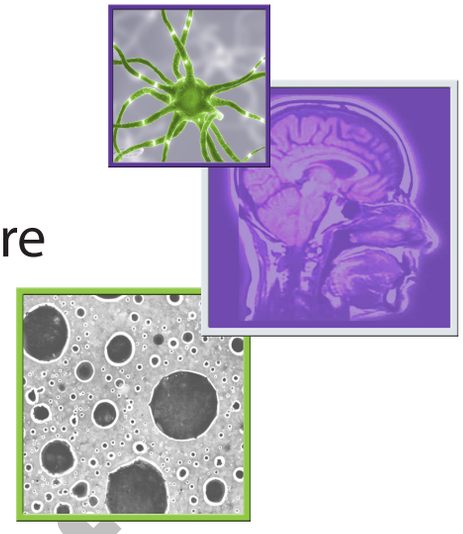
REVIEW

Molecular prognostic factors in glioblastoma: state of the art and future challenges



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Practice Points

- Glioblastoma multiforme (GBM) is the most frequent and lethal tumor of the CNS, for which curative therapies are not available. Classic prognostic factors such as patient's age and performance status, together with tumor characteristics, including grade and molecular features, predict survival in GBM patients.
- The methylation status of the *MGMT* gene promoter and mutation in *IDH1* and *IDH2* genes are among the most promising prognostic biomarkers in GBM.
- GBMs may be stratified into four molecular subtypes – classical, mesenchymal, proneural and neural – each displaying different underlying genetic alterations and gene expression signatures. The assessment of the subtype of each GBM might be important while designing therapeutic approaches.
- A variety of putative prognostic biomarkers have been identified in adult GBM patients. Some examples include the presence of mutations or the expression levels of receptor tyrosine kinases (RTKs), growth factors and intracellular targets (e.g., PI3K); miRNA gene signatures; and serum concentrations of the YKL-40 protein. More recently, the expression of *HOX* and cancer stem cell-associated genes, and loss of chromosome 10 were suggested as novel putative biomarkers predictive of survival in adult GBM, but further studies are required to validate their value.
- Mutations in the *H3F3A* gene are specific to pediatric GBMs, highlighting that pediatric and adult GBMs present a distinct underlying biology. *H3F3A*^{K27M} mutant tumors have a significantly shorter overall survival than *H3F3A*$^{G34R/V}$ or wild-type tumors.
- The fast-accumulated knowledge on new putative biomarkers of GBM aggressiveness and prognosis holds reason for both optimism and caution. While many of these biomarkers have been validated in independent studies, their clinical applicability to highly heterogeneous GBMs is still limited.

SUMMARY Gliomas account for the majority of primary tumors of the CNS, of which glioblastoma (GBM) is the most common and malignant, and for which survival is very poor. Despite significant inter- and intra-tumor heterogeneity, all patients are treated with a standardized therapeutic approach. While some clinical features of GBM patients have already been established as classic prognostic factors (e.g., patient age at diagnosis and

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Karnofsky performance status), one of the most important research fields in neuro-oncology today is the identification of novel molecular determinants of patient survival and tumor response to therapy. Here, we aim to review and discuss some of the most relevant and novel prognostic biomarkers in adult and pediatric GBM patients that may aid in stratifying subgroups of GBMs and rationalizing treatment decisions.

Tumors of the CNS comprise of a broad variety of entities, which range from benign to highly malignant. Typically, their classification is based on their location and histopathological features [1]. Gliomas are the most frequent CNS primary tumors in adults, whose main histological subtypes include astrocytomas, oligodendrogliomas and ependymomas. Astrocytomas represent approximately 70% of all diagnosed gliomas, and are graded from I to IV according to the WHO [1]. Of these, glioblastoma multiforme (GBM; WHO grade IV) is the most frequent and lethal, accounting for more than 50% of all glial tumor types, with an estimated global incidence rate of approximately five per 100,000 people/year [2]. GBMs are characterized by rapid growth and diffuse invasiveness of the adjacent brain parenchyma, and their histopathological features include cellular polymorphism, mitotic activity, nuclear atypia, vascular thrombosis, microvascular proliferation and marked necrosis [1]. Despite several efforts, the treatment for GBM remains mostly palliative, with a median survival of only 15 months [3]. Standard treatment uses a combination of maximum surgical resection, radiation, and concurrent and adjuvant chemotherapy with the alkylating agent temozolomide [3]. Molecular stratification with biomarkers predictive of patient response and outcome may prove crucial in rationalizing treatment decisions. Currently, the most consistently reported and best-established prognostic factors include patient age and performance status, tumor grade and histology, and extent of surgical resection (Box 1) [4–6]. Age is among the most consistent variables associated with GBM patient survival, as older patients fare worse than young patients [4,5,7]. In addition, patients that present higher Karnofsky performance status scores have increased overall survival and better responses to chemotherapy [5–7]. Among the intrinsic tumor characteristics, gliomas of higher WHO grades of malignancy typically have shorter survivals than those of lower grades [8]. Clinically, the extent of tumor surgical resection has also been reported as crucial on influencing GBM patient prognosis, as more complete resections are associated with better outcomes [4,6,7]. In this sense, these classic factors must be clearly assessed when assigning patients for randomized clinical trials, but may also be crucial in aiding clinicians in the refinement of treatment decisions. Nonetheless, in the last decade, studies have identified molecular features that might be prognostically valuable [9–32]. The current most relevant prognostic biomarkers in GBM are summarized in Figure 1 and Box 2, and the most promising ones will be discussed.

MGMT promoter methylation status

The methylation status of the *MGMT* gene promoter region has been shown by many studies as one of the most promising prognostic biomarkers in GBM, although it has not yet reached worldwide clinical applicability [21,33]. *MGMT* encodes a DNA repair enzyme that removes alkyl groups from the O^6 position of guanine, an important site for DNA alkylation after treatment of tumor cells with alkylating agents. If left unrepaired, these lesions trigger cytotoxicity and apoptosis leading to cell death [34]. Two groups showed that epigenetic silencing of *MGMT* by promoter methylation induced loss of *MGMT* expression [35,36], and, therefore, reduced DNA repair activity. Thereafter, Hegi *et al.* showed that this silencing leads to increased sensitivity of the tumor cells to temozolomide treatment [21]. This sensitivity translated into differences in patient survival, with *MGMT* methylation being associated with greater overall survival (median: 21.7 months), as well as higher 2-year survival rates (46%), in comparison with patients with unmethylated *MGMT* (median survival: 12.7 months and 2-year survival: 13.8%). This landmark study suggests that *MGMT* promoter methylation is an independent and favorable prognostic biomarker in GBM patients, and predictive of response to temozolomide [21]. A follow-up study by Stupp *et al.* evaluated adult patients with newly diagnosed GBM, who were treated with standard radiotherapy or radiotherapy combined with concomitant and adjuvant temozolomide [37]. In this study, the methylation status of *MGMT* was evaluated in 206 patients from both cohorts and revealed to be a strong predictor of patient outcome and response to

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chemoradiation [37]; patients with a methylated *MGMT* promoter not only presented longer survivals than patients with unmethylated *MGMT*, but also seemed to benefit more from combined chemoradiotherapy [37]. The value of *MGMT* methylation status is also supported by a recent clinical trial comparing radiotherapy and temozolomide-based treatment in elderly patients [38]. This reported an association between good outcome and *MGMT* methylation in the temozolomide cohort, but not in the radiotherapy cohort [38]. These results were further supported by another study showing that elderly GBM patients presenting with methylated *MGMT* promoters and treated with temozolomide had a significantly longer survival than those who did not present with *MGMT* promoter methylation, or those on the radiotherapy branch irrespective of *MGMT* promoter methylation [39]. Thus, the authors of both reports state that treatment decisions for elderly GBM patients would be aided by assessing *MGMT* promoter methylation [38,39]. A meta-analysis performed by Olson *et al.* that included 20 different studies and a total of 2018 patients, showed that silencing of *MGMT* expression was highly associated with improved overall survival in patients receiving adjuvant chemotherapy, a mild association in patients that received adjuvant radiotherapy, and no benefit in those submitted to surgery alone [40]. Nonetheless, other reports have not supported a statistically significant association between *MGMT* methylation and survival. For example, a study by Costa *et al.* that analyzed the methylation status of *MGMT* in a set of 90 GBM patients treated with postoperative temozolomide-based chemoradiation, observed a trend for longer progression-free and overall survival in GBM patients presenting with *MGMT* promoter methylation, but the differences did not reach statistical significance [15]. Similar results were observed in other studies and reviewed by Costa *et al.* [15]. Another study by van der Bent *et al.* also showed that the methylation status of *MGMT* did not present prognostic significance in GBM patients, and was not able to predict the responses to adjuvant procarbazine, lomustine and vincristine chemotherapy [41]. This is mainly due to the heterogeneity of the study participants, not only with respect to grade, histology and treatment, but also analysis of *MGMT* mRNA expression, methylation status and protein levels [15]. Moreover, sample classification as methylated or unmethylated for a certain gene is still debatable, as the relationship between the CpG methylation at individual sites, overall CpG island methylation and their effects on gene silencing is highly dependent on the location within the gene [42]. Bady *et al.* evaluated the relationship between *MGMT* expression, the specific location of CpG methylation, and the outcome of patients treated with alkylating agents [43]. In this study, two regions of methylated CpG's negatively correlated with *MGMT* gene expression, and were strongly associated with patient survival [43]. This is consistent with *MGMT* expression silencing via CpG methylation, leading to sensitization to alkylating agents [43]. Similarly, Shah *et al.* also identified three regions of methylated CpGs on *MGMT* that correlate with favorable patient progression-free survival, within a population of 44 GBM patients treated with radiotherapy and concomitant and adjuvant temozolomide [44].

Box 1. Selected clinical prognostic markers for glioblastoma.

Classic prognostic factor

- Age [4,5,7]
- Performance status [4–7]
- Mental status [4]
- Symptoms [143]
- Extent of surgical resection [4,6,7]
- Tumor location [5]
- Histological grade [8]

Although the accumulated knowledge on *MGMT* has increased in the last few years, its true clinical significance remains unclear. In fact, *MGMT* methylation status is not yet typically used by clinicians to aid in therapy decisions. Therefore, it is still important to conduct novel clinical trials in prospectively followed patients, and by investigating different drugs and dosages. Indeed, MGMT depletion using pseudosubstrates, such as O^6-(4-bromophenyl) guanine or O^6-benzylguanine, may be able to improve GBM patient response to temozolomide therapy. If so, overcoming temozolomide resistance due to *MGMT* promoter methylation will be a major advance in GBM therapy. In the next few years our understanding of *MGMT* prognostic value and its ability to predict tumor response to different therapies will be very much improved due to the ongoing clinical trials, which may definitively establish it as a major biomarker for GBM patient management.

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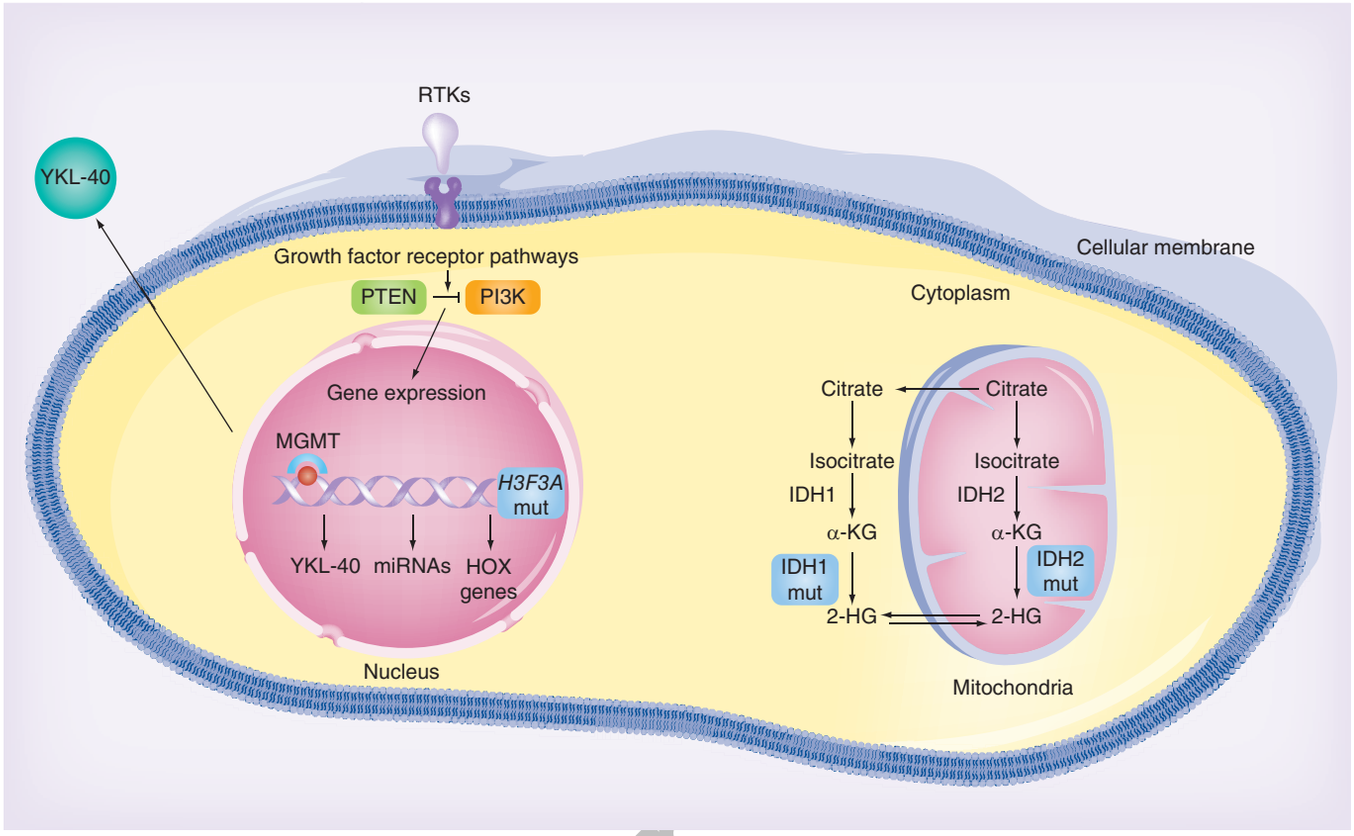


Figure 1. Current putative prognostic biomarkers in glioblastoma. The deregulation of receptor tyrosine kinase pathways leads to aberrant intracellular signaling, including the PI3K pathway, that, among other effects, induces the transcription of genes responsible for sustaining several cancer hallmarks (e.g., *HOX* genes). Loss of *PTEN* function by mutation or loss of heterozygosity has been correlated with poor glioblastoma (GBM) patient survival. *MGMT* expression and promoter methylation levels are markers of GBM patient prognosis. Mutant IDH1^{R132H} and IDH2^{R172K} enzymes are able to convert α-KG into 2-HG, and GBM patients with tumors presenting *IDH* mutations have longer survival. Deregulation of several miRNAs has been implicated in both the initiation and progression of GBM, and many have been reported to present prognostic value. YKL-40 is produced by GBM cells and released into the serum, where its levels are predictive of an aggressive phenotype and associated with poor overall patient survival. Mutant H3F3A^{K27M} is highly specific to pediatric GBM, and associates with worse prognosis in these patients. Green and orange boxes indicate loss or increased function, respectively. Blue boxes indicate mutant IDHs and H3F3A.
2-HG: 2-Hydroxyglutarate; α-KG: α-ketoglutarate; *H3F3A* mut: Mutant *H3F3A*; RTK: Receptor tyrosine kinase.

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IDH mutations

Recent genomic studies revealed the presence of mutations in *IDH1* and *IDH2* genes (*IDH* when referring to both) as important prognostic factors for GBM [28,45,46]. These NADP-dependent enzymes are able to catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate, with the simultaneous production of NADPH [47]. High-throughput sequencing studies of GBM revealed a new *IDH1* mutation, consisting of changing a guanine to an adenine at position 395 of the gene (G395A), thus leading to the replacement of an arginine with a histidine at residue 132 of the protein (R132H) [28]. This heterozygous and somatic mutation was found in 12% of all GBM patients. Similarly, the evaluation of the *IDH2* exon sequence revealed a point mutation changing a guanine to an adenine at position 515 of the gene (G515A), causing the substitution of an arginine to a lysine at residue 172 (R172K). This is analogous to the R132 residue in *IDH1* [45]. *IDH* mutations are highly frequent in secondary GBM (up to 80%), but are rare in primary GBM (less than 10%) [45,48]. Importantly, *IDH* mutations occur more frequently in younger patients, and are associated with greater patient survival [45,49,50]. In addition to demonstrating the capacity of *IDH1*

mutations on distinguishing anaplastic astrocytomas and GBMs into clinically meaningful prognostic subgroups, a report by Hartmann *et al.* showed that the distribution of *IDH1* mutations is associated with patient's age, thus impacting the prognosis of high-grade astrocytoma patients [51]. Interestingly, in grade III anaplastic astrocytoma patients over 60 years old, the absence of *IDH1* mutations was associated with low overall survival, which was comparable with the overall survival of grade IV GBM patients with wild-type *IDH1* [51].

IDH1 and *IDH2* mutations are mutually exclusive and generally associate with specific genetic and clinical characteristics, when compared with gliomas that present wild-type *IDH*. Particularly, it was shown that *IDH* mutations and amplification of *EGFR* in GBMs are mutually exclusive events [50], and that *IDH* mutations are often associated with the methylation of the *MGMT* gene promoter [50,52]. However, these associations are yet to be clarified as they might represent a direct consequence of the mutant IDH activity, or alternative markers for epigenetic changes in tumors presenting *IDH* mutations [53]. Therefore, the understanding of the link between common genetic events and *IDH* mutations in GBMs might provide insights into their roles on gliomagenesis [45,54]. Dang *et al.* showed that cells presenting the $IDH1^{R132H}$ mutation have the capacity to reduce α-ketoglutarate into 2-hydroxyglutarate, while converting NADPH to $NADP^+$, which contributes to tumorigenesis [55]. Some hypotheses have been raised on how the mutant IDH may contribute to gliomagenesis; for example, as dioxygenases require α-ketoglutarate as cofactors and its structure is similar to 2-hydroxyglutarate, the former may compete for the binding site of dioxygenases, thus inhibiting their activity [56]. An example of dioxygenases critical in the context of cancer are PHDs, which are dependent on α-ketoglutarate, and is responsible for the negative regulation of HIF-1α; a transcription factor that stimulates tumor growth under hypoxic conditions by modulating apoptosis, cell survival and angiogenesis [57]. In addition, the production of 2-hydroxyglutarate by mutant IDH inhibits Jmjc domain-containing histone demethylases [58] and TET 5-methylcytosine hydroxylases [56], leading to altered genome-wide histone and DNA methylation. Mutant IDH may also contribute to gliomagenesis by increasing DNA methylation, an effect termed glioma-CpG island methylator phenotype (G-CIMP; as discussed in the 'Glioma CpG island methylator phenotype' section).

Although the understanding of *IDH* mutations is far from complete, its incorporation into prognostic models might possibly improve the clinical management of a subset of glioma patients. Furthermore, a recent study by Songtao *et al.* evaluated the response of 86 secondary GBMs to temozolomide treatment, and associated several markers of GBM (including *IDH* mutations and *MGMT* promoter methylation status) with overall and progression-free survival [59]. In this study *IDH* mutations were found in 73.4% of patients, and an association between these mutations and higher progression-free survival was implied [59]. These authors found that patients presenting *IDH* mutations and *MGMT* promoter methylation had a better response to temozolomide treatment, and that *IDH* mutations may increase chemosensitivity in secondary GBMs [59]. In addition, the possibility of therapeutically targeting mutant IDH proteins is conceptually feasible, and might be a strategy to specifically target tumor cells. In fact, very recent studies used small molecule inhibitors of the most common IDH mutants in acute myeloid leukemia ($IDH2^{R140Q}$) [60] and in gliomas ($IDH1^{R132H}$) [61]. The treatment of a patient-derived oligodendroglioma cell line harboring

Box 2. Selected molecular prognostic markers for glioblastoma.

Molecular prognostic marker

- *MGMT* promoter methylation [21,38,40,43,44]
- *IDH1* and *IDH2* mutations [28,45,49,59]
- Loss of chromosome 10 [27,123]
- Activation of the PI3K/AKT pathway [14,29,96]
- Aberrant p53/RB pathway [12,32,129]
- *HOX* genes signature [19,26]
- *HOXA9* overexpression [17,19]
- *CHI3LI* (*YKL-40*) expression [22,27]
- Single miRNA/miRNA expression signatures [67–77]
- *EGFR* expression/*EGFR* mutation (*EGFRvIII*) [9,20,25,26,84–87]
- *PTEN* expression (wild-type) [25]
- Molecular signatures [30,54]
- *MET* overexpression [23]
- High expression of angiogenic genes (e.g., *VEGF* and *VEGFR*) [30,94]
- Stem cell-like gene expression signatures [10,11,24,26,119–122,137]
- Activation of MAPK members [29]
- Glioma-CpG island methylator phenotype [52,63]
- *NFKBIA* deletion [13]
- *PTEN* and *DLL3* expression [30]
- *H3F3A* mutation [31]

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IDH1^{R132H} with the small molecule inhibitor AGI-5198 reduced growth in soft agar, and inhibited the growth of xenograft tumors derived from that cell line [61]. At the genome-wide level, several genes associated with glial differentiation were found to be upregulated and to have lost repressive histone marks at their promoter, indicating the putative capacity of IDH1 mutant inhibitors to erase histone modifications [61]. In the acute myeloid leukemia study, the treatment with AGI-6780 of an erythroleukemia cell line expressing *IDH2*^{R140Q} lowered the 2-hydroxyglutarate production nearly to physiological levels, and the treatment of patient-derived samples induced differentiation of leukemic blasts in samples harboring *IDH2*^{R140Q} [60]. However, one must take into account the fact that, although promising, these targeted drugs will be a limited approach in primary GBM patients due to the low frequency of these mutations, as well as due to the high intratumor heterogeneity that characterizes these malignancies. In the future, when IDH-targeting drugs become available [62], it will be interesting to determine if the use of these IDH-targeting drugs, individually or combined with other therapies, presents a significant anti-tumor effect in established gliomas. Despite the well-established relevance of *IDH* mutations in the prognosis of secondary GBM and lower-grade gliomas, their use in the prognostication of primary GBM patients is limited by their low frequency.

Molecular subtypes of GBM

Verhaak *et al.* stratified 200 GBMs from The Cancer Genome Atlas (TCGA) into four molecular subtypes – classical, mesenchymal, proneural and neural – each displaying different underlying genetic alterations and expression signatures [54]. The classical subtype was defined by displaying the most common genomic aberrations of GBM, with 93% of samples displaying chromosome 7 amplifications and chromosome 10 deletions, 95% showing *EGFR* amplification, and 95% with homozygous deletion on the *Ink4a/ARF* locus [54]. The mesenchymal subtype was mainly characterized by high expression levels of *CHI3L1* (or *YKL-40*) and the *MET* proto-oncogene [30]; *NF1* mutation or deletions were also found to be characteristic of this mesenchymal GBM [54]. Hallmarks of the proneural subtype include *PDGFRα* amplification, *IDH1* mutations, loss of heterozygosity and mutations at *TP53*. Importantly, the proneural subtype was associated with younger age and longer survival [54]. The neural subtype was defined by the differential expression of neuronal markers, such as *GABRA1*, *SLC12A5*, *NEFL* and *SYT1* [54]. This molecular classification is relevant because each GBM subtype responds differently to treatment [54]. For example, while aggressive treatment protocols significantly delayed mortality in GBM patients with classical and mesenchymal subtypes, and a tendency for a longer outcome was observed in the neural subtype, patients with proneural GBMs do not seem to benefit from this highly aggressive therapeutic approach [54]. In this sense, some of the genetic events underlying the different GBM subtypes could be used to stratify patients and rationalize treatment decisions, ultimately contributing to more personalized therapies. Moreover, publicly available resources, such as TCGA or Oncomine®, which systematically integrate genomic and clinical data from large cancer patient datasets, have been critical in exploring a wide spectrum of genomic alterations characteristic of each tumor type and evaluating their clinical value.

Glioma CpG island methylator phenotype

A very recent report by Turcan *et al.* suggested that the accumulation of 2-hydroxyglutarate might inhibit the α-ketoglutarate-dependent dioxygenase family of enzymes, which in turn will cause histone and DNA hypermethylation – termed the G-CIMP – that results in epigenetic deregulation [63]. Noushmehr *et al.* reported that 24 out of 272 GBMs from the TCGA dataset were G-CIMP-positive tumors [52]. Of these, 21 were classified within the proneural expression group, which accounts for 30% of all the proneural GBMs in that dataset [52]. Moreover, these authors reported a significantly increased survival for proneural G-CIMP-positive GBM patients in comparison with proneural G-CIMP-negative patients, and indeed to all other nonproneural GBM patients [52]. In Cox multivariate analysis, G-CIMP remained a significant predictor of patient outcome after adjusting for patient age, tumor recurrence status and primary versus secondary GBM status [52]. Of note, G-CIMP-positive tumors were associated with recurrent or secondary tumors, and strongly associated with the *IDH1* mutation [52]. In fact, this last association is in agreement with the report of Turcan *et al.,* showing that the mutation of a single gene – *IDH1* – is sufficient

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to establish the G-CIMP by remodeling the methylome, which results in the reorganization of the transcriptome [63]. The single introduction of mutant *IDH1* into primary human astrocytes induced the alteration of specific histone marks and extensive DNA hypermethylation, as well as the reshaping of the methylome in a way that resembles the alterations observed in G-CIMP-positive lower-grade gliomas [63]. Moreover, the epigenomic alterations induced by mutant IDH1 activate important gene expression programs that characterize the G-CIMP-positive proneural GBMs, but not other proneural GBMs, and predict increased survival [63]. In this sense, the authors argue that *IDH1* mutation is the molecular basis of the G-CIMP [63]. Considering the frequent co-occurrence of G-CIMP and *IDH* mutations, which is also a putative prognostic biomarker, future studies are necessary to clarify if the putative prognostic value of G-CIMP is independent of the *IDH*.

miRNAs

ncRNAs have recently emerged as important players in the deregulation of signaling pathways and gene expression in several tumor types, including GBMs. Indeed, the transcriptome is vastly more complex than initially anticipated at the time of the first genome-wide studies; for example, the number of noncoding transcripts is four-times higher than coding sequences. Of all ncRNAs, miRNAs are the most extensively studied, and are key regulators of several biological processes through negative control of gene expression at the post-transcriptional level [64]. Alterations in miRNA genes have been implicated in the initiation and progression of several cancers, either as tumor suppressors or oncogenes depending on their target genes [65]. Specifically, deregulation of these miRNAs has been detected in GBM, with a wide variety of functional roles in cell proliferation, apoptosis, cell cycle regulation, invasion, angiogenesis and glioma stem cell behavior [66]. Many reports have been published describing the ability of miRNAs to predict GBM patient survival [67–77]. For example, Ben-Hamo and Efroni studied five independent datasets and identified a gene–miRNA network comprising of *p38* and its associated miRNA miR-9, which can stratify GBM patients into prognostic subgroups [67]. Other studies focused on the miR-10b expression in human glioma tumors and cell lines, and showed that increased expression correlates with increased glioma grade [78,79], and also with increased expression of the G-protein RhoC and the urokinase receptor uPAR, which have been implicated in migration and invasion [78]. Survival of GBM patients with high miR-10b expression was significantly shorter than those patients with low miR-10b expression [68]. In addition, miR-10b downregulates the expression of several tumor suppressor genes, and is associated with poorer GBM patient survival [69]. Silber *et al.* report that miR-124a is significantly downregulated in grade III and IV astrocytomas relative to non-neoplastic brain tissue [80]. More recently, in a retrospective review of 119 GBM samples, the downregulation of miR-124a was associated with poor patient prognosis [70]. Additionally, another study showed reduced expression of miR-451 in GBMs compared with normal brains [81], suggesting that miR-451 might be a tumor suppressor in the brain. Godlewski *et al.* later reported that high levels of miR-451 are associated with a poorer survival of GBM patients in the TCGA dataset [73].

In addition to studies addressing the prognostic value of individual miRNAs, others have tried to define miRNA expression signatures that may have higher discriminatory power concerning the prediction of GBM patient survival. Zhi *et al.* evaluated the expression profile of 200 miRNAs in a set of 84 astrocytoma samples of different WHO grades, and 20 normal adjacent tissue samples and reported a seven-miRNA (miR-24, miR-21, miR-30c, miR-124, miR-181b, miR-137 and miR-106a) differential expression signature in astrocytoma samples in comparison with normal adjacent tissue [74]. Importantly, this finding was validated in an independent set of 40 astrocytomas and 40 matched tissue samples [74]. Additionally, the authors observed an association between the downregulation of miR-137 and advanced state of the disease, and the low expression of miR-181b and miR-106a, and the high expression of miR-21 were significantly associated with shorter patient survival, independent of other clinicopathological factors [74]. Therefore, these authors suggest that miRNA profiling may be a powerful prognostic and diagnostic marker in astrocytomas [74]. Another study evaluated the levels of 365 miRNAs in eight GBM and four anaplastic astrocytomas, revealing 16 candidate markers associated with glioma progression, of which miR-196a and miR-196b presented the most significant differences [71]. High expression of miR-196 was shown to

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be an independent prognostic factor in a set of 39 GBM patients [71]. This result was reinforced by a recent study that evaluated the expression of miR-196b in 198 glioma tissues [72]. Functional analysis of miR-196b showed that it is a promoter of cellular proliferation, and, as such, its levels are inversely correlated with GBM patient survival [72]. A study performed by Srinivasan *et al.* assessed the miRNA expression data of 222 GBM patients from the TCGA dataset, and found that a ten-miRNA expression signature was an independent predictor of patient survival [75]. This expression signature was also able to segregate GBM patients into low- and high-risk cohorts [75]. Of the ten-miRNA signature, seven were found to be in the high-risk group (miR-31, miR-222, miR-148a, miR-221, miR-146b, miR-200b and miR-193a) and three were in the low-risk group (miR-20a, miR-106a and miR-17-5p). These are thought to either inhibit or promote several traits of cancer cells [75]. Another study by Zhang *et al.* performed whole-genome miRNA expression profiling in 82 Chinese GBM patients [76]. The authors identified a five-miRNA signature, comprising of miR-181d, miR-518b, miR-524-5p, miR-566 and miR-1227, that was able to predict patient survival [76]. Patients scoring high with the five-miRNA signature presented poorer overall and progression-free survival when compared with patients presenting low-risk scores [76]. Moreover, this signature was found to be independent of other prognostic factors [76]. Lakomy *et al.* evaluated the expression of eight miRNAs (miR-21, miR-128a, miR-181c, miR-195, miR-196a, miR-196b, miR-221 and miR-222), as well as the methylation status of *MGMT* promoters in a group of 38 patients with primary GBMs [77]. In addition to the significant associations between the methylation status of the *MGMT* promoter and longer overall and progression-free survivals, the authors also found that the expression of miR-195 and -196b was negatively correlated with overall survival. Moreover, miR-181c in combination with miR-21 was highly sensitive and specific in the prediction of tumor progression within 6 months of diagnosis. However, the remaining miRNAs (miR-128a, miR-196a, miR-221 or miR-222) presented no prognostic or predictive value in GBM patients [77]. Importantly, of all the miRNAs reviewed, miR-196b and the miR-181 family have been more consistently reported to be of relevance in glioma. However, when evaluating miRNA expression profiles, special attention should be paid to the non-neoplastic reference due to variations on basal miRNA expression levels inherent to each individual, or to the fact that commercial references are usually RNAs pooled from several non-neoplastic tissues [82]. Nevertheless, the studies presented here demonstrate not only the potential for using single miRNA genes or miRNAs signatures in the prediction of patient outcome, but also how miRNAs may be crucial to the understanding of GBM biology and to the development of new therapeutics.

Growth factor signaling pathways

Overexpression or mutations in receptor tyrosine kinases (RTKs), growth factors and intracellular RTK targets greatly contribute to the tumorigenic process, and may represent important prognostic factors in GBM. *EGFR* is frequently amplified in GBM, and approximately 50% of these express the truncated form *EGFRvIII*, which is constitutively active and induces cell proliferation, survival and motility [9,83]. *EGFR* amplification and *EGFRvIII* mutants were associated with increased aggressiveness, and pointed to by some authors as prognostically valuable in GBM, as they are associated with shorter patient survival [9,20,25,26,84–87]. However, it is important to highlight that other authors state that these *EGFR* alterations did not associate with survival [88,89]. Similar to *EGFR*, the expression of *PDGFR* was reported to be frequently altered in GBM. Specifically, the phosphorylation of the PDGFRα subunit was associated with shorter survival in recurrent GBM patients [90], while other studies stated that the amplification of *PDGFR*α did not predict GBM patient survival [91,92]. Another RTK frequently altered in GBM is *MET*, which was rarely found amplified in GBM (only ~5% of GBMs), but presented a high frequency of overexpression (~29%) [23]. Moreover, these authors found that *MET* overexpression was associated with GBM shorter patient survival time [23]. In addition to RTKs, soluble growth factors are also important during tumorigenesis; an important example in GBM is VEGF, which is a prominent angiogenic factor [93]. Specifically the VEGF-A isoform, the best characterized isoform, was reported to be more frequently expressed in higher glioma grades, and was associated with poor GBM patient prognosis [93,94].

Moreover, the abnormal expression of intracellular targets of RTK signaling may associate with GBM patient prognosis. In particular, NF-κB

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is a transcription factor that is activated by the EGFR pathway [95]. The NFKBIA is a repressor of *NF-κB*, which was shown to be deleted in up to 24% of GBMs [13]. The deletion of *NFKBIA* was associated with poor GBM patient prognosis [13]. Interestingly, the authors found a pattern of mutual exclusiveness between *NFKBIA* deletion and *EGFR* amplification, and that the restoration of *NFKBIA* expression lessened the malignant phenotype and increased susceptibility to chemotherapeutic treatment in GBM cell lines [13].

The activation of the PI3K pathway is frequently deregulated in cancer, including GBMs. The pathway activation is associated with increased tumor grade, decreased apoptosis and poor patient outcomes [14,29]. In addition, several of the pathway intermediates *per se* presented prognostic significance. For example, phosphorylated AKT was reported as a biomarker of poor outcome in GBM patients [29,96]. The decreased expression of PTEN (at RNA or protein levels), or loss of heterozygosity of chromosome 10q, which encompasses the *PTEN* gene, were reported as indicators of shorter GBM patient survival [97]; however, this is still controversial [98]. Another recent example is the prognostic value of RAF kinase inhibitor, the expression of which was reported to associate with longer overall survival of GBM patients [99]. In the future, novel studies aiming to understand how the integrated analysis of several molecular components of growth signaling pathways may help clarify the true prognostic value of these biomarkers and lead to their integration into the clinical management of GBM patients.

Serum biomarkers of prognosis

Access to primary tumor samples is essential in evaluating tumor-specific genetic and epigenetic features. Biopsy, debulking or serial sampling may be difficult in the scenario of GBM, and moreover, a variety of imaging modalities are used to monitor tumor progression and response to treatment. MRIs can show an increase in tumor volume up to four weeks following completion of radiotherapy. In 50% of cases this is due to an increase in vascular permeability (treatment related) – an effect called pseudoprogression [100] – and might not necessarily translate into poor treatment response. This confounder [101], in addition to the unfeasibility of multiple tumor sampling during the course of the malignancy [102,103], clearly highlights the need for establishing less invasive predictive and prognostic markers. Serological markers mirroring tumor properties might be very good candidates. Serological biomarkers that correlate with patient survival in GBM include cathepsin D [104], AHSG [105], MMP-9 [22] and YKL-40, which is the most widely studied [22,27,106–109]. A study conducted by Tanwar *et al.* evaluated gene expression microarray data of glioma tumor tissue and showed that the most highly expressed gene was *YKL-40* [106]. The role of YKL-40 is not well-established; evidence suggests it may be implicated in cell differentiation, angiogenesis and proliferation, decreasing apoptosis and extracellular matrix remodeling [110]. Serum concentrations of YKL-40 seem to be a strong predictor of an aggressive phenotype in GBM [22,106]. Moreover, increased expression has been associated with glioma grade, shorter time to progression, resistance to radiotherapy and poor patient overall survival [22,107–109]. However, for the establishment of YKL-40 serum levels as a prognostic marker, further prospective studies with repeated measurements of YKL-40 levels before and after surgery are required. The high reproducibility of YKL-40 measurements in serum, as well as the fact that this biomarker is already well established for routine use, indicates that its inclusion in clinical practice should be relatively straightforward, and might provide crucial information on tumor progression and patient survival. In general, the use of serum biochemical markers that correlate with the biological traits of the tumor may be important during the design of treatment strategies and evaluating response to treatment. Equally important, these biomarkers may be able to detect disease progression or relapse early.

Histone mutations

GBM occurs comparatively less frequently in children than in adults but still remains a devastating disease with an incidence of 0.5 per 100,000 in Europe. Presenting symptoms, neurological sequelae, radiological and histological appearances are identical in both adults and children. What is particularly unique to the pediatric setting, however, is the occurrence of diffuse intrinsic pontine glioma; a form of malignant glioma specific to the pons and which, due to its location, is a challenge to treat. Pediatric tissue samples are scarce relative to adult counterparts and it has, therefore, been difficult to draw definitive conclusions about the underlying biology. As a result, they have been viewed as virtually indistinct from adults, contributing to a universal

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treatment strategy of surgery, radiotherapy and chemotherapy with temozolomide.

An increasing number of molecular profiling studies had hinted at the distinct underlying biology of pediatric cases [111–115], which was definitively proven with the identification of specific mutations in the *H3F3A* gene [116,117]. These were the first mutations described in histone genes in cancer and are highly specific to pediatric GBM. The *H3F3A* gene encodes the histone variant H3.3, and the mutations produce the amino acid substitutions glycine to arginine or valine at position 34 (G34R/V) or substitution of lysine to methionine at position 27 (K27M). Diffuse intrinsic pontine gliomas may also harbor K27M mutations in the gene encoding histone H3.1, *HIST1H3B* [117]. G34R/V mutant tumors peak at approximately 13–14 years, and are restricted to the cerebral hemispheres, while K27M mutant tumors arise at 6–7 years and are located in the pons and midline structures, especially the thalamus [31,118]. Although difficult to separate from the effects of anatomical location, K27M mutant tumors have a significantly worse overall survival than G34R/V or wild-type tumors [31,118]. This is clinically important, as thalamic tumors are currently treated on supratentorial protocols, although they may instead need to be considered along with diffuse intrinsic pontine glioma in terms of novel molecularly targeted therapies.

Other putative prognostic factors

In addition to the abovementioned prognostic biomarkers, other molecular characteristics of GBM have been suggested in some studies to associate with patient survival. Some examples include abnormal *p53* and *RB* functions, expression of cancer stem cell markers [119–122], loss of chromosome 10 [27,123], codeletion of 1p/19q [124,125] and activation of *HOX* genes [17,19,26].

The p53 and RB tumor suppressor pathways are frequently altered in GBM (~80%). Nonetheless, their prognostic value is still controversial, with some studies reporting an impact of *p53* pathway in patient survival [12,126], while others did not replicate this [127,128]. Similarly, decreased expression of the *RB* gene in GBM has not been clearly established as a prognostic factor [32,129,130]. In addition, wild-type *p16* was associated with improved survival of GBM patients treated with chemoradiotherapy [131], while the homozygous deletion of *p16* was associated with poor survival in male GBM patients [132].

Studies have shown the presence of a subpopulation of cells within GBM, termed glioma stem cells, that present abnormal characteristics regarding proliferation and differentiation [133]. This population of cells was associated with tumor recurrence [134] and therapy resistance [11], and characterized by several stem cell markers, including CD133, CD44, ID1, Nestin and SOX-2 [135]. Several studies have tried to correlate the expression of these markers with GBM patient prognosis, but no consistent associations have currently been established [136]. For example, some studies report an association between CD133, Nestin, cJun, CD44 and ID1 expression and GBM patient poor prognosis [10,119–121,135,137], while others suggest an association of CD133 and SOX-11 expression with longer prognosis [24,122]; other studies report no effects on GBM patient survival due to the expression of Nestin, CD133 and CD15 [138,139]. The contradictory findings regarding the clinical relevance of these putative stem cell markers in GBM warrants further investigation.

The clinical relevance of some chromosomal copy number aberrations has also been investigated in GBM. Loss of chromosome 10 is highly frequent in GBM [27,123], and emerged as an important influence on global changes in the tumor gene expression, being reported as the most important copy number alteration for GBM classification and associated with a negative prognosis in GBM [27]. In addition, although codeletions of 1p/19q in oligodendrogliomas have been established as clinically relevant prognostic markers associated with increased patient survival time [140], these codeletions are uncommon in GBM, and the studies concerning their prognostic value in GBM have currently reported controversial findings [124,125].

Homeobox genes have also been recently studied in the context of glioma, particularly GBM. These genes encode transcription factors that play critical roles during normal development and differentiation [141], and have been found to be deregulated in cancer [141]. Recently, the expression of several *HOX* genes was found altered in gliomas [142]. A subsequent report identified the expression of a *HOX*-dominated gene cluster in GBM, enriched for stem cell-like properties, as an independent predictor factor for shorter survival time in patients treated with radiotherapy and concomitant chemotherapy [26]. Costa *et al.* showed that *HOXA* genes are differentially activated in GBM when compared with lower-grade gliomas and normal brain tissue, and identified

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GBMs with an abnormal chromosomal domain of transcriptional activation that includes the *HOXA* cluster [17]. This gene cluster is reversibly regulated by the PI3K pathway via an epigenetic mechanism regulating the levels of histone H3 lysine 27 trimethylation [17]. Of all *HOXA* genes, *HOXA9* expression was predictive of GBM poor patient survival in two independent datasets, and was shown to have proproliferative and antiapoptotic functions in GBM cells [17]. More recently, Gaspar *et al.* showed that pediatric GBM cell lines resistant to temozolomide present a coordinated expression of several *HOX* genes, of which *HOXA9* and *HOXA10* are crucial effectors, and also suggested that the *HOX*-enriched signature is regulated by the PI3K pathway [19]. Importantly, pediatric patients with high-grade gliomas that express *HOXA9* and *HOXA10* had significantly shorter survival [19]. Overall, these studies suggest some *HOXA* genes may be prognostically valuable in both pediatric and adult GBM patients.

Conclusion

In conclusion, the work on prognostic factors in GBMs provides reasons for both optimism and caution in dealing with this highly malignant cancer. To date, the most relevant and still promising biomarker of prognosis in adult GBM patients is the status of *MGMT* promoter methylation, which has frequently been associated with patient survival and therapeutic responses. The multiplicity of techniques available to evaluate *MGMT* methylation status (including methylation-specific PCR) allows its routine establishment in the clinics, but, of equal importance, these methods may be applied in formalin-fixed paraffin-embedded tissues that is the standard format of samples deposited in tumor banks. New putative biomarkers, such as the expression levels of *HOXA* genes and the presence of *IDH* mutations, may be performed in this sample format using routine techniques such as immunohistochemistry or PCR followed by sequencing, respectively. However, the evaluation of *IDH* mutations in patient prognostication is limited to secondary GBM or lower-grade gliomas due to its low frequency in primary GBM (<10%). Concerning the pediatric setting, mutations of the *H3F3A* gene are as highly important, and may be established in the prognostication of these patients. Nonetheless, true clinical benefit will most likely only be seen with careful patient selection based on the presence of such biomarkers within clinical trials (the so-called precision medicine model). The increasing integration of molecular and clinical data through contemporary bioinformatics tools, will hasten the introduction of such biomarkers into the clinic leading to tailored treatment according to molecular subgroups. This also allows timely identification of patients unlikely to respond to standard therapies, permitting rapid entry into clinical trials, while avoiding the adverse unnecessary side effects of ineffective therapies. The challenge ahead is to identify and truly translate their relevance into effective, targeted drug therapies as well as to continue the discovery of further molecular markers of GBM.

Future perspective

The true clinical benefit of prognostic markers in GBM will probably only be perceived upon careful selection of patients based on the evaluation of tumor biomarkers and their integration in clinical trials. The cumulative integration of molecular and clinical data due to developments in bioinformatics tools will most certainly lead to the faster introduction of molecular biomarkers into the clinical routine, and thus to patient-tailored treatments according to the molecular alterations of GBM subgroups. Critically, this will conceptually allow the timely identification of patients that may not positively respond to conventional therapeutics, allowing their informed choice of entering clinical trials, while being spared the side effects and significant costs of ineffective therapies. The challenge in the neuro-oncology field for the next decade is to discover novel biomarkers of prognosis and therapy response, and to translate and integrate this knowledge into the development of targeted and effective therapies.

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- ▪▪ of considerable interest

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