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Review: Molecular pathology in adult high-grade gliomas: from molecular diagnostics to target therapies

K. Masui[*], **T. F. Cloughesy**[‡,§], and **P. S. Mischel**[*,†,§]

[*]Department of Pathology and Laboratory Medicine, David Geffen University of California at Los Angeles School of Medicine, Los Angeles, California, USA

[†]Department of Molecular and Medical Pharmacology, David Geffen University of California at Los Angeles School of Medicine, Los Angeles, California, USA

[‡]Department of Neurology, David Geffen University of California at Los Angeles School of Medicine, Los Angeles, California, USA

[§]Department of Henry Singleton Brain Tumor Program, David Geffen University of California at Los Angeles School of Medicine, Los Angeles, California, USA

Abstract

The classification of malignant gliomas is moving from a morphology-based guide to a system built on molecular criteria. The development of a genomic landscape for gliomas and a better understanding of its functional consequences have led to the development of internally consistent molecular classifiers. However, development of a biologically insightful classification to guide therapy is still a work in progress. Response to targeted treatments is based not only on the presence of drugable targets, but rather on the molecular circuitry of the cells. Further, tumours are heterogeneous and change and adapt in response to drugs. Therefore, the challenge of developing molecular classifiers that provide meaningful ways to stratify patients for therapy remains a major challenge for the field. In this review, we examine the potential role of MGMT methylation, *IDH1/2* mutations, 1p/19q deletions, aberrant epidermal growth factor receptor and PI3K pathways, abnormal p53/Rb pathways, cancer stem-cell markers and microRNAs as prognostic and predictive molecular markers in the setting of adult high-grade gliomas and we outline the clinically relevant subtypes of glioblastoma with genomic, transcriptomic and proteomic integrated analyses. Furthermore, we describe how these advances, especially in epidermal growth factor receptor/PI3K/mTOR signalling pathway, affect our approaches towards targeted therapy, raising new challenges and identifying new leads.

Keywords

high-grade gliomas; integrated analyses; molecular markers; predictive factors; prognostic factors; target therapies

Correspondence: Paul S. Mischel, Lya and Harrison Latta Professor of Pathology, The David Geffen UCLA School of Medicine, 10833 Le Conte Avenue, Los Angeles, CA 90095-1732, USA. Tel: +1 310 794 5223; Fax: +1 310 267 2058; pmischel@mednet.ucla.edu.

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Introduction

Malignant gliomas are the most common type of primary brain cancer. They arise from the constituent glial cells of the brain, or their precursors, and diffusely invade surrounding brain, making curative surgical resection almost impossible. Diffuse gliomas are classified on their morphological appearance and similarity to astrocytic or oligodendroglial cells and graded on a scale of II–IV with increasing malignancy, according to the World Health Organization (WHO) classification [1]. The high-grade gliomas, anaplastic gliomas (WHO grade III) and glioblastoma (GBM, WHO grade IV), have a dismal prognosis, with median survivals of a few years for the former and 9–12 months for the latter [2]. On rare occasions, patients with GBM survive greater than 3 years and are referred to as GBM long-term survivors [3,4]. Several clinical and histopathological elements are reportedly associated with a better prognosis for GBM, including young age, good performance status, gross total resection, adjuvant treatments, giant-cell subtype and oligodendroglial differentiation [1,5–9]. The heterogeneity of patient outcomes points to the insufficiency of a classification based on histopathology. That is, as a group, the diagnostic categories provide information about the group, but limited information about an individual patient. More importantly, in an era of therapies designed to target specific molecular lesions, a classification based on histopathology does not provide sufficient insight for patient stratification. Therefore, great efforts have been made to incorporate new information about the molecular landscape of gliomas into novel classifications that may potentially guide treatment. Importantly, this is an evolving process. Classifications based on molecular abnormalities, which will be discussed below, are internally consistent and in general correlate well with morphological criteria. However, these classifiers have yet to reach the level of sophistication and depth that will enable patient stratification.

During the last few decades, the discoveries of several genetic alterations and aberrant signalling pathways have made a considerable contribution to our understanding of the genesis and biology of gliomas (Figure 1). One of the significant spin-offs from this is that genetic profile can be of prognostic or predictive importance [10,11]. This is an important step towards developing specific treatments for the individual, that is, the concept of personalized medicine [12,13]. Here, we provide a brief look at what is known about the molecular aspects of high-grade gliomas, including prognostic/predictive markers and emerging new concepts, that are useful for the stratification of patients and an adequate evaluation of the potential efficacy of therapeutic agents.

Distinctive molecular features and their prognostic and predictive significance for high-grade gliomas (Table 1)

O-6-methylguanine-DNA methyltransferase (*MGMT*)

The DNA-repair enzyme *MGMT* gene, located on 10q26, has been a subject of interest owing to its association with response to alkylating drugs [14, 15]. In tumours, the cytosines in the *MGMT* promoter CG dinucleotides (CpG)-rich sites often carry a methyl group that silences its expression relative to normal tissue [15]. Of interest, epi-genetic abrogation of

MGMT augments sensitivity to one of the most-used alkylating agents for GBM, temozolomide (TMZ), which damages DNA by methylating the O-6 position of the guanine [16,17].

The MGMT status can be tested by methylation-specific polymerase chain reaction (MS-PCR), which is based on bisulphite conversion of unmethylated cytosines into uracils [18]. The assay can be performed on formalin-fixed paraffin-embedded (FFPE) tissue [19]. Further, real-time MS-PCR allows for quantification with high throughput [20]. Other techniques have also emerged, including methylation-specific pyrosequencing [21] and methylation-specific multiplex ligation dependent probe amplification (MS-MLPA) [22], but the value of immunohistochemistry (IHC) in the assessment of *MGMT* methylation is controversial [23].

About 40% of primary GBM and over 70% of secondary GBM display epigenetic MGMT silencing [24,25], although these frequencies vary [15,26]. *MGMT* promoter methylation is also observed in anaplastic gliomas, ranging from 50% to 80% [27–29]. The relationship between MGMT and TMZ was revealed by a prospective trial as a strong and independent predictive factor for GBM patients [6,17,30]. Subsequent studies confirmed that *MGMT* promoter hypermethylation can also be a prognostic factor for patients with GBM [31,32], including elderly patients [33]. In anaplastic gliomas, *MGMT* methylation seems to be a favourable prognostic marker independent of therapy (radiation or chemotherapy) [28,34]. Anaplastic gliomas with hypermethylated-*MGMT* exhibit a survival benefit when treated with radiation alone [32]. In ana-plastic oligodendroglioma (AO), *MGMT* promoter methylation is also prognostic, but does not have predictive value [34]. *MGMT* promoter methylation is associated with improved outcome in patients with anaplastic astrocytoma (AA) and anaplastic oligoastrocytoma (AOA) treated with TMZ at recurrence [35]. These results might be related to associations with other prognostic markers in these tumours, including 1p/19q deletions [34,36,37] and *IDH1* mutation [38,39].

Isocitrate dehydrogenase 1/2 (*IDH1/2*)

One of the significant recent discoveries in the field of GBM is a recurrent mutation in the active site of cytosolic NADP-dependent isocitrate dehydrogenase (*IDH1*) [40]. Subsequently, mutations in mitochondrial NADP-dependent isocitrate dehydrogenase (*IDH2*) gene have also been reported in around 3% of gliomas [41]. The *IDH1* gene, located on chromosome 2q33, encodes an enzyme which catalyses the oxidative carboxylation of isocitrate to a-ketoglutarate, resulting in the reduction of NADP to NADPH [42]. *IDH2* on chromosome 15q26 produces an enzyme that plays the same role in mitochondria [43]. Several theories for the tumorigenic potential of mutant IDH1/2 protein have been promulgated; the mutation can decrease the amount of NADPH necessary for cellular protection from oxidative stress [39,44] or produce a decrement in a-ketoglutarate, which degrades HIF-1a, a promoter for tumour growth and angiogenesis [45]. However, heterozygous *IDH1/2* mutations may be consistent with a gain of function, and the mutant enzyme was reported to increase the formation of 2-hydroxyglutarate, which might be tumorigenic [46,47]. Despite their tumorigenic potential, *IDH1/2* mutations are associated with young patient age, secondary GBM and longer overall survival [40].

Detection of *IDH1/2* mutations relies on direct sequencing, including pyrosequencing [38,48], or single strand conformation polymorphism [49]. An alternative method, derived cleaved amplified polymorphic sequence, utilizes mismatched primers for endonuclease-based detection of mutations in codon 132 of *IDH1* [50]. Another approach is melting curve analysis performed on real-time PCR products [51], allowing for rapid and sensitive analysis easily accessible in the diagnostic laboratory. An *IDH1* R132H (93% of glioma-associated *IDH1* hotspot mutations) mutation-specific antibody suitable for FFPE tissue is now available for IHC [52,53].

IDH1 mutations appear to be found almost exclusively in grade II and III gliomas and the secondary GBMs that arise from them. Over 80% of secondary GBMs possess an *IDH1* mutation, and anaplastic gliomas also show high frequencies of *IDH1* mutation (AA: 69.2% and AO: 86.1%) [44]. In contrast, *IDH1* and *IDH2* mutations are rarely detected in primary GBMs, with a frequency of 3–7% [38,39,44,54–56]. The original studies describing these mutations in GBMs [55], AAs [28] and AOs [29] demonstrated a better outcome for tumours with mutation than for those without. Furthermore, a recent trial provides evidence that *IDH1* status is more prognostic for overall survival than standard histological criteria, the order from relatively favourable to poor outcome being: AA with mutation, GBM with mutation, AA without mutation and GBM without mutation [57]. These findings suggest that the classification of anaplastic glioma and GBM, and thus patient management, should include *IDH1* status. While *IDH1/2* mutations signify a more favourable prognosis across all grades of glioma, attempts to demonstrate a link between these mutations and benefit from a specific treatment have so far failed [29], and the role of *IDH* mutations as predictive indicators remains to be defined.

The *IDH1/2* mutation story has two important consequences. First, it provides a powerful link between genetic alteration and the biochemical phenotype of cancer. In leukaemia, *IDH1/2* mutations result in a hypermethylated phenotype, which impairs haematopoietic differentiation [58]. The recent finding that *IDH1* mutations are tightly linked with the CpG island methylator phenotype (G-CIMP) across all glioma tumour grades [59], raises the possibility that *IDH1* mutations may also be disrupting global methylation patterns in diffuse gliomas. Future studies will be needed to determine if *IDH1* mutations similarly limit differentiation and promote tumour stem-cell self-renewal. Second, the strong association between *IDH1/2* mutations and grade II/III gliomas and secondary GBMs and its relative exclusion from primary GBMs provide a critical insight – primary and secondary GBMs are fundamentally not the same disease. The recent finding that *IDH1*-mutant and *IDH1*-wild type GBMs contain non-overlapping sets of molecular events and that *IDH1*-mutant tumours preferentially arise from oligodendroglial precursors involved in frontal cortex maturation further suggest a different cell of origin for *IDH1*-mutant gliomas [60]. These results also raise a critical question. If *IDH1/2* mutations are a rare event in the majority of primary GBMs, which are phosphatidylinositol 3′; kinase (PI3K) hyperactivated, what are the links between PI3K hyperactivation and altered cellular metabolism in primary GBMs?

Chromosome 1p/19q

Oligodendroglial tumours exhibit frequent loss of heterozygosity (LOH) on the short arm of chromosome 1 (1p) and the long arm of chromosome 19 (19q) [61]. Although putative tumour suppressors on 1p and 19q have been evaluated and no tumorigenic genes definitely identified thus far [62–66], a recent exome sequencing study of oligodendrogliomas revealed inactivating mutations of the *CIC* gene (*homologue of the Drosophila gene capicua*) on 19q and the *FUBP1* gene (*far-upstream element binding protein 1*) on 1p in a substantial fraction of oligodendrogliomas, providing important insights into the pathogenesis, diagnosis, prognosis and treatment of these tumours [67]. One of the notable findings with 1p/19q LOH is the favourable response to chemotherapy in oligodendroglial tumours with this co-deletion [10].

Generally, 1p/19q loss is assessed with PCR-based LOH assays [68] or fluorescence *in situ* hybridization (FISH) [69]. FISH uses fluorescence-conjugated probes directly on tissue sections, and is favoured by neuropathologists, because tissue architecture is preserved. MLPA is another option and detects multiple copy number changes in a single analysis [70]. Other techniques, such as array comparative genomic hybridization (CGH), provide an estimate of copy number at all chromosomal loci and can detect losses as well as polysomies [71].

Losses of 1p and 19q are detected in up to 90% of oligodendrogliomas and 50–70% of AO [72], whereas 30–50% of oligoastrocytomas, 20–30% of AOA, and less than 10% of diffuse astrocytic gliomas, including GBM, demonstrate this aberration [73]. The role of 1p/19q loss in AO has been studied, confirming the prognostic and possibly predictive role of these markers in the setting of first-line chemotherapy [74,75], although their relationship to second-line chemotherapy is less clear [76,77]. Furthermore, favourable outcome may be independent of the type of adjuvant therapy [28,74,75]. In contrast to AO, the frequency of 1p/19q deletions among GBMs is low, and any association with outcome is complex. 1p/19q deletions alone did not appear to associate with relatively good survival in one series of patients with GBM [78], but in another study using CGH some GBM long-term survivors showed combined loss of 1p and 19q [79]. Conversely, deletions involving 1p and 19q seem to display shorter survival in a small number of GBM, possibly due to genomic instability [80]. As 1p/19q LOH in anaplastic gliomas is significantly correlated with prognosis and chemosensitivity, and given the difficulties associated with histopathological classification, genetic testing should be performed routinely for these tumours [81].

Epidermal growth factor receptor (EGFR) and PI3K-Akt pathway

Overexpressed or mutated tyrosine kinases contribute to the development and progression of many tumours [82]. This is also the case with gliomas, and *EGFR* (7p12) amplification is a hallmark of GBM, specifically primary tumours [83]. About 50% of *EGFR*-amplified GBM express a ligand-independent truncated mutant variant, EGFRvIII, which is characterized by genomic deletion of exons 2–7, resulting in a constitutively active oncogenic form [84,85]. The subsequent strong and persistent activation of downstream PI3K signalling provides advantages for cell survival, proliferation, and motility [86–88]. There are additional missense mutations in the extracellular domain of *EGFR* in GBM, which are oncogenic and

mutually exclusive to EGFRvIII [89]. These findings suggest a broader spectrum of *EGFR*-activating mutations in GBM, the clinical importance of which is not currently understood.

By cytogenetics, *EGFR* amplification usually manifests as double-minutes, small fragments of extrachromosomal DNA that can be effectively detected by FISH. FISH can also provide information about genetic heterogeneity within tumours [85]. Other techniques such as real-time PCR can be used to identify and quantify *EGFR* amplification [90]. MLPA analysis allows for simultaneous and semi-quantitative copy number analysis [91]. IHC with an antibody specific for *EGFR*vIII is claimed to detect tumour cells with this variant [92], although the value of EGFR IHC is less clear [93]. In addition, a real-time RT-PCR assay has been developed for quantification of *EGFR*vIII expression in FFPE samples [94].

Increased EGFR-mediated signalling is observed in about 30% of gliomas [95,96] and 60% of GBMs [12], and the Cancer Genome Atlas (TCGA) project identified copy number alterations and/or amplification of *EGFR* in 45% of GBMs [97]. *EGFR* amplification and *EGFR*vIII associate with high-grade malignancy and may provide prognostic information [1,92]; *EGFR*vIII overexpression and *EGFR* amplification have been promoted as indicators of poor survival in GBM [98,99]. Additionally, *EGFR*vIII has been reported to be an indicator of poor prognosis in GBM and AA [85,100–102], and also poor response to radiation therapy and chemotherapy [103,104]. Conversely, other studies have reported that these abnormalities lack any association with survival in GBM [105,106], or that *EGFR* amplification may confer improved survival in elderly patients [107,108].

Activation of the PI3K pathway is significantly linked to increasing tumour grade, decreased levels of apoptosis, and adverse outcome in human gliomas [109,110]. Multivariate analysis suggests that elevated expression of p-Akt is related to poor prognosis in GBM [111]. p-MAPK also appears to be an independent marker of outcome in GBM, associated with increased resistance to radiation therapy [110]. One of the key mesenchymal signature genes, *Chitinase 3-like 1* (*YKL-40*), which potentially activates Akt and MAPK [112,113], is predictive of poor outcome in *EGFR*vIII-negative patients, but not in *EGFR*vIII-positive patients [114]. Raf kinase inhibitory protein expression correlates with tumour grade and is a marker for good prognosis in high-grade gliomas [115]. Low PTEN (phosphatase and tensin homologue deleted in chromosome 10) RNA or protein levels, or LOH, portend decreased survival in patients with GBM [116,117]. Also, *PTEN* mutation, although not having a prognostic impact on patients with GBM, is a negative prognostic factor in AA [108].

p53 and Rb pathway

The *TP53* tumour suppressor gene, located on 17p13, is frequently mutated or deleted in human tumours, including gliomas [118,119]. p53 can execute diverse cellular programmes, such as cell cycle arrest, DNA repair, apoptosis, autophagy, differentiation, senescence and self-renewal [120,121]. The retinoblastoma (Rb, 13q14) pathway is also a key cell cycle regulatory complex at the G1 checkpoint. *CDKN2A*, located on 9p21 and deleted in many cancers, encodes the p16 protein, a key inhibitor of the cell cycle via Rb pathway signalling, and its homozygous deletion is associated with WHO grade III or IV gliomas [1]. Gliomas often display mutations in the ARFMDM2-p53 and p16INK4A-CDK4-RB tumour suppressor pathways [122,123]. Primary GBM often displays loss of the *INK4A/ARF* tumour

suppressor gene locus along with *PTEN* mutation and *EGFR* amplification/mutation, and secondary GBM shows frequent mutations of *TP53* [124].

The relevance of p53 to the treatment and outcome of patients with high-grade glioma has remained controversial. Some studies have shown that p53 status, assayed either by expression or mutation analysis, is correlated with (relatively good) outcome [125,126], while others have demonstrated no prognostic impact in anaplastic gliomas and GBM [127–130]. Also, *MDM2* amplification, although infrequent, has been shown by some to be predictive of poor outcome [125,131], whereas others have observed no prognostic value [132]. p53 status might cooperate with other prognostic variables; for example, *TP53* mutation has been linked to low *MGMT* mRNA expression [133], although this does not correlate with *MGMT* promoter methylation [134]. Loss of *CDKN2A*, *CDKN2B*, or *RB* or *CDK4* amplification, disrupting the Rb pathway, has been shown in AA to associate with decreased survival [135,136]. Conversely, p16 appears to be associated with improved survival in patients treated with chemotherapy and radiation [137]. Overall, it appears that the prognostic impact of p53 and Rb aberrations is at best marginal.

Emerging biological markers, entities and integrative concepts for high-grade gliomas

Cancer stem-cell markers

Cumulative data indicate that clonal populations of tumour cells display heterogeneity with respect to proliferation and differentiation [138,139]. Recent concepts of cancer development propose that a minor population of cancer stem cells or stem-like cells derived from GBM contribute to tumorigenicity [140] and therapeutic resistance [141,142]. Previous reports showed diverse stem-cell marker expression within gliomas, including CD133, nestin, Sox-2, Musashi-1, LHX2, Bmi-1, CXCR4, Flt-4/VEGFR-3 and CD105/Endoglin [143–145]. However, it remains uncertain what role stem-cell markers might have in predicting glioma development, progression or outcome.

In vitro cancer stem-cell generation and presence of CD133+/Ki67+ cells are reportedly indicators of disease progression and poor clinical outcome [146]. Several studies also provide clinical evidence for the involvement of a tumour stem-cell phenotype in the progression and chemoradiotherapeutic resistance of GBM, including CD133 (prominin-1) [146–148], nestin [147,149,150], BMI-1 [151], Notch1 [152] and Wnt/β-catenin-related molecules [153]. However, other studies do not support these findings [154,155].

MicroRNA

MicroRNAs (miRNAs) are short (19–24 nucleotides) nonprotein-coding RNAs that regulate gene expression at the post-transcriptional level. These tiny RNA molecules can function as key regulators of a wide variety of biological processes, including cell proliferation, cell differentiation, apoptosis and development [156]. Given the pivotal regulatory role of miRNAs in a wide range of processes, several miRNAs seem to act as tumour suppressors or oncogenes in the biology of cancers, including GBM.

Increasing numbers of miRNAs have been shown to target important pathways that drive GBM, including tyrosine kinase signalling [157], cell cycle progression [158–161], gliomagenesis via stem-cell regulation [159,162,163] and malignant progression [164]. Furthermore, there are differences in miRNA expression patterns among gliomas of different grades, and miRNAs can be useful for subclassifying GBM in a manner that facilitates more accurate prognosis and treatment decisions [165,166]. These include miR-196 [167] and miR-21, miR-181b, miR-106a [168].

GBM with oligodendroglial component (GBMO)

Glioblastoma represents the most malignant type of diffuse astrocytic tumour, but a subset of GBM shows focal oligodendroglial features, suggesting that some GBM may also have an oligodendroglial origin [169,170]. The presence of an oligodendroglial component in GBM appears to be an important prognostic factor, outcome being better for GBMO than for classic GBM [171]. An oligodendroglial component is detected in 10% of GBM, and these patients are significantly younger and survive longer [172]. Furthermore, patients with GBMO treated with post-operative chemotherapy and radiotherapy have a better prognosis than is reported for GBM in modern chemoradiation series [172]. An oligodendroglial component is also associated with improved survival in patients with AA [173], although this lost power in a multivariate analysis [174]. These results support the concept that GBM and AA with an oligodendroglial component have a better prognosis than pure GBM and AA respectively.

What factors may have a prognostic impact in GBMO? LOH on 1p and 19q is significantly associated with GBMO [173]. In a study using CGH and FISH, four discrete cyto-genetic subtypes of GBMO with different outcomes have been proposed: an ‘astrocytic’ subtype characterized by +7/-10; an ‘oligodendroglial’ subtype with -1p/-19q, an ‘intermediate’ subtype showing +7/-1p and an ‘other’ subtype having none of the former aberrations [175]. However, others have found no association between cytogenetics, histopathology, and outcome in GBMO [176]. The designation of AOA with necrosis as GBMO remains a controversial move, which has yet to be backed by a clear understanding of how GBMO overlaps GBM on a genetic basis, but the clinical relevance of this histopathological phenotype needs to be addressed in future studies of high-grade gliomas [1,177,178].

Clinically relevant subtypes of GBM by integrated analyses (Table 2)

One recent approach to the molecular characterization of GBM utilizes a combination of molecular signatures, as opposed to individual markers. Previous molecular studies have identified important genetic events in GBM: (i) dysregulation of growth factor signalling via receptor tyrosine kinase (RTK) aberrations; (ii) activation of the PI3K pathway; and (iii) inactivation of the p53 and Rb tumour suppressor pathways [123]. These findings can be integrated with data from the TCGA Research Network, which was established to generate a comprehensive catalogue of genomic abnormalities driving tumorigenesis [97]. Utilizing gene expression profiles generated from a large series of GBMs, unsupervised clustering identifies four molecular subgroups: Proneural, Neural, Classical and Mesenchymal [179]. In line with previous mRNA-based studies on GBM [180], a proneural signature appears to be associated with a better clinical outcome than a proliferative or mesenchymal signature.

In addition, an analysis of epigenetic changes from TCGA samples identified the existence of a proportion of GBM tumours with highly concordant DNA methylation at a subset of loci, indicative of G-CIMP [59]. G-CIMP-positive GBMs are associated with specific clinical and genetic features, showing a favourable prognosis among all GBMs and among the proneural subset [59]. These data could provide a scheme that integrates genomic, epigenomic and transcriptomic elements in a molecular stratification of GBM. Additionally, proteomic analysis of GBM samples revealed three patterns of expression and activation of proteins in glioma-relevant signalling pathways: (i) EGFR activation associated with amplification and mutation of the receptor; (ii) platelet-derived growth factor (PDGF)-pathway activation that is primarily ligand-driven; and (iii) loss of NF1 expression, all of which provide insight into glioma biology and therapeutic strategies [181].

The current status of molecular therapies targeting the EGFR/PI3K/mammalian target of rapamycin (mTOR) signalling pathway in high-grade gliomas

Despite contradictory findings on the prognostic utility of *EGFR* amplification, advances in our understanding of the EGFR/PI3K pathway in GBM suggest that targeted therapeutics could be used to treat tumours with specific aberrations. Additionally, mTOR acts through the canonical PI3K pathway to mediate cell growth and proliferation via two distinct complexes, mTORC1 (mTOR with PRAS40, raptor, mLST8/GbL) and mTORC2 (mTOR with rictor, mSIN1, protor, mLST8) [182]. mTORC1 has been identified as a critical step in glial transformation and, through its substrates such as S6K1 and 4E-BP1, integrates growth factor signalling with cellular metabolism underscoring its value as a cancer cell target [183,184]. mTORC2 regulates Akt signalling in a rapamycin-insensitive manner, placing mTOR as both a downstream target and an upstream activator of Akt [185]. Thus, mTOR is an important integrator of multiple signalling cascades, which could be therapeutically targeted (Figure 2).

In the clinic, EGFR tyrosine kinase inhibitors and the allosteric mTOR inhibitor rapamycin (and its derivatives), have failed to show durable efficacy as monotherapies [186–190]. However, some of these trials have emphasized the importance of EGFR and mTOR signalling in GBM, validating their role as targets, while also demonstrating the relative ease by which GBMs develop compensatory resistance mechanisms to maintain signal flux to critical downstream effectors. These clinical experiences suggest that tumour cell susceptibility to targeted therapeutics is greatly affected by context-dependent oncogene addiction and acquired resistance. Initial results with the EGFR tyrosine kinase inhibitors, gefitinib and erlotinib, indicate relatively low response rates of 10–15% [188,191]. However, expression of the constitutively active mutant *EGFR*vIII sensitizes tumours to EGFR inhibitors, but only if the PTEN tumour suppressor protein is intact. In fact, loss of PTEN uncouples the inhibition of EGFR from the inhibition of downstream PI3K signalling, demonstrating that PTEN loss is a critical factor in promoting resistance to EGFR inhibitors, in part, because maintained PI3K signal flux is maintained in PTEN-deficient tumours [186]. Concurrently, cells with high levels of EGFR coupled with low levels of activated Akt were proven more likely to respond to small molecule tyrosine kinase inhibitors [188].

These studies indicate that intact regulation of PI3K signalling appears to be critical for effective response to EGFR. Furthermore, mTORC1 appears to be an effector of EGFR inhibitor resistance through PTEN loss or RTK activation [192,193]. Indeed, preclinical studies have demonstrated that dual EGFR/mTOR inhibition was effective at targeting EGFR-activated PTEN deficient tumours [193–195]. In contrast, some PTEN-intact malignant glioma patients relapse after a relatively short window of clinical response to EGFR kinase inhibitors. Notably, it was reported that upon treatment with EGFR inhibitors, other RTKs such as c-MET and/or PDGFR were co-activated, engaging PI3K to maintain downstream pathway activation, despite EGFR inhibition [196]. Also, a recent report shows that AKT inhibition induces the expression and phosphorylation of multiple RTKs, effects due partly to mTORC1 inhibition and partly to a FOXO-dependent activation of receptor expression [197]. Altogether, these findings suggest that nongenetic adaptations in tumour cells result in resistance to treatment and teach a critical lesson; there are many paths towards resistance, whereas a few critical mediators must be inhibited for sensitivity.

Understanding the complex role of mTOR in regulating signal transduction is critical to developing more effective mTOR-targeted therapies, even though studies in human patients with recurrent malignant glioma have failed to demonstrate consistent responses to rapamycin and its analogues [190,198–200]. Rapamycin treatment leads to Akt activation, presumably due to the loss of negative feedback for attenuating PI3K signalling, which is associated with significantly shorter time-to-progression [187]. PI3K pathway reactivation after rapamycin treatment suggests that dual PI3K/mTOR inhibitors function by preventing PI3K signalling reactivation and more effectively targeting mTORC2 (and mTORC1) signalling. A dual PI3K/mTOR inhibitor (PI-103) was indeed efficacious at blocking the growth of GBM cells, independent of PTEN status [194]. Dual PI3K/mTOR inhibitors may also suppress extracellular signal-regulated kinase signalling activation through mTORC1 inhibition and a PI3K-dependent mechanism [201].

In GBM, mTORC2 signalling is less well understood than mTORC1 signalling. mTORC2, which is activated by PI3K, phosphorylates Akt on serine 473 (Ser473), thereby promoting Akt activity. mTORC2 also activates additional kinases, including serum glucocorticoid-induced protein kinase (SGK) and protein kinase Ca, all of which may play important roles in regulating cell proliferation and growth. mTORC2 activity has been shown to be relevant to glioma cell proliferation, motility and gliomagenesis [202] and is required for growth of EGFR/PI3K-activated gliomas in a drosophila model [203]. These results raise the possibility that mTORC2 signalling is essential for glioma growth, particularly in the context of enhanced PI3K signalling [203]. We recently demonstrated that mTORC2 is frequently activated in GBM and that *EGFRvIII* can potently stimulate mTORC2 and GBM growth and survival, by activating NF-kB through SGK1. We also showed that mTORC2 is involved in feedback activation of Akt in rapamycin-treated patients, implying a need to inhibit both mTORC1 and mTORC2 in order to achieve a better clinical response. Such clinical trials are currently under way. However, a previously unsuspected role for mTORC2 in mediating chemotherapy resistance has also been indentified. EGFRvIII-expressing GBMs are exquisitely resistant to cisplatin [204]. We have shown that mTORC2 mediates chemotherapy resistance through mTORC2/SGK1-mediated activation of NF-kB. Genetic or

pharmacologic inhibition of mTORC2 reverses GBM cell resistance to cisplatin, TMZ and etoposide. These results strongly suggest a critical role for drugs that target both mTORC1 and mTORC2, including in combination with chemotherapy [205].

mTOR also plays a critical role in integrating cellular metabolism with signal transduction. Class 3 PI3K (vps34) is reported to provide an amino acid sensing mechanism to activate mTORC1 signalling through a process that is independent of class I PI3K and its canonical signalling pathway [206]. This observation suggests that class 3 PI3K signalling (to mTORC1) could facilitate escape from mTOR or dual class I PI3K/mTOR inhibitors. mTORC1 has also emerged as a critical downstream effector of the tumour suppressor liver kinase B1. Liver kinase B1 is thought to suppress tumours by negatively regulating mTORC1 signalling through AMP-activated protein kinase. A study by our group has demonstrated that the AMP-activated protein kinase agonist, AICAR, effectively blocks the growth of EGFR-activated GBM, primarily by inhibiting lipogenesis [207]. We have also shown that EGFR signalling promotes activation of a transcriptional regulator of fatty acid synthesis, SREBP-1 [208,209]. Importantly, abundant EGFR signalling makes GBM cells more dependent on fatty acid synthesis, and consequently interruption of fatty acid synthesis causes massive apoptotic cell death in tumours with abundant EGFR signalling. Further investigations have uncovered an EGFRvIII-activated, PI3K/SREBP-1-dependent tumour survival pathway acting through the low-density lipoprotein receptor (LDLR) [210]. Targeting LDLR with the liver X receptor agonist GW3965 caused inducible degrader of LDLR-mediated LDLR degradation and increased expression of the ABCA1 cholesterol efflux transporter, potently promoting tumour cell death in a GBM model. Thus, understanding interactions between cellular metabolism and mTOR signalling, as well as EGFR signalling through the PI3K/Akt and RAS/extracellular signal-regulated kinase pathways, may pave the way for developing more effective treatment strategies.

Conclusion

The classification of malignant gliomas is changing profoundly; novel schemes are being developed around data on their genomic landscape from the TCGA [97] and from next generation sequencing [40]. While we await an ideal scheme that successfully matches the molecular profile of a malignant glioma to its targeted therapy, further advances gleaned from these methodologies, plus epigenetics, proteomics and an understanding of oncogenic miRNAs, will move the field forward to its goal of personalized medicine for patients with malignant glioma.

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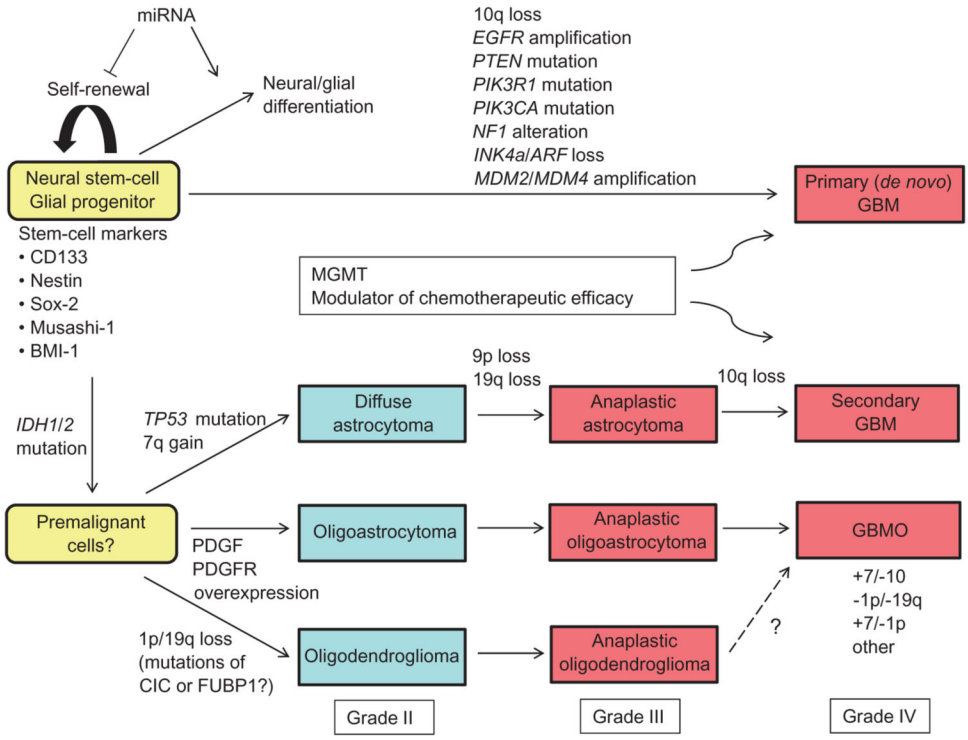

Figure 1.

Proposed genetic alterations, aberrant signalling pathways and therapeutic modulators in human high-grade gliomas.

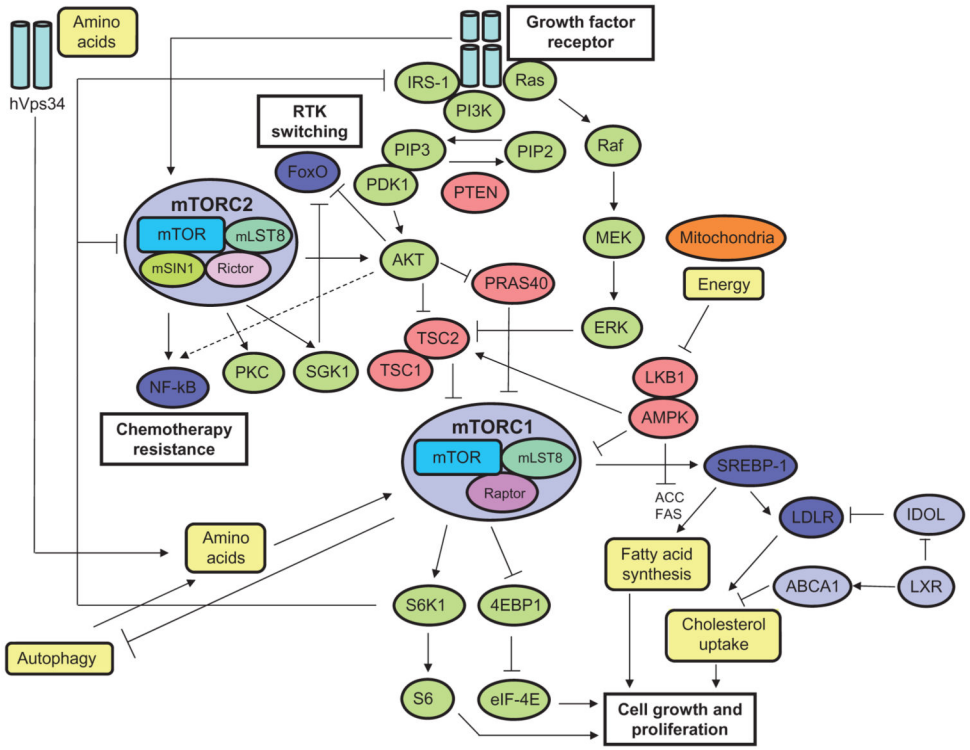

Figure 2.

mTORC plays a key role in integrating signal transduction and metabolic pathways in glioblastoma.

Table 1

rognostic/predictive molecular markers in high-grade gliomas

	MGMT ***methylation***	**IDH1/2** ***mutation***	***lp/19q codeletion***	***EGFR and PI3K pathway****	***p53 pathway mutation***	***Rb pathway mutation***	***Stem-cell markers****	***miRNA****
stic glioma III)	Prognostic (AA, AO, AOA)	Prognostic (AA, AO)	Prognostic/predictive (AO)	Negatively prognostic (AA)	Marginal	Marginal	Negatively prognostic?	Negatively prognostic?
astoma (Grade IV)	Prognostic/predictive	Prognostic	Prognostic?	Negatively prognostic/predictive? †	Marginal	Marginal	Negatively prognostic?	Negatively prognostic?

y, a prognostic marker is considered to allow for estimating the outcome in a treatment-independent manner, whereas a predictive marker is of value in the context of a specific therapy.

factors could benefit us in the prediction of aggressive nature of gliomas.

t be predictive for molecular targeted therapies.

plastic astrocytoma; AO, anaplastic oligodendroglioma; AOA, anaplastic oligoastrocytoma.

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Table 2

Integrated view of gene expression and genomic alterations across glioblastoma (GBM) subtypes

	Proneural [179,180]	Neural [179]	Classical [179]	Mesenchymal [179,180]	Proliferative [180]
Morphology	Astrocytic or oligodendroglial	N/A	N/A	Astrocytic	Astrocytic
Markers	Oligodendroglial and proneural development	Neuronal	Neural precursor and stem cell (Nestin, Notch, SHH)	CHI3L1 (YKL40), CD44, VEGF, Inflammatory (TNF, NF-kB)	PCNA, TOP2A
Distinct neural cell types	Oligodendrocytic	Neuronal	Astrocytic	Cultured astroglia, microglia	Neural stem cell or transit amplifying cell?
Chromosome gain/loss	High 4ql2 amplification (*PDGFRA*), low7pll.2 amplification (*EGFR*) and 10q23 loss (*PTEN*)	Chr 7 gain and chr 10 loss	Chr 7 gain with chr 10 loss, high 9p21.3 deletion (*CDKN2A/B*) with low 13ql4 deletion (*RBI*)	17ql 1.2 deletion (*NF1*)	Chr 7 gain and chr 10 loss
Representative mutated genes	*TP53, IDH1* (mutually exclusive with *PDGFRA* abnormality), *PIK3CA/ PIK3R1*	Not representative	*EGFRvIII, TP53* (−)	*NF1, PTEN*	N/A
Patient age	Younger	Older	Older	Older	Older
Secondary GBM	Represented	Not represented	Not represented	Not represented	Not represented
Prognosis	Longer than average survival (not statistically significant)	Shorter than average survival	Shorter than average survival	Shorter than average survival	Shorter than average survival
Intensive treatment	Not alter survival	Efficacy suggested	Reduce mortality	Reduce mortality	N/A
G-CIMP tumour* [59]	Represented	Not represented	Not represented	Not represented	N/A
Proteomic tumour class† [181]	PDGF class?	N/A	N/A	NF1 class?	N/A

*G-CIMP tumours have a high frequency of *IDH1* mutation and characteristic copy number alterations and are associated with younger age and improved survival.

†EGFR-cocluster class shows intermediate expression levels of Proneural and Mesenchymal signatures. Chr, chromosome; N/A, not available.