with a fourfold higher risk of mortality.[46] However, no correlations were drawn between the presence of CSF ctDNA and glioma grade, length of disease or previous therapy.[46] Interestingly, there was no detectable level of CTCs in the CSF of 90% of patients with detectable ctDNA,[46] supportive of a higher detection sensitivity to ctDNA, compared with CTCs.

A limitation, however, is the low quantity of ctDNA in the plasma of patients with glioblastoma, compared with other systemic malignancies, likely due to the presence of the BBB.[48] In one study, the plasma ctDNA yield was undetectable in over 90% of patients with glioma (n=27) when analysed via PCR.[45] Despite this, next-generation sequencing of the plasma of 419 patients with primary brain tumours, including glioblastoma, demonstrated the presence of detectable levels of ctDNAs in 50% of patients,[49] with a recent study yielding a detection rate of 51% in patients with primary glioblastoma.[50] Indeed, the discrepancies in ctDNA yield might be due to differences in isolation/detection technique used by various laboratories. While the concentration of ctDNA is essential for ease of detection, it is vital that techniques are developed with sufficient sensitivity and specificity to detect the presence of ctDNA at even the smallest amounts. It is also unknown whether an association exists between quantity of ctDNA and glioma malignancy. Research on the ctDNA levels of patients with low-grade and high-grade glioma and large-scale correlation analyses on ctDNA concentration and glioma grade are necessary.

Nonetheless, the collective results of the above studies show the promising potential of ctDNA to be used as a biomarker for glioma diagnosis and progression. The likely abundance of ctDNA in bodily fluids such as CSF and peripheral blood means that the genetic profile of patients with glioma can be analysed via safe and minimally invasive techniques, such as liquid biopsy and lumbar puncture.

microRNAs

A combination of tissue-specific characteristics, oncogene and suppressor functions prize miRNAs as one of the most valuable biomarker candidates for gliomas. In fact, miRNAs have been reported to encompass a third of all non-coding RNAs in glioblastoma EVs.[51] Via permutation tests and Cox regression analyses, miRNA expression profiles have previously facilitated the prognostic substratification of intrinsic glioblastoma subtypes. Li

and associates[52] successfully translated miRNA expression profiles into a risk score for patient survival and found that patients with glioblastoma attributed to lower risk scores survived for significantly longer than those with higher risk scores. Additionally, expression of exosomal miR-21, miR-222 and miR-124-3p has been shown to be significantly higher in patients with high-grade gliomas, compared with those with low-grade gliomas and healthy controls.[53] miRNAs may also be valuable in measuring response to therapy. For example, serum miR-181 level has been shown to correlate with response to temozolomide in patients with glioblastoma,[54] and miR-21, miR-222 and miR-124-3p levels have been shown to be dramatically decreased in postsurgery blood samples of patients with glioma.[53]

In particular, miR-21, a known oncogene, is overexpressed in human glioblastoma cell lines and tumour tissue.[55 56] Interestingly, serum microvesicles of patients with glioblastoma also had elevated miR-21 levels, compared with controls.[37] A meta-analysis analysing the diagnostic efficacy of miR-21 for glioblastoma attributed it to a pooled sensitivity of 0.82 and a specificity of 0.94.[57] Hence, miR-21 might be an accurate diagnostic biomarker. Its expression has also been reported to increase in high-grade gliomas, compared with low-grade gliomas, potentially via inhibiting critical proapoptotic gene expression.[56] In support of this, miR-21 knockdown inhibited glioblastoma cell proliferation in vitro and solid tumour formation in vivo.[55] miR-21 expression was also inversely correlated with glioblastoma patient survival.[55] The functions of miR-21 extend towards the efficacy of glioma therapy—miR-21 downregulation boosted the proapoptotic effect of temozolomide in glioblastoma cells.[58] Similarly, miR-21 overexpression in a human primary glioblastoma cell line (U87MG) was found to significantly inhibit temozolomide-mediated apoptosis via a Bax/Bcl-2 dependent pathway.[59] Given the above evidence and its high abundance within glioblastoma EVs,[41] miR-21 may be a powerful clinical biomarker of gliomas, with high specificity and sensitivity.

Proteins

Proteins present in bodily fluids, including serum, CSF and urine, can potentially be used to diagnose gliomas and measure response to therapy. However, little validation exists in this field. Present in 20%–25% of glioblastoma cases,[60] a focus has been on EGFRvIII, a constitutively activated form of EGFR that promotes oncogenesis.[10 61] The clinical value of EGFRvIII is still uncertain; however, studies have attempted to delineate its potential as a biomarker. Microvesicles shed from glioblastoma cells have been revealed to distinctively express higher levels of EGFRvIII, enabling the differentiation of tumour-related microvesicles from host cell microvesicles.[32] Interestingly, in the same study, microvesicle EGFRvIII expression declined in a dose-dependent manner with increasing concentration of temozolomide and also significantly declined on administration of geldanamycin (antitumour Hsp90 inhibitor) in the T103 mouse model of glioblastoma.[32]

Anti-EGFRvIII antibody titres were also shown to increase to over fourfold greater than baseline levels in a phase II clinical trial investigating the effects of the EGFRvIII-targeting rindopepimut vaccine in patients with glioblastoma.[62] However, a recent study by Felsberg *et al*[63] involving 106 EGFR-amplified patients with glioblastoma did not deduct major differences in EGFRvIII expression with progression, recurrence or survival. Hence, the clinical value of EGFRvIII remains unclear.

Serial analysis of gene expression and northern blot hybridisation demonstrated YKL-40 (also known as Chitinase 3-like 1) overexpression in glioblastoma versus normal brain tissue.[64] A recent meta-analysis involving eight studies further associated YKL-40 upregulation with a significantly worse overall survival in patients with glioblastoma.[65] Serum YKL-40 levels have been correlated to the presence of radiographically active glioblastoma and anaplastic astrocytoma.[66] Patients with glioblastoma and anaplastic astrocytoma with no radiographic evidence of tumour had significantly lower YKL-40 levels compared with respective patients with active tumour.[66] These findings were reciprocated in another study involving 343 patients with anaplastic glioma, whereby patients with no radiographic disease progression had markedly lower serum YKL-40 levels than patients with disease progression.[67] Furthermore, patients with glioblastoma who had undergone partial resection had increased YKL-40 levels relative to patients who had undergone total resection.[68] The association between YKL-40 and glioma progression and survival has been demonstrated by further studies,[69 70] supporting the potential of YKL-40 to serve as a clinical biomarker for glioma. However, some studies suggest the contrary, reporting no association between serum YKL-40 concentration and progression-free survival,[71] as well as tumour volume.[72] Additional larger studies should be conducted to validate YKL-40 as a potential glioma biomarker, as there is no consensus on its clinical value.

Notably, matrix metalloproteinase (MMP)-9 in serum and CSF measured in patients with glioma were associated with prognosis and treatment response. Increased MMP-9 levels in the CSF of patients with recurrent glioma treated with doxycycline signalled a failed response to treatment.[73] Serum MMP-9 levels in patients with high-grade glioma were shown by ELISA to be upregulated following tumour resection.[66] This speculation is likely a result of increased inflammation in response to surgery, as MMP-9 levels are increased during inflammation.[74] Interestingly, patients with glioblastoma with radiographically evident disease had significantly higher serum MMP-9 expression, compared with patients with radiographically undetectable disease.[66] MMP-9 therefore shows potential as a biomarker for glioblastoma progression, although further research is much needed.

Increased haptoglobin expression levels have been demonstrated to be proportional to increasing tumour grade, being higher in glioblastoma samples than diffuse

astrocytomas.[75] However, this correlation was applied to only astrocytomas and glioblastomas, and not other lower-grade gliomas. Haptoglobin overexpression in tumours implanted in mice resulted in a worse prognosis.[75] Despite these results, a later study by van Linde *et al*[71] demonstrated no significant association between serum haptoglobin concentration and progression-free survival. They further reported no significant differences in serum haptoglobin concentration following chemoradiation.[71] Research on haptoglobin as a glioma biomarker is still in its infancy.

A2-Heremans-Schmid glycoprotein (AHSG) levels have been correlated to overall survival in a cohort of 72 patients with glioblastoma.[76] Patients with lower AHSG expression had a significantly lower median survival time, compared with the controls.[76] However, another study determined no significant association between serum AHSG level and progression-free survival.[71] Clearly, the biomarker potential of AHSG remains uncertain, although variations in data might be pinpointed to differences in collection times, sample characteristics and assays.[77]

Multiple reaction monitoring and ELISA recently detected S100A8 and S100A9 upregulation in glioblastoma sera.[78] S100A8/A9 transcript levels were also predictive of poor prognosis. Patients with increased transcript levels of S100A8/A9 had a significantly lower median survival time.[78] S100A8/A9 levels were also upregulated to a higher degree in glioblastoma, compared with WHO grade III glioma.[78] There is potential for S100A8/A9 to serve as biomarkers for glioma classification and prognosis, although studies in this field are limited and should be further expanded on.

CONCLUSIONS AND FUTURE DIRECTIONS

The molecular and cellular complexity of gliomas significantly limits diagnosis and treatment. This is further compounded by immense intertumoural and intratumoural heterogeneity, and collectively impacts on the ability to develop effective and reliable biomarkers of disease. To date, there are no approved biomarkers of glioma for clinical practice. There is an urgent need for biomarkers that (1) aid in diagnosis and patient stratification, (2) identify true disease recurrence, and (3) indicate response to treatment. Biomarker candidates exist in the form of CTCs, EVs, nucleic acids and proteins, but studies are scattered and have provided conflicting evidence. Notably, research on EVs has offered the most compelling data supporting their capacity to differentiate between glioma grade, assess response to therapy and indicate the extent of patient immune responses. Additionally, the miR-21 oncogene has demonstrated potential, with its ability to be detected with relatively high specificity and sensitivity, and its expression having been associated with increasing glioma grade, decreased patient survival and decreased response to chemotherapy. Nonetheless, there needs to be a more focused and indepth approach to biomarker analysis. There is an abundance of research covering a multitude of biomarker candidates, but detailed studies focused on each of the candidates per se, are lacking. It would be interesting to establish follow-up investigations to studies that have demonstrated significant associations between biomarker contenders and outcomes of glioma. Large-scale single-cell RNA sequencing would be a useful technique to identify differences and similarities in molecular expression between patients, 'healthy control' postmortem specimens and within single tumours. Novel technologies, such as single-molecule arrays (Simoa), would additionally be beneficial in detecting proteomic molecules within sera/plasma with incredible sensitivity and specificity. These would serve as valuable steps in identifying potential biomarkers for glioma.

Contributors LKK researched the topic and wrote the first draft. KD and MH provided input in regard to surgical approaches and surgical management in addition to editorial input. TJO and DW provided intellectual input and reviewed drafts of the manuscript. MM designed the concept and assisted LKK with overall approach, provision of intellectual input and editorial content.

Funding This study was funded by Brain Foundation Australia.

Competing interests None declared.

Patient consent for publication Not required.

Provenance and peer review Not commissioned; externally peer reviewed.

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