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Chapter 1

Molecular Advances in Glioblastoma Neuropathology

Jens Schittenhelm and Marco Skardelly

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Abstract

Glioblastoma is the most frequent and malignant brain tumor with a wide variety of morphological appearances. For a long time, the tumors were classified either as primary ("de novo") glioblastomas that develop rapidly in elderly patients or as secondary glioblastomas with clinical or histological progression from low-grade diffuse astrocytoma or anaplastic astrocytoma. Recent data from the comprehensive genetic characterization of these tumors identified a number of common and diverging alterations and pathways that allow future stratification of glioblastomas into several age-dependent biological subgroups. While the histological classification of diffuse gliomas based on the WHO grading scheme is still necessary, the use of additional meaningful immunohistochemical (and mutation-specific) markers, such as IDH R132H, ATRX, and H3F3A K27M, has improved routine diagnosis. In recent years, the spectrum of clinically relevant molecular markers has expanded. The utility of MGMT, ATRX, TERT, H3F3A and LOH 1p/19q in predicting prognosis and response to therapy in routine diagnostic settings is discussed.

Keywords: neuropathology, histopathology, immunohistochemistry, molecular classification, glioma

1. Introduction

Gliomas are diffusely growing neoplasms of the central nervous system that present high rates of morbidity and mortality. They are the most frequent CNS neoplasms, accounting for approximately 29% of all CNS neuroepithelial tumors [1]. In contrast to almost all other brain tumors, such diffuse gliomas are characterized by extensive, diffuse infiltration of tumor cells into the brain parenchyma—the neuropil. This infiltration is so extensive that even past attempts with radical resection of a hemisphere were not successful, as the tumors reemerged on the

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contralateral hemisphere [2]. Because of their similarity with non-neoplastic glial cells, these tumors are considered to be of astrocytic and/or oligodendroglial lineage and do not include the biologically different group of ependymomas [3]. The most common primary and malignant brain tumor among this group is glioblastoma, widely known by its acronym "GBM." The tumor was originally designated as "glioblastoma multiforme" because of the extensive variability of tumor histologies. However, some specific (and rare) entities have been isolated from this umbrella term, and individual glioblastomas can also appear quite monomorphic in histology. For this reason, the term "multiforme" is no longer in use following the WHO 2007 classification [4].

Glioblastomas are preferentially located in the subcortical or deep white matter of the cerebral hemispheres, but they may be observed in any other region of the brain, including the cerebellum and spinal cord [3]. Upon initial presentation, less than 2% of the tumors show multiple, clearly distant lesions [5]. In our institution, we prefer to use the term "multifocal" for such lesions without apparent MRI and histological continuum and the term "multicentric" for tumors with radiologically or macroscopically visible distinct tumor centers that have developed from a single lesion. Conventional radiological modalities tend to underestimate the extent of diffuse infiltrative glioma growth. Tumor cells are usually present even outside the peritumoral areas of low density in CT and hyperintensive regions on T2-weighted images in MRI [6]. Not surprisingly, the radiological distinction between multifocal and multicentric gliomas is slightly uncertain. The most extreme example of diffuse infiltrative glioma growth is represented by gliomatosis cerebri. This diagnosis requires the involvement of at least three cerebral lobes, usually bilaterally [3]. Because gliomatosis lacks molecular differences between more circumscribed tumors, this entity will most likely be removed in the next WHO revision. Unlike secondary/metastatic brain tumors, gliomas usually respect the blood-brain barrier, and extraneural metastasis, which is extremely rare, occurs due to ventriculo-peritoneal shunts [6]. In contrast, cerebrospinal fluid spread of glioblastoma cells is occasionally observed, but it is still far less common than in ependymomas or childhood primitive neuroectodermal tumors. Common routes of spread of glioblastoma include the fornix, corpus callosum, anterior commissure, and radiatio optica because of the high affinity of tumor cells to myelinated structures [7]. Symmetric tumors spread across the corpus callosum are called "butterfly gliomas." Tumors that reach the dura often show marked desmoplasia, leading to a firm texture that resembles gliosarcoma or meningioma [8].

2. Clinicopathological parameters of glioblastoma

Glioblastomas represent approximately 65% of all astrocytic or oligodendroglial neoplasms [1]. Incidence rates, estimated to be up to 4.6 per 100,000 people, tend to vary by region, with generally higher numbers in developed countries, increasing with patients' age and showing a slight predominance for males [9]. The vast majority of tumors are observed in adults, with a mean age at diagnosis of 61 years. However, GBM can be found in children, and due to their lower incidence, these tumors are often grouped together with anaplastic astrocytomas and intrinsic pontine gliomas as high-grade (i.e., WHO grade III and IV) tumors. As a matter of

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fact, comparing clinical and molecular data from such cohorts with adult tumors is difficult. Generally, a younger age of onset in non-pediatric GBM is one of the strongest predictive factors of prolonged survival [10]. Despite this fact, surprisingly, many publications do not include patients' age in multivariate analysis when analyzing biomarkers of patient survival. While almost all GBM occur sporadically, in individuals with Lynch syndrome, a constitutional mismatch repair defect results in an increase of brain tumors, including GBM, among families [11]. Glioblastomas have been reported in other inherited tumor syndromes, including neurofibromatosis type 1, Li-Fraumeni and Turcot-Syndrome and multiple enchondromatosis [3].

Because diffuse astrocytomas show a tendency to progress to a more malignant phenotype during disease progression, these tumors end up as the so-called secondary glioblastomas, (10–15% of all glioblastomas). However, the vast majority of glioblastomas (85–90%) develop without a precursor lesion—the so-called "de novo" or primary glioblastoma [12]. Based purely on histology, primary and secondary glioblastomas cannot be distinguished and it is expected will be rather separated in future WHO classification that glioblastomas will be separated by their molecular profile, than their clinical history (this will be discussed in more detail below). While there is no doubt that oligodendrogliomas undergo a similar malignant tumor progression as astrocytic neoplasms, there is still debate about how many of these truly develop into glioblastomas. As there is some overlap in preoperative imaging of GBM with solitary metastases or lymphomas, intraoperative cytology and frozen sectioning may help in the rapid diagnosis and decision making in neurosurgical procedures. In many cases, nuclear atypia, uneven cell distribution, and the presence of fibrillary processes should guide diagnosis. However, cellularity in frozen sections is often underestimated as a result of artifactual spaces (**Figure 1**).

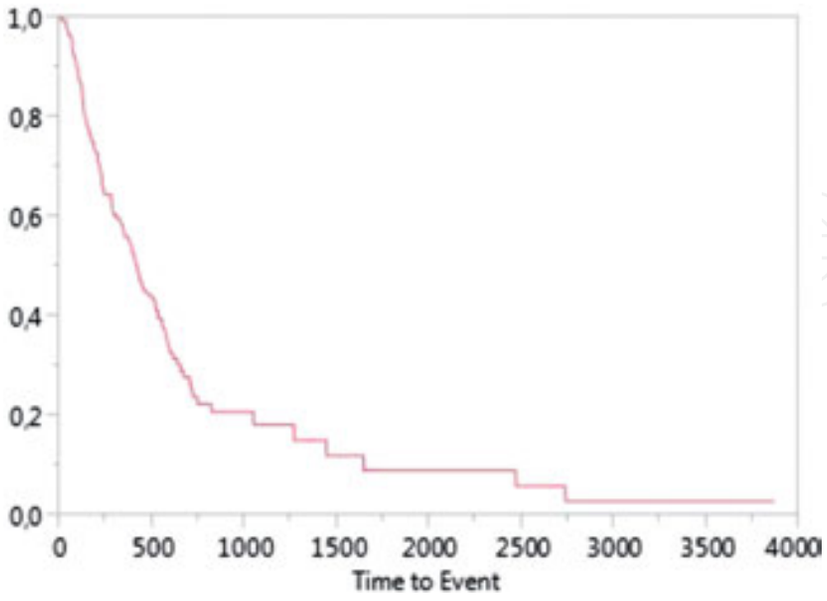


Figure 1. Glioblastomas have a poor prognosis. Kaplan–Meier overall survival Tübingen glioblastoma cohort (n = 205, median survival: 417 days, all cases IDH-wild-type, age <45 years, primary cases).

In newly diagnosed glioblastoma, current therapeutic strategies include surgery (from biopsy to gross total resection, depending on location, which itself is a prognostic marker [13]) followed by concomitant radiotherapy with temozolomide (TMZ) and up to 6 cycles of TMZ maintenance therapy in patients ≤65 years with a median overall survival (OS) of 14.6 months [14].

In addition to the well-established prognostic factors of survival in GBM patients, such as age, extent of resection (EOR), Karnofsky performance scale (KPS), and the treatment modality, molecular alterations (i.e., IDH mutation and MGMT gene promoter methylation, which we will discuss in more detail below) represent not only new significant prognostic factors of survival but also predictors of therapeutic responses in subgroups of GBM. MGMT gene promoter methylation status is a strong prognostic marker of survival in GBM with a median OS of 12.6 months in unmethylated and 23.4 months in methylated GBM treated by concomitant radio-chemotherapy with TMZ. Moreover, MGMT gene promoter methylation status also serves as a predictor of the response to TMZ therapy. In patients >65 years of age exclusive radiotherapy or chemotherapy is indicated according to the MGMT gene promoter methylation status. Patients with methylated GBM most benefit from monotherapy with TMZ, and patients with unmethylated GBM demonstrated the highest survival profit by exclusive radiotherapy [15, 16]. Recurring tumors with a methylated MGMT promoter (i.e., the silencing of the antagonistic effect of this repairing enzyme on alkylating chemotherapy) may thus benefit from a second round of treatment with TMZ [17]. Approximately 30–35% of glioblastoma samples contain a methylated MGMT promoter [18]. However, tumor treatment in diffuse gliomas lacks a persistent therapy response [19], which is why there is an increase of studies with direct (“personalized”) targeting of driver mutations in glioblastomas. Alternative treatment approaches include the application of oncolytic parvoviruses to induce tumor cell cycle arrest and cell death [20], the application of low-energy alternating electric fields that affect dividing cells' viability [21], and the application of local hyperthermia induced by superparamagnetic iron oxide nanoparticles coated with hydrophilic polymers subjected to an alternating magnetic field [22].

One of the primary mechanisms related to treatment failure and tumor recurrence in GBM, even in targeted therapies, might be attributed to intratumoral molecular heterogeneity [23] and the presence of a subpopulation of cancer stem cells that contribute to tumor propagation and tumor maintenance through their ability to self-renew and differentiate [24].

2.1. Histologic tumor classification

Although serious advances in the neuroimaging of glioblastomas have been made in the past, histopathologic evaluation of neurosurgically removed tumor specimens is still required for definite classification and subsequent molecular stratification. In 1979, the World Health Organization (WHO) issued a publication for the classification of tumors of the central nervous system. This included a grading scheme based on the malignancy of tumor behavior. The grading of CNS tumors is performed with a four-tiered score, which ranges from grade II to

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grade IV in astrocytic/oligodendroglial tumors, to separate the histologic continuum of diffuse gliomas according to their expected clinical behavior. Grade IV has the worst prognosis and is reserved for glioblastoma [3].

On macroscopic view, the necrotic center of the tumor is often surrounded by a macroscopically visible gray rim and surrounded by yellowish-grayish texture blending into the surrounding white matter. Black hemorrhagic streaks and thrombosed veins are typically observed in glioblastomas [4]. Glioblastomas' tumor borders are usually diffuse, but rare cases (especially giant cell glioblastomas and gliosarcomas) can be very circumscribed, mimicking a carcinoma metastasis.

The astrocytic heritage of the glioblastoma is best appreciated in cases with prominent eosinophilic cytoplasm of pleomorphic tumor cells resting on a fibrillary background, but this is not the rule for all tumors [25]. As expected in a malignant tumor, marked nuclear atypia and elevated mitotic activity is common. The presence of microvascular proliferations, necrosis or both are required to determine the diagnosis of GBM [3, 4]. Intraoperative consultation with cryosection and smears can provide an initial histological diagnosis and the grade of malignancy. Therefore, intraoperative consultation is useful for neurosurgeons to a) confirm the region of interest in stereotaxic surgery, to b) decide the EOR in relation to the risk of developing new neurological deficits in patients with tumors in eloquent regions, and to c) distinguish between tumor infiltration and reactive astrocytosis.

In absence of these hallmarks, tumors must be classified as anaplastic astrocytomas, even when subsequent molecular data indicates that such tumors are underclassified glioblastomas [26]. On average, three pseudopalisading necroses are present in a glioblastoma specimen. Pseudopalisading cells are usually less proliferative and exhibit higher rates of apoptosis due to hypoxic conditions. More than half of the palisades show a central vascular lumen, and in approximately 20%, intravascular thrombosis is also observed [27]. The presence and extent of necrosis is an adverse prognostic factor [3]. Vascular proliferation in the form of glomeruloid bodies in glioblastomas is observed more frequently than in tumors from any other organ system [28]. Vascular proliferations tend to accumulate in the peripheral region of high cellularity corresponding to the contrast-enhancing ring observed in radiological images [3]. In addition to intrinsic tumor growth, the so-called secondary properties ("Scherer signs") indicate the presence of glioblastoma: perineuronal satellitosis, subpial growth along cortical surfaces and perivascular and intrafascicular growth along myelinated fibers in white matter tracts, mostly characterized by small undifferentiated cells [29]. In the spinal cord, tumor cells might extend into the subarachnoid space [4].

Tumor appearance can be so heterogeneous that diagnosis is often based on tissue patterns rather than individual tumor cell morphology. The 2007 WHO classification recognizes two distinct morphological variants, the giant cell glioblastoma and the gliosarcoma. The giant cell glioblastoma is often subcortically located, and the aptly named cells are regarded as a type of regressive change and harbor a high frequency of Tp53 mutations [3]. Diagnosis requires the presence of giant, often multinucleated cells in more than 50% of tumor cells that can be associated with reticulin deposits [30]. These tumors need to be distinguished from the more benign subependymal giant cell astrocytoma or pleomorphic xanthoastrocytoma. Gliosarcoma

consists of often densely interwoven malignant glial and mesenchymal components and account for up to 2% of all glioblastoma samples. The alternating reticulin-free glial and reticulin-containing mesenchymal deposits can be additionally visualized through GFAP immunohistochemistry [3]. While the OS in giant cell glioblastoma is somewhat better, data from a large retrospective study (and others) did not show significant differences for gliosarcomas [31]. Currently considered as a pattern, not a morphological variant, gliomas can show focal areas of epithelial differentiation that range from the positive immunoreactivity of epithelial antigens to adenoid or squamous formations, leading to the misdiagnosis of carcinoma [32]. Among this group, the epithelioid GBM stands out with a younger age of onset and a high percentage of therapeutically relevant BRAF V600E hotspot mutations, and it is very likely that this tumor will become a third glioblastoma variant in the upcoming 2016 WHO classification [32]. These closely packed tumors have variably lipidized, small- to medium-sized cells with rounded cytoplasmic profiles, eosinophilic cytoplasm without stellate processes, and the absence of interspersed neuropils [33].

Small cells with little cytoplasm can appear so monomorphous that small cell glioblastomas mimic anaplastic oligodendrogliomas. Such tumor cells intermingled with gemistocytes are more likely observed in glioblastomas developing from a previous lower-grade gemistocytic astrocytoma. However, the tumors show the same aggressive course as primary GBM [34]. In some tumors, the nucleus-to-cytoplasm ratio is so high that sharply demarcated hypercellular tumor nodules with evidence of neuronal differentiation are present. These clonally expanded, often myc-amplified, so-called PNET components have a high risk of cerebrospinal fluid dissemination [35]. Some tumors may show prominent perivascular rosettes resembling anaplastic ependymomas but usually lack the more uniform roundness of ependymal tumor cells. Tumor cells can be elongated and arranged in fascicles so that upon first viewing them, a sarcoma comes to mind. In up to 15% of the tumors, perinuclear halos around nuclei in glioblastomas may resemble oligodendrogliomas on first viewing; however, the tumor nuclei usually lack the monotonous roundness of true oligodendrogliomas. Such tumors are often called glioblastoma with oligodendroglial component because initial studies suggested that these tumors might have a better prognosis than standard glioblastoma. Comprehensive reviews, including molecular analysis, indicated a pathogenetically heterogeneous group (including misdiagnosed oligodendrogliomas) without a prognostic role [36]. Another occasionally encountered pattern is the presence of adipocyte-like tumor cells that are clearly of astrocytic origin. These tumors are not genetically distinct from conventional glioblastoma [37]. In some cases, the xanthomatous/lipomatous changes are so prominent that the glial nature of these tumors is obscured [38]. Other morphologic variants include granular cell astrocytoma, which is characterized by large, PAS-positive cells with a degenerative granular lysosomal content. These look similar to the benign granular cell tumor of the pituitary stalk [39]. Metaplastic transformation can be so strong that chondroid and osseous formations in gliomas are possible [40]. Rare cases may show melanotic differentiation [41] or a rhabdoid phenotype with focal loss of INI-1 as observed in atypical teratoid/rhabdoid tumors (**Figure 2**) [42].

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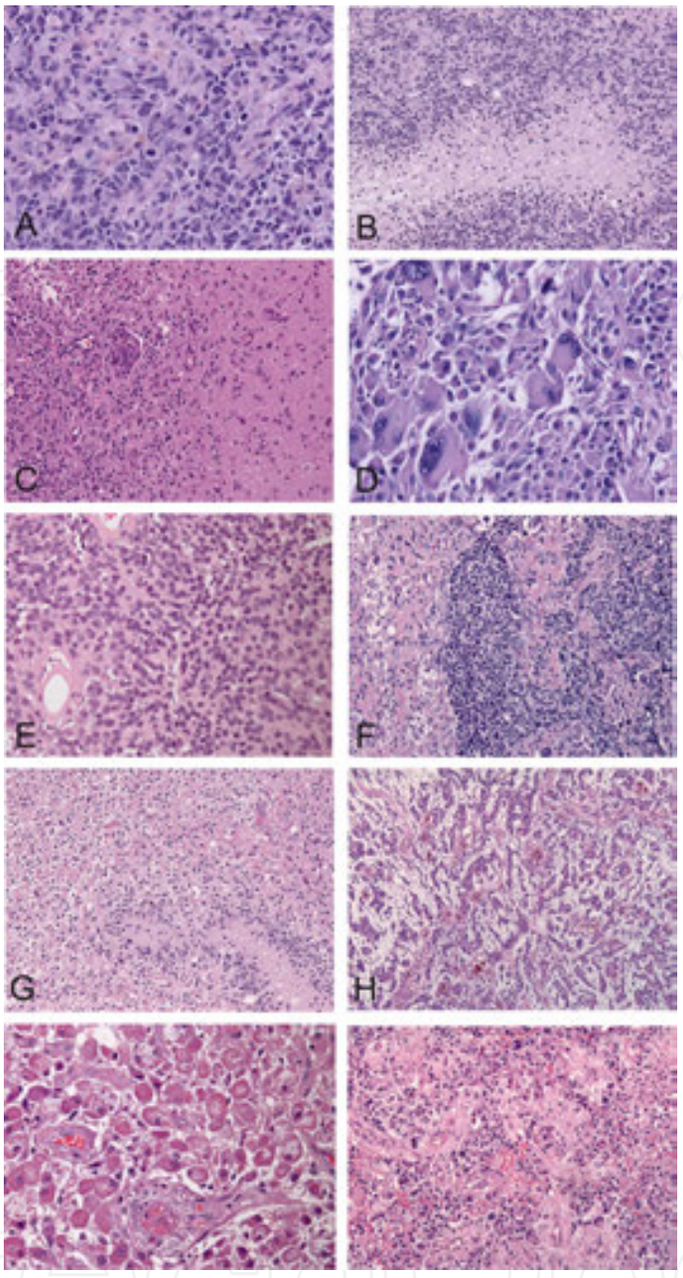


Figure 2. Glioblastoma histology consists of an anaplastic glial tumor with increased cellularity and mitotic activity (A), areas of pseudopalisading necrosis (B). The tumor diffusely invades the brain and has proliferating vessels (C). Morphological variants include giant cell glioblastoma (D), small cell glioblastoma (E), glioblastoma with PNET component (F), with oligodendroglial component (G), with adenoid features on a myxoid background (H), granular cell glioblastoma (I), and epithelioid glioblastoma (K).

2.2. Immunohistochemistry

In many instances, the diagnosis of GBM is straightforward in histology. However, confirmation of the diagnosis with a routine immunohistochemistry panel is mandatory for laboratory quality and improves diagnostic accuracy. Furthermore, some stains have not only diagnostic but also a prognostic role, and their results should be communicated back to clinicians.

The immunoprofile of the glial markers GFAP and EAAT1 in GBM is similar to astrocytomas [43]. In the vast majority of glioblastomas, these antigens are strongly expressed in the cytoplasm of the tumor cells, but they may be occasionally lacking (especially in small cell glioblastomas). The alternatives S-100, WT1, and MAP2 are less specific. Strong MAP2 and WT1 immunoreactivity in cytoplasmatic cell processes is observed in 90% of glioblastomas and is helpful in discriminating GBM from oligodendrogliomas [44, 45]. Vimentin is very unspecific and has no diagnostic value in brain tumors. Cytokeratin expression in glioblastomas (especially in giant cell glioblastomas and glioblastomas with epithelial differentiation) is an important diagnostic pitfall and should not lead to the erroneous diagnosis of carcinoma metastasis [32]. Dot-like EMA immunoreactivity is less frequently observed in GBM than in ependymomas, where usually more than 5 EMA-positive dots per high-power field are observed [46].

Identifying axons with neurofilament stains within the tumor can support the diffuse growth of GBM. In gliosarcomas, GFAP is lacking in reticulin-rich, sarcomatous areas. Usually 15–25% of the nuclei are MIB-1 positive, but the tumor proliferation rate varies greatly between individual GBM. We have observed gemistocytic tumors with less than 5% positive cells and small cell glioblastomas with 90% proliferating cells [25]. Recurrent tumors with history of previous radiation may show little proliferating activity. Because of inconsistent laboratory techniques and varying evaluation methods, MIB-1 immunoreactivity has little prognostic relevance. Nuclear Tp53 immunoreactivity in primary GBM is less present than in astrocytomas and their GBM recurrences, but it may be considerably high in giant cell glioblastomas. Tp53 expression alone is not an independent prognostic marker, but in combination with a methylated MGMT promoter, p53 nuclear staining in more than 50% of tumor cells indicates a less favorable course, similar to GBM with an unmethylated MGMT promoter [47]. It is noteworthy, that not all p53 immunoreactive tumors contain mutations in the Tp53 gene [48] and molecular determination of p53 mutation status in GBM does not correlate with patients' outcome [49]. Several studies have attempted to obtain the MGMT status by immunohistochemistry for MGMT protein expression, but results are hampered by diverging cut-off values and poor correlations with clinical outcome. MGMT immunohistochemistry therefore should be avoided unless there is a consensus with clinical data [50]. Microglial markers, such as CD68- and CD163-positive cells, are regularly found in GBM and can be very widespread, especially in tumors with granular cell components, and must be distinguished from demyelinating lesions.

The NADP-dependent enzymes IDH1 and IDH2 catalyze the conversion from isocitrate to alpha-ketoglutarate. Mutations of the catalytic center in brain tumors result in the accumulation of the oncogenic metabolite D-2-hydroxyglutarate [51]. Because IDH1 mutations are associated with a significantly more favorable outcome in GBM, confirmed in several independent studies, IDH analysis has become the major biomarker in neuropathology practice [52, 53]. The prognostic role of IDH1/2 mutations becomes obvious in WHO grade IV IDH-positive glioblastomas because they show a better prognosis than WHO grade III IDH-negative anaplastic astrocytomas [54]. The upcoming 2016 WHO tumor classification therefore separates glioblastomas according their IDH status. The vast majority of IDH1 hotspot mutations

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lead to a distinct amino acid substitution on codon 132 (Arg132His), for which a mutation-specific antibody is available [55]. This antibody, however, does not recognize other non-canonical IDH1 and IDH2 mutations, and negative staining results do not imply an IDH wild-type status. IDH1 R132H antibody expression is found in 4% of primary and in 71% of glioblastomas with a lower-grade precursor [55]. In elderly GBM patients above 65 years, the incidence of IDH mutations is rare—one study reported only 2 positive cases out of 167 samples, accounting for the unfavorable prognosis of this age class [56]. Another important clinical aspect is the association of epileptic seizures in IDH1 mutant tumors (**Figure 3**) [57].

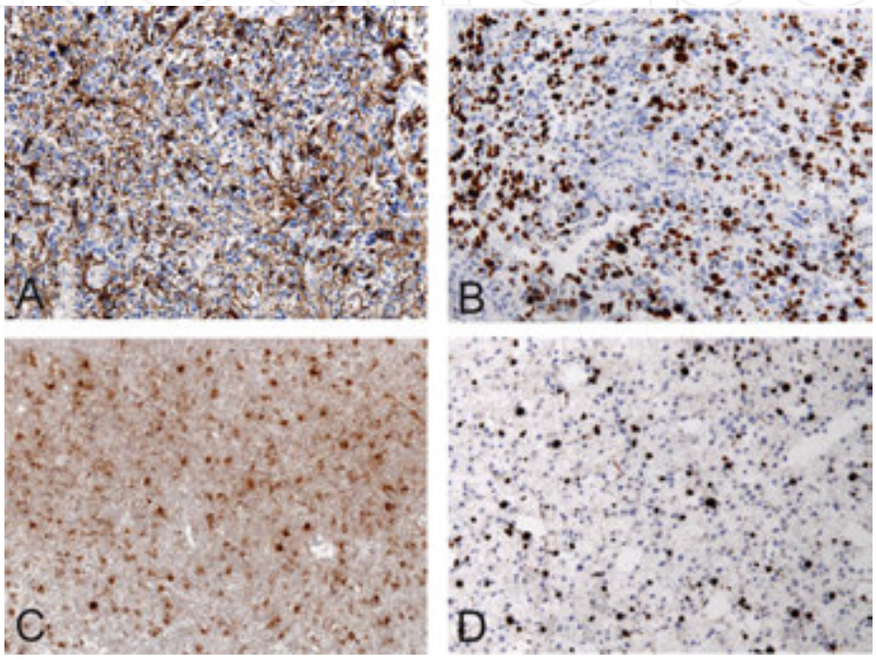


Figure 3. Immunohistochemistry: (A) Limited GFAP expression in a glioblastoma with an oligodendroglial component. The absence of an IDH mutation and partial 19q deletion excluded pure oligodendroglioma in this case. (B) Elevated proliferative activity (MIB-1 index) in the same case. (C) Prognostically relevant IDH1 R132H expression in a glioblastoma arising from a grade II astrocytoma seven years ago. The same case shows nuclear ATRX loss in tumor cells (D).

Some studies aimed to identify antigens to detect the tumor subpopulation with stem cell-like properties, i.e., the cells that have a marked capacity for proliferation, self-renewal, and differentiation. In glioblastoma, the most widely propagated marker for cancer stem cell ability is CD133 along with SOX2 and nestin [58]. However, recent studies indicate that CD133-negative tumor cells may also have stem cell abilities and that different subtypes associated with divergent molecular glioblastoma profiles, discussed below, may exist [59].

2.3. The genetic landscape

Like other tumors, GBM shows an accumulation of several epigenetic and genetic alterations in neoplastic cells within the brain. Recent data from the comprehensive multiplatform genomic characterization of GBM samples identified a number of common and diverging

alterations and indicate that glioblastoma consists of biologically heterogeneous subgroups with similar histological appearances. The pilot project of The Cancer Genome Atlas Consortium in 2008 analyzed more than 20000 genes in 90 primary glioblastomas and identified the most common somatic genetic alterations/mutations: Tp53 (42% of the tumors mutated), PTEN (33%), NF1 (21%), EGFR (18%), RB1 (11%), and PI3K-pathway genes (7–10%) [60]. Interestingly, Tp53 mutations are found in almost all GBM with a giant cell component [61]. In addition, pediatric glioblastomas also show a high frequency of Tp53 mutations, as in up to 60% of tumors examined [62].

The most surprising finding was the discovery of isocitrate dehydrogenase (IDH) mutations that occurred in younger patients; these mutations are always heterozygous and are associated with an increase in OS [63, 64]. So far, several hotspot mutations were identified in the catalytic active centers in IDH1 R132, IDH2 R140, and R172 codons, and the most common, R132H, comprises over 80% of all mutations observed to date. The IDH1/2 mutations are present in 50–88% of the so-called secondary glioblastomas (the same point mutation is always present in lower grade precursor tumors, and in cases of a IDH1 R132C mutation, strongly associated with an astrocytoma phenotype). In contrast, IDH1 mutations are observed in only 3–7% of primary glioblastomas and gliosarcomas [54, 65]. In these tumors, IDH2 mutations are virtually absent, except for a single reported case. There is ongoing discussion of whether anaplastic astrocytomas that are IDH1/2 wild-type should be considered glioblastomas without vascular proliferation and/or necrosis because they show the same clinical course and also contain similar genomic alterations as primary GBM. Because IDH1 R132H also represents a tumor-specific antigen, immunotherapy trials currently aim at vaccination to induce antitumor immunoreactions [66].

The activation of receptor tyrosine kinases (RTKs) and the associated RAS/PI3K signaling pathway are common events in GBM. Approximately 45–50% of all primary GBM show high-level genomic amplification of epidermal growth factor receptor (EGFR). Among 30–50% of these amplified cases, intragenic rearrangements with the deletion of exons 2–7, the EGFRvIII variant is detected as a late event. The resulting overexpression leads to the constitutive activity of the receptor in the absence of its ligand and trigger the downstream pathways, resulting in increased cellular proliferation and radiation resistance of the tumor [67]. GBM with small cell morphology are often EGFRvIII-positive tumors [34]. Another important fact is that EGFR mutations in GBM significantly differ from those mutations found in other cancer types, such as non-small-cell lung cancer, where mutations are often located in the intracellular domain [26].

Like EGFR, other RTKs, such as PDGFR and MET, are often amplified in GBM. Platelet-derived growth factor receptor α amplification is significantly enriched for pontine tumors and the pediatric GBM cohort. These tumors are often grouped by the German Cancer Research Center (DKFZ) tumor methylation profiles as RTK class I tumors, while EGFR amplified tumors are classified as RTK-II tumors [68]. Approximately 40% of all PDGFR amplified tumors contain an intragenic deletion of exons 8 and 9 in PDGFRA, which induces an aggressive growth phenotype. Detailed cellular analysis in GBM samples showed that independent focal amplification of PDGFR α and EGFR could coexist in the same tumor [69].

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Other common oncogenic alterations in GBM involve the extended PI3K-AKT-mTor and RAS-MAPK pathways. Mutations of the important primary negative regulator PTEN are found in up to 30% of GBM, and the RAS antagonist neurofibromin 1 (NF1) is mutated in 15% of all primary tumors. NF1 germline mutations result in neurofibromatosis type 1, and although rare in comparison to the prevalence of pilocytic astrocytoma, the occurrence of GBM in NF1 syndrome has been reported in a handful of cases [70]. The loss of NF1 is usually associated with concomitant Tp53 mutations, and the additional deletion of PTEN results in the progression of astrocytoma to GBM [48]. Interestingly, PTEN loss and subsequent PI3K-AKT pathway activation results in increased expression of the programmed death ligand-1 (PD-L1), which in turn contributes to GBM immunoresistance and immune escape [71]. Consequently, studies with immune-checkpoint inhibitor treatment in GBM are currently ongoing, although there are conflicting data on the prognostic role and predictive role of PD-L1 gene and protein expression in glioblastoma [72].

Up to 78% of GBMs show alterations of the retinoblastoma (Rb)/CDKN2A-p16^{ink4a} pathway [73]. This pathway plays a central role in proliferation and cell cycle regulation, and alterations include deletions and point mutations in several involved genes. Interestingly, the Rb promoter is far less methylated in primary GBM than in secondary GBM, consistent with the observation that the CDKN2A-p16^{ink4a} gene is affected by the commonly observed chromosomal 9q loss in primary GBM [74]. Although CDKN2A deletion is associated with tumor progression of astrocytomas to GBM and is one of the key molecular marker used to determine a "classical GBM," its prognostic role remains to be elucidated.

Mutations in the promoter region of the telomerase reverse transcriptase (TERT), the catalytic subunit of the telomerase complex, are found in 70–80% of GBM. Similar high frequencies in brain tumors are only observed in oligodendrogliomas [75]. The presence of a TERT mutation in IDH1/2 wild-type tumors is associated with poor outcome and indicates underdiagnosed glioblastoma in a small specimen with otherwise low-grade glioma [76]. TERT itself may also contribute to glioma genesis, as there is evidence that the TERT SNP genetic rs2736100 may influence glioma risk, although it is not a prognostic marker itself [77]. Glioblastomas that do not carry a TERT mutation were recently designated as "triple negative" tumors, i.e., lacking, IDH mutation, 1p/19q codeletion and TERT mutation, again highlighting the importance of these three markers for tumor stratification. TERT mutations are rare in pediatric glioblastomas, where whole exome sequencing recently identified H3F3A mutations, often combined with Tp53 and ATRX/DAXX alterations [78]. The H3F3A mutations are concentrated on two hotspots, K27M and G34V/R, which are mutually exclusive. Both show distinct clinicopathological tumor profiles. H3F3A K27M mutations alter the di- and tri-methylation state of endogenous histone H3 at the Lys27 position and are mainly found in pontine and thalamic tumors [79]. In contrast, the mainly supratentorially located H3F4A G34 mutant tumors presented as a histopathologically heterogeneous group of neoplasms, overlapping classical GBM with central nervous system primitive neuroectodermal tumors (CNS-PNET) [80]. There is growing evidence that G34 mutations may have an alternative mechanism to drive MCYN overexpression for tumor growth [68]. While K27M mutant tumors show a very unfavorable course, the three-year survival rate without this mutation in the pediatric cohort is approxi-

mately 70% [81]. Alpha-thalassemia C-linked mental retardation (ATRX) mutations are found in approximately 30% of pediatric GBM and in 6% of adult glioblastoma. Interestingly, in pediatric GBM, ATRX mutations occur around a hotspot near the carboxy-terminal helicase, while they are widely distributed across the gene in adult GBM [82].

ATRX and its binding partner DAXX (death-associated protein) belong to a complex with a role in regulating chromatin remodeling, nucleosome assembly and telomere maintenance. ATRX mutant tumors are associated with alternative lengthening of telomeres, the so-called ALT phenotype [78]. Because nuclear ATRX is diminished in tumors with the ALT phenotype, ATRX immunohistochemistry has become useful in identifying potential IDH mutants, H3F3A alterations or secondary GBM (usually showing ATRX loss and being mutually exclusive of LOH 1p/19q) [83]. Furthermore, retrospective analysis of ATRX in samples from the NOA-04 clinical trial showed a survival benefit of ATRX mutant tumors [84].

2.4. Epigenetics and molecular profiling

The DNA repair enzyme O-methylguanine-DNA methyltransferase (MGMT) removes alkyl groups from the O^6 position. Methylation of the MGMT promoter region results in decreased MGMT activity, which in turn results in decreased tumor resistance to alkylating agent therapy with TMZ, and is therefore a predictive molecular marker [36]. Usually, MGMT is determined in formalin-fixated paraffin-embedded specimens, and approximately 40% of all primary GBM carry a methylated promoter [18]. Less than 15% of gliosarcomas have a methylated phenotype [65]. In pediatric glioblastomas, approximately half of the tumors are methylated, mainly due to the association between H3F3A mutations and the methylated MGMT profile, but the prognostic and predictive role of MGMT methylation in children remains a matter of controversy [62, 68]. MGMT analysis is essential for almost all clinical studies and one of the most requested molecular analyses in routine neuropathology practice.

Unsupervised hierarchical clustering of GBM identified four tumor subclasses called proneural (characterized by mostly IDH/Tp53 mutations, PDGFRα amplifications, PI3K pathway dysregulation), classic (large-scale EGFR amplification, PTEN, and CDKN2A loss), mesenchymal (NF1, Tp53, and CDKN2A alterations), and neuronal (currently no specific genetic alterations). As expected from the IDH mutation data, the patients with a proneural tumor profile were younger and showed the best outcome [60]. The proneural group exhibited a robust hypermethylated glioma-CpG island methylator phenotype (G-CIMP) that is also present in most secondary GBM indicating a common gliomagenesis for IDH-mutated GBMs [85]. Glioma stratification according the IDH status therefore has widely replaced the previous clinic-based separation of primary and secondary GBM. Computational modeling to predict the temporal sequence of driver events during tumorigenesis indicates that most non-GCIMP mesenchymal GBMs arise from a PDGFA-driven proneural-like precursor though additional NF1 loss [86]. However, assignment with established glioblastoma subtype classifiers becomes difficult in cases with substantial tumor heterogeneity and may change further during patient treatment [73].

Glioblastoma intratumoral heterogeneity contributes to therapy resistance. This is exemplified in a whole genome sequencing report that showed not only extensive mutational and copy-

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number heterogeneity within the primary tumor but also uncovered the recurrence of a double-minute chromosome converging on the KIT/PDGFRA/PI3K/mTOR axis, superseding the IDH1 mutation in dominance in a mutually exclusive manner [87]. Despite targeted therapy with imatinib, the patient succumbed to progressive disease. Another good example is the recent discovery that EGFRvIII mutant cells are expressed only in a fraction of GBM cells of EGFR amplified tumors and enhance the proliferative activity of their neighboring EGFR wild-type tumor cells though cytokine secretion [88]. Because the majority of GBMs exhibit the activation of three or more RTKs, this highlights the need for the combined approach of several specific inhibitors for successful treatment.

Although there is increasing knowledge of divergent molecular alterations in histologically similarly appearing glioblastoma specimens, clinical decision-making on molecular alterations of glioblastoma subtypes is still limited. Notable exceptions include the determination of chromosomal allelic losses in 1p/19q in younger GBM patients, as such tumors respond far better to a combined Procarbazine-CCNU-Vincristine (PCV) therapy regimen [89]. The 1p/19q co-deletion is strongly associated with oligodendroglial tumor morphology and will become a diagnostic marker to be reassigned in future glioblastoma with this signature into the anaplastic oligodendroglioma group [26]. Up to 75% of co-deleted tumors also show either additional IDH1 or IDH2 mutations [90]. This combined molecular signature is so robust and remains visible in tumor recurrences, even in cases with increased intratumoral heterogeneity, and overlaps with "proneural" expression profile of the TCGA genomic landscape [91].

2.5. Conclusion

Glioblastomas have an extensive variety of histological appearances and divergent immunohistochemical and molecular profiles, making diagnosis somewhat difficult for those who are not familiar in working with brain tumors. The histological classification of diffuse gliomas based on the latest WHO grading scheme is a prerequisite to optimal decision-making regarding patient treatment. In addition to core features (microvascular proliferations, necrosis, and secondary structures of Scherer), the clinically relevant pattern and variants (gliosarcoma, giant cell glioblastoma, epithelioid, small cell GBM, and GBM with PNET component) should be clearly depicted in neuropathology reports. Immunohistochemistry and molecular biology have contributed to an improved classification and were shown in some cases to be of prognostic value. A panel of different antibodies is very helpful in securing the diagnosis and avoids potential differential diagnostic pitfalls. The advantages and limitations of the most commonly used antibodies, such as GFAP, WT1, MAP2, MIB-1, P53, IDH1R132H, and ATRX, in GBM have been outlined. The GBM subtype, patient's age, tumor location, and staining results subsequently guide a staged approach to therapeutically relevant molecular analysis, such as the 1p19q codeletion, MGMT promoter methylation, H3F3A screening, TERT promoter and IDH hotspot mutations. The classic concept of primary and secondary glioblastomas has been challenged by the discovery of clinically divergent molecular GBM cohorts, providing a good example of "convergent evolution showing a similar phenotype of genotypically different tumor cells" [92]. The implementation of these additional molecular markers into routine diagnoses has already started, including the routine determination of

MGMT gene promoter methylation status to guide therapy and the re-classification of tumors for appropriate treatment according to LOH1p/19q analysis, and it is expected to further evolve. The heterogenous landscape within and across GBMs underscores the difficulty in developing multimodal targeted therapies and is also a challenge to stratify patients for clinical trials. However, the recent identification of recurring driver mutations as illustrated here provides a rationale to identify tumor-specific peptides and antibody targets that may improve glioblastoma treatment.

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Author details

Jens Schittenhelm[1,2*] and Marco Skardelly[1,2]

*Address all correspondence to: jens.schittenhelm@med.uni-tuebingen.de

1 Department of Neuropathology, Institute of Pathology and Neuropathology, Germany

2 Department of Neurosurgery, University Medical Clinic, University of Tübingen, Germany

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