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REVIEW ARTICLE

A comprehensive overview on the molecular biology of human glioma: what the clinician needs to know

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Abstract
The molecular biology of human glioma is a complex and fast-growing field in which basic research needs to meet clinical expectations in terms of anti-tumor efficacy. Although much effort is being done in molecular biology research, significant contribution to the quality of life and overall survival still lacks. The vastness of molecular biology literature makes it virtually impossible for clinicians to keep up to date in the field. This paper reviews some practical concepts regarding glioma tumorigenesis from the clinician's perspective. Five main aspects are discussed: major intracellular signaling pathways involved in glioma formation; genomic, epigenetic and transcriptomic relevant features of glioma; the prognostic and predictive values of molecular markers according to the new WHO classification of glial tumors; the importance of molecular and cellular heterogeneity in glioblastoma, responsible for its therapy resistance; and the interaction between glioma and the immune system, in view of the novel and promising targeted therapies.

Keywords Glioma · Glioblastoma · Malignant glioma · Molecular biology · Epigenetics · Transcriptomics

Introduction

Human glioma comprises a group of heterogeneous primary brain neoplasms that, from a basic histopathologic perspective, can be readily classified in two domains. First, low-grade glioma (LGG), which seems to benefit from maximal safe resection, eventual reoperation and adjuvant radiation and/or chemotherapy [1], and second, high-grade glioma (HGG), which also benefits from maximal resection and mandatory postoperative chemoradiation [2]. However, whereas therapy for LGG is currently able to extend life expectancy over 10–15 years on average from diagnosis [3], aggressive treatment against glioblastoma (GBM) yields a modest 14-month overall survival [4].

Reasons for this poor prognosis include the infiltrative nature of malignant glioma, which precludes disease cure even in the event of supramaximal resection, and its inherent resistance to radiation and chemotherapy [5]. This resistance seems to be linked to intricate biochemical and signaling pathways alterations driven by genetic determinants within the heterogeneous tumoral cells and their microenvironment [6, 7].

The molecular biology of human glioma is a very complex and fast-growing field in which basic research needs to meet clinical expectations in terms of anti-tumor efficacy and patient survival. At present, much effort is being done in molecular biology research yet without significant contribution to the quality of life and overall survival [8]. The vastness of basic research literature regarding the molecular biology of glioma makes it virtually impossible for clinicians to follow and deeply understand the enormous complexity within the field.

The aim of this paper is to review some practical basic and advanced concepts about the molecular biology of human glioma formation from the perspective of the

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clinician involved in the treatment of these tumors. We selected five main aspects: tumorigenesis linked to major intracellular signaling pathways; some genomic, epigenetic and transcriptomic relevant features of human glioma; the transition from pure morphology to molecular markers in classifying gliomas and their prognostic and predictive values; the importance of molecular and cellular heterogeneity in GBM, responsible for its resistance to therapies; and the interaction between glioma and the immune system, in view of the novel and promising therapies directed to specific checkpoints, the efficacy of vaccinations and the role of oncolytic viruses.

Human glioma tumorigenesis

Molecular concepts involved in glioma formation

Glioma is the most common primary tumor of the central nervous system, a heterogeneous group with distinct histopathological characteristics that usually presents as a diffuse and aggressive intracerebral mass. Gliomas originate from glial progenitor cells, that develop and grow resembling astrocytic and/or oligodendroglial lineages [9]. As in many cancer types, there are no clearly defined etiologic factors, except in two circumstances. First, certain gene mutations within the context of some genetic diseases seem to predispose to glioma formation. These genetic syndromes include neurofibromatosis type 1 (in which the NF1 gene is affected), neurofibromatosis type 2 (NF2), tuberous sclerosis (TSC1, TSC2), von Hipple Lindau syndrome (VHL), Li-Fraumeni syndrome (p53), Turcot syndrome (APC, hMLH1, hMSH2, PMS2), Gorlin syndrome (PTCH1), and Cowden syndrome or multiple hamartoma (PTEN). Second, glioma tumorigenesis related to gene damage has been observed following the ionizing radiation used in radiotherapy treatments [10].

According to the molecular mechanisms involved in the oncogenic process, two main pathogenic drivers are described: oncogenes and suppressor genes. Oncogenes, that were discovered through the study of retroviruses [11], under normal conditions, they function as proto-oncogenes, which code for proteins that regulate cell functions like differentiation or growth. These proto-oncogenes become true oncogenes induced by specific mutations or cellular deregulation processes, causing cells to proliferate without the need for intracellular signal activation, just by the mere upregulated gene activity. Contrarily, and also under usual conditions, suppressor genes continuously act as tumor inhibitors. Noticeably, both alleles of a suppressor gene need to be inactivated to promote tumorigenesis. A single mutated copy of the suppressor gene can be inherited but may have no phenotypic manifestation in cancer development. Many oncogenes and suppressor genes are involved in glioma formation as described below.

Epigenetic changes, that is, chemical modifications of the nucleic acids without changing the nucleotide sequence, have been recognized as relevant features in the process of glioma formation. These changes, that may affect the DNA packing proteins or the nitrogenous bases, are capable of silencing or activating specific genes, like for example, oncogenes or suppressor genes. In general, epigenetic changes appear as gene methylation or acetylation, which usually provoke gene silencing or activation, respectively [12]. For example, histone methylation can be a mechanism for silencing some tumor suppressor genes involved in glioma formation [13].

Interestingly, the result of some gene mutations can be assessed and measured as so-called *oncometabolites*. A percentage of human gliomas carry mutations in the isocitrate dehydrogenase 1 (IDH1) and 2 (IDH2) genes. These genes code for enzymes involved in the Krebs cycle, transforming isocitrate into alfa-ketoglutarate. When mutated, IDH1 acquires the capability of transforming isocitrate into 2-hydroxyglutarate (2HG), an oncometabolite that accumulates in the cells promoting the impairment of some epigenetic-related enzymes, resulting in newer DNA methylation profiles involved in tumorigenesis [14].

Another relevant molecular aspect of glioma tumorigenesis is the concept of *cancer stem cells*. It has been demonstrated that the adult brain harbors neural stem cells (NSC) able to produce both neural and glial progenitors [15]. Due to their inherent self-renewing capacity, NSCs are candidates to be transformed into cancer stem cells, therefore, becoming a source of future cancer cells. However, it has been reported that also specialized cells can enter a dedifferentiation process and acquire stem cell-like features [16, 17]. Moreover, the cell division program followed by these cancer stem cells, can be symmetric, that is, a cancer stem cell would produce two new cancer stem cells, or asymmetric, obtaining a cancer stem cell and another differentiated cancer cell. Whether it is the symmetric or the asymmetric division scheme responsible for maintaining oncogenic cells growing in glial tumors is still under study [15] (Fig. 1).

Importantly, the cell division cycle can be arrested or paused in a G0 phase and cells can enter in a somehow quiescence state. *Quiescent* tumor cells may rapidly re-enter the cell cycle upon specific and fine-tuned cell signaling. Studies have shown that glioma stem cells can enter the quiescent state under the influence of some molecular factors like pRB, p53, ncWnt, PTEN and FoxO, and, conversely, they may exit the G0 state induced by other factors such as Akt, mTORC1, cWnt, Notch, CDK3/cyclin C [18]. The state of quiescence of certain glioma cell subpopulations is currently regarded as an extremely relevant factor involved in tumor resistance to chemo or radiation therapy, as discussed below.

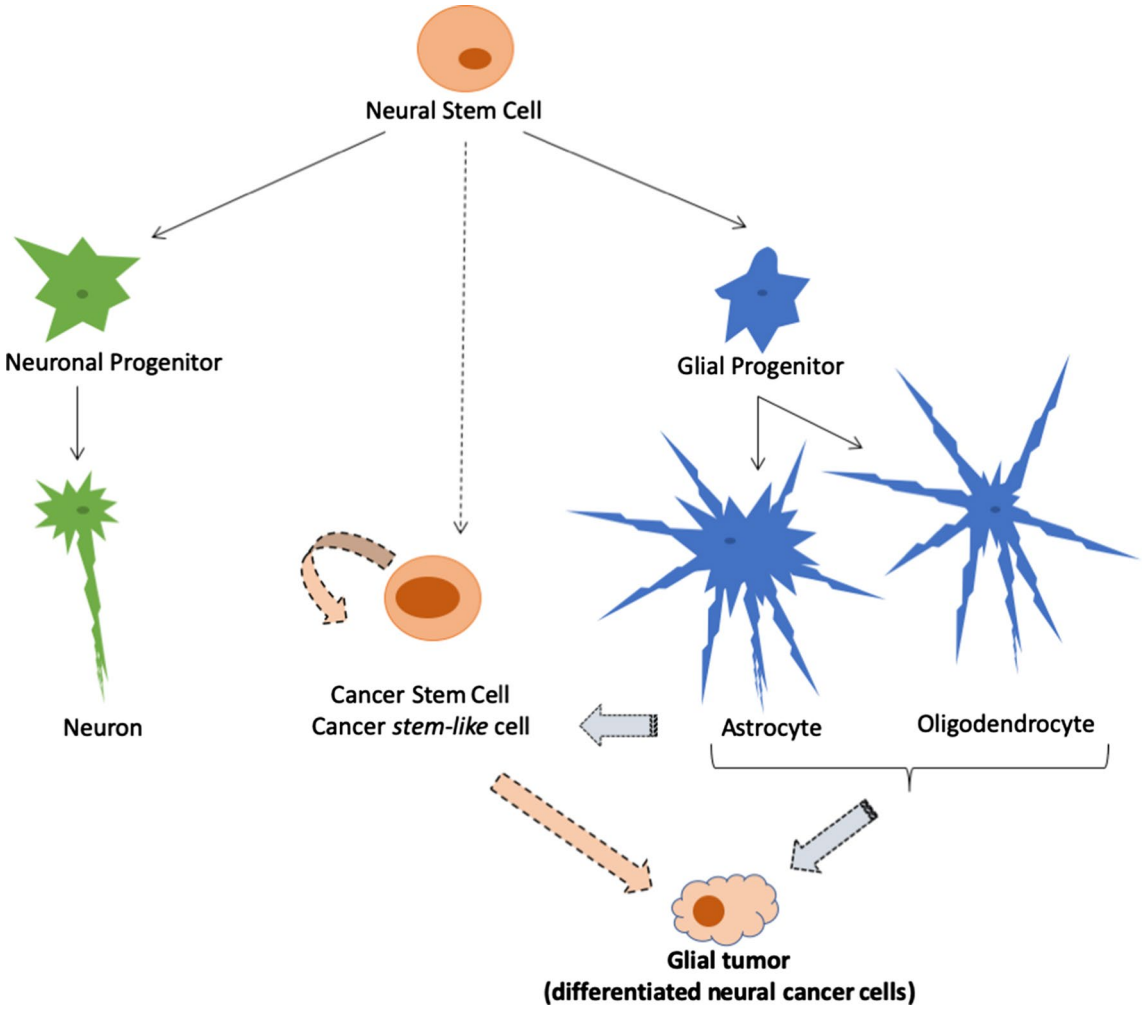

Fig. 1 Scheme of plausible glial tumor origin attending to cell division. A neural stem cell can differentiate into a neuronal or a glial progenitor, which would form neurons and astrocytes or oligodentrocytes respectively, but also, neural stem cells may transform into cancer stem cells or cancer stem-like cells, which in turn would divide symmetrically to form new cancer stem cells, or in an asymmetrical way to produce glial tumor cells. On the other hand, also differentiated cells like astrocytes and oligodendrocytes can dedifferentiate to form new cancer stem cells, or maintain some differentiated characteristics giving rise to glial tumor cells

Major signal pathways involved in glioma formation

Figure 2 depicts some pathogenic mechanisms involved in glioma formation in which specific intracellular signaling pathways are affected. Analysis of large amounts of information from the *TCGA* (*Tumor Cancer Genome Atlas*) database indicates that GBM tumorigenesis is driven by three main signaling pathways alterations: the RB pathway, the TP53 pathway, and the PTEN/NF1/RTK pathway [15].

RB is a well-known tumor suppressor gene which encodes for a protein that regulates the G1/S cell cycle checkpoint through sequestering E2F and inhibiting its oncogenic activity [19]. Mutations in RB or its pathway (p16INK4a and CDK4) result in an increased mitotic activity, which may lead to malignant transformation [20].

TP53 encodes the tumor suppressor p53, also known as the *guardian* of the genome. In response to DNA damage, this protein pauses the cell cycle, and if such damage cannot be repaired, the cell initiates apoptosis [21, 22]. This gene, the most frequently impaired gene in human cancer overall, is known to be altered in many astrocytoma cells of both LGG and HGG [23, 24], which results in primordial astrocytoma cells to evade apoptosis [25].

The PTEN/NF1/RTK pathway controls cell growth in glioma. Some tyrosine kinase receptors (RTKs) are the well-known EGFR and PDGFR, which promote cell growth via activation of RAS, MAPK and PI3K among others [15]. The tumor suppressors PTEN and NF1 are the negative regulators of PI3K and RAS activity, respectively, and modulate the cell cycle entry of NSCs [26, 27]. Research in murine NSCs has shown that inactivation of TP53 and PTEN leads

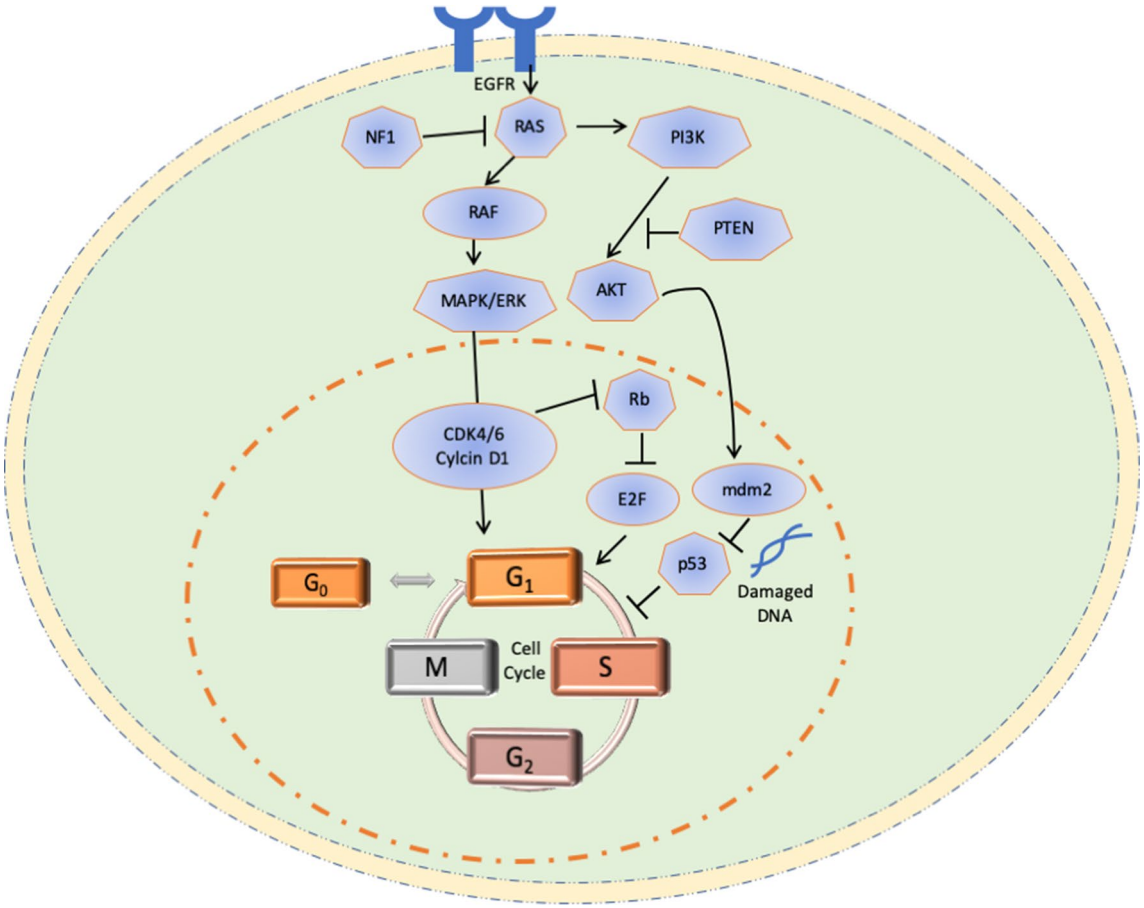

Fig. 2 Schematic representation of signaling pathways involved in glioma formation. Transmembrane tirosin kinase receptors such as EGFR can dimerize and activate different pathways like the RAS/MAPK or PI3K signaling pathways. In this context, proteins like NF1 or PTEN can act as inhibitors over the Ras or PI3K signaling, respectively. The RAS/MAPK/ERK pathway can act over CD4/6 and Cyclin D1 affecting cell cycle progression. This complex phosphorylates Rb causing E2F release, which in turn drives G1 to S phase transition. Downstream of PI3K signaling, AKT transduces signal through mdm2 inside the cell nucleus, leading to p53 degradation. Importantly, p53 is able to arrest the cell cycle upon detection of DNA damage. Other factors, not depicted here, are responsible for the cell cycle to enter the G_0 phase. *EGFR* Epidermal growth factor receptor, *NF1* Neurofibromin 1, *RAS* RAS proto-oncogene family, *PI3K* Phosphatidylinositol 3-kinase, *MAPK* mitogen-activated protein kinase, *ERK* extracellular signal-regulated kinase, *AKT* serine/threonine-protein kinase AKT, *Rb* Retinoblastoma-associated protein, *E2F* E2F transcription factor, *CDK4/6* cyclin-dependent kinases 4 and 6, *mdm2* E3 ubiquitin-protein ligase Mdm2, *p53* cellular tumor antigen p53

to the development of HGG, mediated by the oncoprotein MYC [28]. Moreover, ablation of PTEN in NF1 and TP53-deficient mice generates highly invasive GBM [29], therefore, suggesting a cooperative effect of these oncogenic and tumor-suppressive pathways.

Genomic, epigenetic and transcriptomic features of human glioma

The current advance of *omic's* (genomic, epigenomic, transcriptomic, proteomic, metabolomic) techniques provide a better understanding of the gene alterations involved in cancer development. Particularly, the *TCGA* initiative has incorporated an extensive analysis of a large number of different types of cancers, including gliomas [30]. This massive input of data links genetic alterations, epigenetic changes, and multiple expression analyses with clinical data including treatment, overall survival and even pathological findings [31–33]. In recent years, numerous studies have used these platforms of massive molecular analysis, with a strong potential to clarify biological processes embedded in glioma pathophysiology, to elucidate the heterogeneity observed in GBM [34–37]. This has allowed to establish the main pathways altered in glioblastoma and to associate them with mutations or copy number alterations in EGFR-PTEN-PI3K, NF1-RAS-BRAF, MDM2-MDM4-TP53 and CDKN2A/B-CDK4-RB1 pathways, among other [37]. A detailed description of each molecular pathways

is, however, beyond the scope of this review and can be found elsewhere [15].

Epigenetic alterations in glioma: epigenetics maps

GBM can be divided into primary and secondary tumors [38]. Although primary and secondary GBM are pathologically identical, primary tumors are more common, exhibit a more aggressive behavior and usually appear in older patients [39]. Moreover, their molecular alterations differ according to their IDH1/2 and EGFR-PTEN mutational status [40]. The newer WHO classification of brain tumors includes molecular analysis of IDH1/2 mutations within the diagnostic procedure, mutations that have proven to influence prognosis more than the mere histologic subgrouping [41]. Interestingly, it has been shown that most LGG lacking IDH1 mutation behave more aggressively than their IDH-mutated counterparts, mimicking the clinical course of a GBM. Similarly, an IDH1-mutated GBM exhibit better prognosis compared to the wild-type variant [42]. One of the main effects of IDH1/2 mutations has been attributed to the generation of a special tumor phenotype called *hyper-methylation*, which generates a global change in the transcriptional profile. This increase in methylation inhibits the expression of multiple genes [43], but also increases the expression of specific genes like PDGFRα, an oncogene known to drive tumorigenesis [44]. Therefore, the methylation profile defines a subgroup of gliomas with special characteristics including better prognosis that, in the case of IDH1/2 mutations, have been called *G-CIMP* (*Glioma CpG island Methylator Phenotype*) and, conversely, non-G-CIMP in IDH1/2 wild-type tumors [43].

Interestingly, G-CIMP tumors belong to the *proneural* transcriptional subgroup (see below), are more prevalent among LGG, display distinct copy number alterations, and are highly associated with IDH1 mutations. Patients with G-CIMP tumors are younger at the time of diagnosis and exhibit significantly better overall outcome [43]. The analysis of methylation profiles has been used to generate interesting *tumor maps* that group together subtypes of gliomas with distinct genetic and clinical characteristics that influence prognosis [36] (Fig. 3). The utility of these epigenetic maps, which can gather up to 100 different brain tumor entities, rely on their potential robust applicability to the differential diagnosis [45].

Transcriptomic GBM subtypes

Recent studies have been able to group GBM into several tumor subtypes based on specific transcriptional patterns. According to the widely-cited papers by Phillips et al. [46] and, more recently, Verhaak et al. [47], analysis of 840 genes (210 signature genes per subgroup) from samples of a large

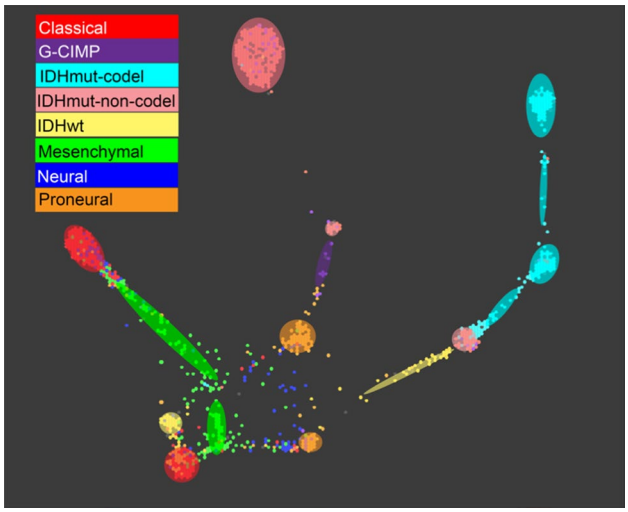

Fig. 3 Tumor map of diffuse glioma. Epigenetic tumor map based on RNA sequencing and DNA methylation data

cohort of GBM patients, divided gliomas into four subgroups termed *classical*, *mesenchymal*, *neural* and *proneural* (Fig. 4a). In parallel, the gene alterations present in each subtype were also characterized, including variations in the number of copies and mutations.

The *proneural* subtype is highly prevalent in mutations/amplifications of PDGFRα and mutations in IDH1/2 and TP53. Additionally, this subtype overexpresses genes associated with the development of oligodendrocytes, like PDGFRα, Sox2 and Olig2. Moreover, these tumors are G-CIMP positive and comprise the majority of secondary GBM, which associates the best prognosis [47] (Fig. 4b). The *classical* subtype exhibits common amplification/mutation of EGFR, as well as the homozygous deletion of CDKN2A and low expression of p16INK4A. The expression of neural stem cell markers, like Nestin, as well as the components of the Notch and Shh (Sonic Hedgehog) signaling pathways are also very prevalent in this subtype [47]. The *mesenchymal* subtype shows an increased prevalence of mutations in NF1, TP53 and PTEN genes, and high levels of mesenchymal cell markers expression, like CHI3L1 (Chitinase-3-like protein 1), MET and CD44. The mesenchymal signature of GBM, that is CD44 expression, and NF-κB activation correlate with poor radiation response and shorter survival [48]. Finally, the *neural* phenotype shows the expression of neuron characteristic markers, like NEFL (neurofilament light), GABRA1 (gamma-aminobutyric acid type A receptor Alpha1 subunit), SYT1 (synaptotagmin 1) and SLC12A5 (Carrier family 12-member 5 solution), with astrocyte and oligodendroglial differentiation markers [47]. Since they carry a transcriptional profile similar to that of non-tumor tissue, this subtype is highly infiltrated by healthy brain cells [48].

A

Gene	Proneural (PL)	Neural (NL)	Classical (CL)	Mesenchymal (MES)
TP53	**54%**	21%	0%	32%
IDH1	**30%**	5%	0%	0%
PTEN	16%	21%	23%	**32%**
NF1	5%	16%	5%	**37%**
EGFR	16%	26%	**32%**	5%
EGFRvIII	3%	0%	**23%**	3%
PIK3R1	19%	11%	5%	0%
RB1	3%	5%	0%	13%
ERBB2	5%	16%	5%	3%
PIK3CA	8%	5%	5%	3%
PDGFRA	11%	0%	0%	0%

B

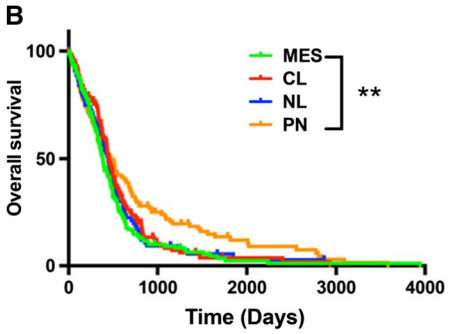

Fig. 4 Characteristics of the different molecular subtypes of GBM. **a** Genomic alterations in the different transcriptomic subtypes of GBM. **b** Overall survival of GBM subtypes. Note the differences between the proneural and mesenchymal subtypes

Hyper-vascularization of GBM

HGG typically exhibit very abundant and aberrant vascular proliferation, which is considered a characteristic feature of GBM. The main factors involved in the angiogenic process are the vascular-endothelial growth factor (VEGF), angiopoietin 1 and 2 (Ang-1 and Ang-2), PDGF, Interleukin-8 (IL-8) and hepatocyte growth factor (HGF) [49]. Although the hyper-vascularization state found in glioma is the result of a complex and poorly understood mechanism, it is postulated that, at the first stages of gliomagenesis, when the rapid tumor growth increases the demand of nutrients and oxygen, glioma cells are grouped around pre-existing vessels (vascular *co-option*), and later, new vessels begin to develop around already formed ones (*neoangiogenesis*). Hypoxia seems to be the main inducer of this process through the activation of HIF-1α (inducible hypoxia factor 1 subunit alpha) [50], which generates a robust angiogenic response partly mediated by increased expression and secretion of VEGF and IL-8. Another mechanism involved is *vasculogenesis*, which consists of the recruitment and differentiation of endothelial progenitors from the bone marrow [51], or via TIE-2 and SDF-1/CXCR4 pathways [52]. Yet, an additional relevant process for GBM vasculature formation is so-called *vascular mimicry*, based on the ability of tumor cells to form functional vascular networks similar to real vessels [53]. In fact, tumor cells are capable of differentiating into endothelial cells [54] and mural cells (pericytes), which are necessary for the generation of microvascular proliferation and tumor growth [55]. The overall result of this entire angiogenic process is the aberrant vasculature, with dilated and tortuous vessels, permeable endothelium, and reduced pericyte coverage found in GBM [55, 56]. This defect in the recruitment and functionality of the pericytes contributes to an abnormal formation of the blood–brain barrier, leading to extravasation of gadolinium-based contrast agents, the radiologic hallmark of most aggressive gliomas [57].

Current glioma classification: from morphology to genetics

Before the publication of the WHO classification of brain tumors 2016 update [58], primary brain tumors were classified merely on the basis of histopathologic criteria, according to the resemblance of the supposed cell of origin. Each tumor was given a I–IV grade designation reflecting the *malignancy* of the lesion [59]. Thus, grade I tumors were considered benign and curable by surgical removal with adjuvant therapy being unnecessary, whereas grade IV tumors carried a poor prognosis despite aggressive surgery and adjuvant chemoradiation. The 2016 update introduced, for the first time, molecular parameters in the diagnostic protocol. This helps to reduce interobserver diagnostic discrepancies among pathologists, make the diagnosis of glioma subtypes more reliable, and standardize treatment schemes for epidemiologic studies and eventual targeted therapies. Molecular parameters have, therefore, changed the diagnostic landscape of diffuse gliomas, which now comprise only three main subgroups (diffuse astrocytoma, midline glioma with H3K27M mutation, and oligodendroglioma) with various grades and variants [58, 60] (see Fig. 5).

The first step in the diagnostic procedure of a suspected glioma is still an adequate histopathological assessment to distinguish glioma from other non-tumoral lesions or even normal brain parenchyma. Once the specimen is judged to be compatible with glioma, four molecular markers are usually determined: IDH mutation, ATRX loss, 1p/19q codeletion and H3F3K27M mutation. First, immune-histochemical analysis of the IDH1-R132H (the most commonly mutated variant of IDH1) and ATRX status (marker of astrocytoma lineage) are determined [61]. In cases in which IDH1-R132H is negative, other IDH1 and IDH2 mutations can be identified by gene sequencing. However, the WHO does not recommend searching for different other IDH mutations in patients older than 54 years harboring tumors with clear astrocytic appearance, ATRX loss, and histologic grade IV

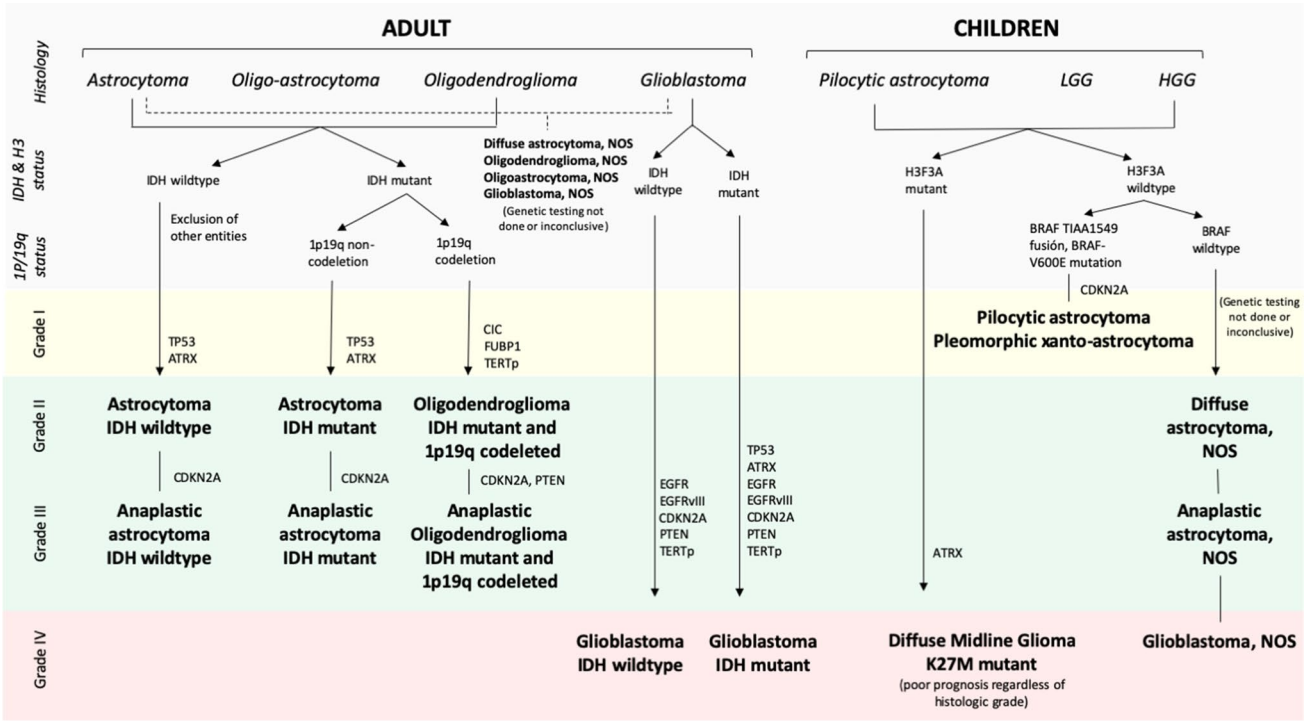

Fig. 5 View of the current classification of adult and pediatric diffuse gliomas according to the status of relevant genetic biomarkers (see text for details). (Modified from Park et al. [60])

appearance, because the chance of finding such mutations is virtually inexistent.

IDH status is key for classifying glioma into IDH-mutated and non-mutated (or IDH-wildtype). The term *NOS* (*not otherwise specified*) refers to patients in which molecular determinations are not performed or yield inconclusive result [61]. IDH mutation is present in 70–90% of grade II and III gliomas, in 85% of secondary glioblastomas, but only in 5% of primary glioblastomas [62]. IDH mutation is very useful to rule out reactive gliosis, a condition in which the mutation needs to be necessarily absent. IDH mutation is a good prognostic factor for all histologic subtypes and grades of diffuse gliomas [62]. Specifically, the absence of IDH mutation within grade II/III gliomas results in poorer prognosis than expected [63]. Moreover, IDH-wildtype grade III gliomas seem to follow a clinical course even poorer than IDH-mutated GBM [64].

When a diffuse glioma is found to be IDH-mutated, the next diagnostic step is searching for 1p/19q co-deletion, usually by detecting a loss of heterozygosity by microsatellite analysis or by fluorescence in situ hybridization (FISH) techniques. This co-deletion is exclusive of oligodendroglial lineage [58] and its presence carries diagnostic, prognostic and predictive value regarding response to treatment [64, 65] (see Table 1).

The 2016 WHO update also includes a new entity, the so-called *midline diffuse glioma with H3K27M* mutation [58]. This glial tumor presents preferentially in children, generally located in the brainstem or thalamus. About 80% of these tumors exhibit histone H3 mutation, in which lysine-27 is replaced by methionine (K27M), within the genes encoding for the H3.3 (H3F3A) or H3.1 (HIST1H3B) histones [66]. These lesions respond poorly to treatment, associate a worsened prognosis compared to non-mutated tumors, and can be regarded as a GBM in terms of biologic behavior [64].

Advantages of adding molecular information to pathology

The advantages of adding molecular information to histopathologic features include improved diagnostic objectivity and provide homogeneity for implementing clinical trials. For example, in IDH-wildtype gliomas, with ATRX loss but negative for 1p/19q co-deletion, it is relevant to know the H3 histone status, since differentiating a mere astrocytic tumor from a diffuse midline astrocytoma carries important prognostic implications [67]. Molecular information may also be useful when confronting LGG, especially when little specimen is available and histopathologic features are unclear. In LGG without IDH mutation, the analysis of genetic alterations of the BRAF gene (duplications, KIAA1549-BRAF fusion or BRAF-V600E mutation) may be a powerful tool. Studies show that 70–80% of children harboring cerebellar pilocytic astrocytomas exhibit KIAA1549-BRAF fusions,

Table 1 A review on the relevant biomarkers in the management of glioblastoma: diagnostic, prognostic and predictive values [65]

Biomarker	Diagnostic?	Prognostic?	Predictive?
IDH1/IDH2 mutation	**Yes** Differentiates between primary and secondary GBM. Also differentiates between glioma and reactive gliosis or other gliomas without IDH mutation like pilocytic astrocytoma, gangli-oglioma or ependymoma IDH status is homogenous within grade II and III gliomas	**Yes** Both IDH1 and IDH2 mutations are favorable, especially in grads III and IV tumors Provides better OS and PFS	**No** Does not provide information for clinical decision making Complete surgical resection is associated with improved survival in patients with IDH1 mutation
1p/19q co-deletion	**Yes** The presence of co-deletion supports the diagnosis but the absence does not rule out an oligoden-droglial tumor. Co-deletion status is homogenous within grade II and III tumors	**Yes** Co-deletion is a strong favorable prognostic factor in anaplastic tumors (and likely in lower grad tumors) receiving radiotherapy and/or alkylating agents	**Yes** Co-deletion is predictive of increased survival in patients treated with PCV added to radiation versus radiation alone
MGMT promotor methylation	**No** However, it helps to differentiate true progression from pseudo-progression in operated and irradi-ated GBM MGMT status is homogenous within gliomas and at the time of recurrence	**Yes** MGMT promoter methylation is a favorable prognostic factor in anaplastic glioma receiving radiotherapy and/or chemotherapy. Improves OS and PFS (with IDH mutation)	**Yes** Predicts good response to chemotherapy with alkylating agents (TMZ) and radiotherapy in newly diagnosed GBM, recurrent GBM, and GBM in the elderly
EGFR mutation or amplification	**No** EGFRvIII variant highly correlates with glioma subtypes	**No** Better prognosis if EGFRvIII plus Ki64 < 20%, or normal PTEN, or MGMT promotor methylation	**No** Vaccines under research
Neuroimaging	**No** No specific imaging feature is diagnostic of GBM. Heterogeneous contrast uptake and surrounding edema are suggestive	**No** Necrosis and extent of edema correlate with worse survival. PET Met-C^{11} uptake indicates worse survival	**No** Correlations between ADC maps and better response to bevacizumab + chemotherapy

and BRAF-V600E mutation is present in 70% of pleomorphic xantoastrocytoma, 30% of ganglioglioma, and 10% of extra-cerebellar pilocytic astrocytomas [68]. In fact, a percentage of BRAF-V600E mutated tumors may benefit from specific kinase inhibitor therapy [69].

Therefore, to avoid the inconclusive and confusing *NOS* designation, the 2016 update of the WHO classification of brain tumors recommends testing at least IDH, 1p19q and H3 status, to correctly classify both adult and childhood diffuse gliomas. The rest of genetic alterations (EGFR, PTEN, TP53, ATRX, CDKNA2…) are characteristic of the various tumor subtypes but are not required for a standardized diagnosis at the present time.

Heterogeneity in glioblastoma

It is widely known that less than 5% of patients harboring a GBM survive longer than 5 years [70]. The main reason for explaining the refractory behavior of GBM is the extensive cellular heterogeneity both across and within tumors. In fact, GBM is currently regarded as a complex group of diseases even within the individual, in which different cellular subclones carry multiple mutations responsible for the intrinsic resistance of the tumor to chemoradiation. This heterogeneity is exhibited via a complex array of cellular and molecular changes.

At the *molecular level*, studies have provided the basis for GBM classification according to transcriptional subtypes [46, 47]. As mentioned above, Phillips et al. [46] defined 3 transcriptional GBM subtypes: proneural, mesenchymal and proliferative, in which expression of certain genes correlated with survival of each type. More recently, Verhaak et al. [47] classified GBM in 4 transcriptional subtypes termed proneural, neural, classical and mesenchymal, also with prognostic significance. In general, the broad distinction between proneural and mesenchymal subtypes carries prognostic implications, and is mainly based on changes at the DNA level, like an amplification of certain genes (EGFR, PDGFA, PIK3CA, CDK4, CDK6) and deletion of others (CDKNA2, PTEN, RB1). The publication in 2013 of the seminal paper by Brennan et al. [37] from the TCGA group, over 500 GBM, provided the most extensive information about the incidence of recurrent mutations present in GBM: PTEN (31%), TP53 (29%), EGFR (26%), PIK3R1 (11%), IDH1 (5%), along with TERT promoter mutation in the majority of GBM. Noticeably, proneural subtypes often harbor mutations in IDH1, TP53 and ATRX, whereas mesenchymal subtypes are usually IDH1 wildtype and harbor NF1 mutations, which again carry prognostic relevance.

Regarding gene mutation, the most important distinction is whether the GBM is IDH1-mutant or IDH1-wildtype. IDH1 mutation is present in 60–80% of grade II and III astrocytomas and oligodendrogliomas, in the majority of secondary GBM, but only in 5–6% of primary GBM [7]. It is interesting that the IDH1-R132H mutant variant leads to the production of the oncometabolite 2HG than can be detected by MR spectroscopy [71], through advanced MRI specific sequences [72]. IDH1 mutant GBM tend to appear in much younger patients, the expected survival is 3 times greater than IDH1-wildtype GBM, and exhibit less peritumoral edema, contrast enhancement, but higher cystic components and frontal lobe affectation [73]. Studies suggest that IDH1 mutation occurs at a very early stage in GBM development and can be considered a primordial alteration affecting the overall prognosis [74]. Moreover, resection of the total IDH1-mutant tumor volume (both the T1 enhancing mass plus the T2 hyperintense volume) leads to a significant improvement of survival in patients harboring IDH1 mutant malignant astrocytomas [75]. A mild survival benefit was obtained, however, when only the T1 enhanced mass was removed in IDH1-wildtype malignant astrocytomas [75], emphasizing the favorable prognostic implication of harboring IDH1 mutations.

Within a particular GBM, there is a subclonal variety of alterations, especially affecting the RTKs, the intracellular pathway involved in cell growth which seems to be highly dysregulated in glioma. Amplification of RTKs include EGFR (60–70%), PDGFRA (12–15%) and MET (5%). However, therapy with RTK inhibitors has failed to provide clinical benefit in these tumors [76], suggesting that the subclonal mosaicism present might be partly responsible for the resistance. The study by Patel et al. [77] showed that cells within the same GBM exhibited different RTKs even at the single cellular level. Moreover, *contamination* of the predominant proneural subtype of a particular GBM with genetic alterations characteristic of other subtypes, like those present in the mesenchymal subtype, significantly worsens the prognosis [37, 47].

At the *cellular level*, a growing body of evidence supports the idea that a fraction of primordial cells within the tumor have stem cell-like properties, so-called *tumor-initiating cells* (TIC) or cancer stem cells, which are thought to be responsible for tumor growth, recurrence and therapy resistance [78–80]. Although we lack specific genetic markers that differentiate TIC from regular GBM cells [7], several molecules have been studied, like Nestin, CD133, SOX2, CD15 or CD44. Interestingly, identification of TIC within specific transcriptional subtypes showed that mesenchymal TIC are relatively radioresistant compared to proneural TIC, and proneural TIC can be converted into mesenchymal TIC when exposed to tumor necrosis-alfa [48]. This suggests that the micro-environment of the tumor somehow affects the transcriptional profile and, therefore, the tumor phenotype at different stages of the disease [48]. Moreover, epigenetic factors also condition the TIC phenotype and drive

the self-renewal power and the tumorigenic potential of TIC [81].

Studies have shown that ionizing radiation enriches TIC populations with increased CD133 positivity which in turn favors reduced apoptosis [80], therefore, promoting resistance to radiotherapy. Others have noted, in animal models, that TIC populations are relatively resistant to temozolomide [77], and treatment with the alkylating agent may actually select cell subpopulations resistant to therapy at the moment of recurrence [7]. Finally, TIC can also *trans-differentiate* into other non-neural lineages like endothelial cells and pericytes, indicating that GBM can actually build its own favorable micro-environment, a perivascular *niche*, in which TIC may survive despite treatment agents [82].

Until further research completely elucidates the specific pathogenic mechanisms of GBM formation, it can be affirmed that the main reasons explaining its resistance to treatment are in fact the two hallmarks of the diseaase: invasiveness, and the remarkable molecular and cellular heterogeneity, both across and within individual tumors.

Immunological microenvironment in glioma

Interactions between malignant glioma and the immune system

GBM has been classically regarded as a tumor capable of escaping any immunological response, partly attributable to the existence of the blood–brain barrier but also due to the lack of lymphatic nodes within the brain parenchyma [83, 84]. Recently, Woroniecka et al. [85] have found that many GBM patients harbor a large number of mature T-cells trapped within the bone marrow, and tumor cells somehow induce T-cells dysfunction through various mechanisms, leading to a condition of frank immune-depression. This state can be reversed after removing the tumor, although it returns at the time of tumor recurrence until the patient reaches a stage in which the immune system is ultimately unable to recover [85].

Role of steroids and extent of resection

The extent of resection is an increasingly recognized prognostic factor of survival in GBM [86, 87]. Patients in which the tumor can be completely resected, the mass effect disappears and the tumor immunosuppressive effect is attenuated. Perioperative steroids used to control peri-tumoral edema, also contribute to the immunologic decline. The study by Díez-Valle et al. [88] has recently shown that steroids can be rapidly and safely reduced and withdrawn within a few days after surgery in a large proportion of patients. However, there is no established optimal steroid dosage and it is common practice to maintain low doses of dexamethasone until the end of radiotherapy. It has been found an inverse association between overall survival and dependency on steroids in a series of newly diagnosed GBM, all of which were good candidates for resection [88]. Therefore, it seems that both malignant glial tumors and steroids enhance an immune-depressive state with a plausible negative impact on survival. Ideally, GBM patients would benefit from both maximum safe resection and rapid steroid tapering and discontinuation whenever possible.

Dendritic cells vaccination, checkpoint inhibitors and oncolytic virus

Although some strategies aimed to enhance the immune response against brain tumors have been tested, at present, none of them have proven efficient for the treatment of GBM. The enormous heterogeneity of GBM cells explains why targeted strategies have failed so far; EGFRvIII vaccine (*Rindopepimut*) is an example of the inability of tumor control despite a robust humoral immune response [89]. Although vaccines obtained from tumor lysates have been shown to increase overall survival several months compared to vaccines against specific antigens [90], phase III trials have not been yet performed, so robust evidence to support them still lacks.

PD-1 and PD-L1 checkpoint inhibitors, such as nivolumab, have been tested in GBM. They were able to enhance the local immune response against the tumor, however, without a clinical benefit for patients [91]. Oncolytic viruses are designed to infect and destroy glioma tumor cells, by activating the immune system against specific tumor antigens. More than 10 different viruses, through different pathogenic mechanisms, are capable of infecting brain tumor cells. Recently, in patients with recurrent GBM, in phase I/II trials, an adenovirus (DNX 2401) [92] and a recombinant poliovirus (PVSRIPO) [93] have been tested, associating a modest response of 20% of patients showing increased OS. Studies with a retroviral replicating vector (Toca-511) have also failed to significantly improve survival in glioma patients according to preliminary trials [94]. Although immunotherapy is a promising tool in GBM management, research and clinical studies are still needed to design tailored therapies for individual patients.

Future directions for targeted therapies

Almost none of the recurrent genomic variants present in GBM have been associated with clear prognostic and predictive value, basically due to the strong cancer cell plasticity, characterized by marked inter- and intratumor heterogeneity, even at the single-cell level and also

between primary and recurrent tumors. The most common targeted therapy is selective inhibition of growth factors RTKs, which seem to benefit many cancer types. Although the RTK/MAPK/PI3K signaling pathway alteration is a hallmark in glioblastoma, targeted therapies have yielded disappointing results [95, 96].

Other targeted therapies aimed to other growth factor receptors (bevacizumab to VEGF, onartuzumab to MET, rindopepimut to EGFRvIII, erlotinib to EGFR, buparlisib to PI3K), to DNA repair and epigenetic modifiers (vorinostat to histone deacetylases, veliparib to PARP), antiangiogenics (bevacizumab to VEGF, cediranib to VEGF1-3 and PDGFR, sunitinib and lapatinib to other targets), and to immune checkpoints (nivolumab to PD1, ipilimumab to CTLA-4, durbalumab to PD-L1, pembrolizumab to PD1) have been tested, primarily in phase I and II trials, without definitive impact on survival, as recently reviewed by Touat et al. [97]. Also, inhibitors of mutant IDH enzymes are promising targeted agents currently under evaluation [98], as well as peptide vaccines [99].

Defining relevant targets in glioma is challenging, partly due to the enormous heterogeneity of the lesions. The concept of *precision medicine* in glioma treatment, driven by molecular stratification, is an interesting, promising and scientifically-based issue. Within this context, the combination of targeted therapies should ideally avoid the emergence of resistant subclones. Agents *more broadly* targeting pathways (such as MDM2 inhibitors), instead of those aimed at single mutation variants, may also be more effective in larger population subsets. Current drugs tested in clinical trials have not been designed specifically for glial tumors and exhibit poor crossing of the blood-brain barrier and tumor penetration. Therefore, future trials directed to glial tumors need to overcome two main challenges: defining relevant molecular drivers and biomarkers, and designing specific and highly bioactive drugs [97].

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Compliance with ethical standards

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