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Tumor Discovery

REVIEW ARTICLE

Targeting glioblastoma invasion and therapy resistance: Emerging trends and molecular pathways

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Abstract

Glioblastoma (GBM) is the most common and aggressive primary central nervous system malignancy. Significant resistance to therapeutic intervention is a core feature of GBM that drives tumor recurrence and underlies the remarkably poor clinical outcomes associated with this disease. This review explores the therapeutic strategies and molecular pathways involved in GBM. Therapy resistance in GBM depends on multiple interconnected macrostructural and biomolecular mechanisms, including aggressive and diffuse invasion, tumor microtube network formation, stem-like cell enrichment, selective neurovascular permeability, an immunosuppressive microenvironment, and a high degree of inter- and intra-tumoral heterogeneity. Collectively, these pathobiological features insulate specific tumor compartments and maintain GBM viability despite significant treatment-induced cellular stress. While there is enthusiasm for addressing GBM therapeutic resistance, the scale of the challenge remains immense. The identified resistance mechanisms extensively interact and can compensate for single-target assaults. Emerging overlaps between neuro-oncology and developmental neurobiology additionally suggest that GBM may exploit therapeutic resistance mechanisms yet to be identified, functioning beyond the current scientific understanding. Thus, the scope and diversity of this problem demand a comprehensive therapeutic approach capable of targeting multiple interacting mechanisms of therapeutic resistance.

Keywords: Glioblastoma; Invasion; Tumor microtube; Cytoskeleton

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1. Introduction

Primary central nervous system (CNS) tumors are rare cancers that arise directly from cells of the brain or the spinal cord. Tumors that arise from the meningeal tissues are also categorized as primary CNS tumors, and in the United States (US), this combined category of tumors represents <1% of cancer diagnoses.[1] Still, these tumors

account for approximately 3% of US cancer deaths, and the disproportionality of this death rate is significant, considering that between 2014 and 2018, only 29.1% of primary CNS tumors were classified as malignant.[2]

Primary malignant CNS tumors arise predominantly from glial cells (80.9%) and are correspondingly called malignant gliomas.[2] Malignant gliomas develop within both the brain and spinal cord, with approximately 98% originating in the brain.[3,4] Glial cells can be categorized as astrocytes, oligodendrocytes, and ependymal cells, whereas gliomas can be categorized as astrocytomas, oligodendrogliomas, and ependymomas.

Glioblastoma (GBM) is categorized as a type of astrocytoma and is the most aggressive and common histological subtype of malignant glioma. GBM is the most diagnosed primary CNS malignancy and represents approximately half (49.1%) of diagnoses in this category of human cancer.[2] This corresponds to a US incidence rate of 3.23/100,000 people, or approximately 13,000 new GBM diagnoses each year.[2]

GBM is a rapidly fatal cancer with an incredibly poor overall survival rate. Analysis of data collected through the Surveillance, Epidemiology, and End Results Program (SEER) at the National Cancer Institute of the US National Institutes of Health (NIH) shows that the median overall survival time for GBM patients in the US was 8 months and the 5-year overall survival rate is approximately 7%[2] between 2001 and 2018. This is notably lower than the 14.6-month median overall survival time reported by Stupp *et al.*[5] SEER's inclusion of data from GBM patients who chose not to undergo treatment – for whom the median survival is reported between 1 and 3 months[6,7] – likely contributed to this statistical discrepancy. However, GBM is known for its heterogeneity,[8-10] and these data aptly highlight the persistent challenge of epidemiologically characterizing a clinical cohort that includes many poorly understood subcategories with readily observable survival differences.

The combination of low incidence and poor survival rate indicates that GBM is not a prevalent disease. At present, it is estimated that there are slightly <19,000 Americans with a documented GBM diagnosis (0.006% of the 2022 US population), and the NIH correspondingly categorizes GBM as a rare disease.[11]

The overall incidence of GBM varies greatly by age, sex, and ethnicity. GBM is known to develop at all ages, but the incidence increases with age and peaks between 75 and 79 years old for the individual sexes (Figure 1A). The average age of GBM diagnosis is 65 years old.[2] Males are 60% more likely to be diagnosed with GBM overall (incidence rate ratio = 1.6) (Figure 1B),[2] but females account for the majority of high-grade gliomas that evolve from lower-grade gliomas (previously categorized as secondary GBM).[12] GBM is more common in individuals of European ancestry,[13] and its incidence in Whites is 98% higher than in Blacks and 144% higher than in Asian/Pacific Islanders (Figure 1C).[2] Socioeconomic status (SES) is also significantly correlated with GBM incidence, and the highest SES is associated with a 45% higher GBM incidence rate than the lowest SES after controlling for self-reported race.[14]

2. GBM risk factors

GBM risk factors are poorly understood. Moderate-to-high dose ionizing radiation to the head – particularly if administered in childhood – is the only environmental risk factor known to be unequivocally associated with the development of primary CNS tumors.[15] Radiation-exposed children are more likely to develop a meningioma than a malignant glioma,[15] and this risk factor accounts for very few cases of either pathology. Other extensively studied environmental risk factors include medications, hormone exposure, diet, body habitus and body mass index, smoking, birth weight, cell phone usage, and electromagnetic field exposure.[16] None of these factors have been conclusively associated with GBM development.[16]

Heritable genetic risk factors are estimated to represent approximately 25% of overall GBM risk, but around 70% of the genetic variance underlying that risk contribution remains to be identified.[17] At present, only 5% of GBMs are observably linked with familial disease,[18] and only 1 – 4% are associated with inherited genetic disorders known to increase glioma risk.[16] Most of these disorders are associated with well-characterized loss-of-function mutations in tumor suppressor genes.[16]

3. Clinical classification of CNS tumors

The aggression, extent, and spread of systemic cancers are commonly assessed and described using the American Joint Committee on Cancer Tumor, node, metastasis (TNM) staging system.[19] In this standardized clinical model, the tumor (T) component describes the size, location, and local invasive capacity of the primary tumor.[19] The node (N) component defines the degree to which cancer infiltrates the lymph nodes that surround the primary tumor.[19] The metastasis (M) component assesses the spread of primary cancer to distant organs or tissues.[19]

The unique environment of the CNS and the correspondingly unique behavior of primary CNS tumors make the TNM staging model poorly suited to describe the behavior of GBM and other CNS cancers. For example, primary CNS tumors can be locally invasive and aggressive,

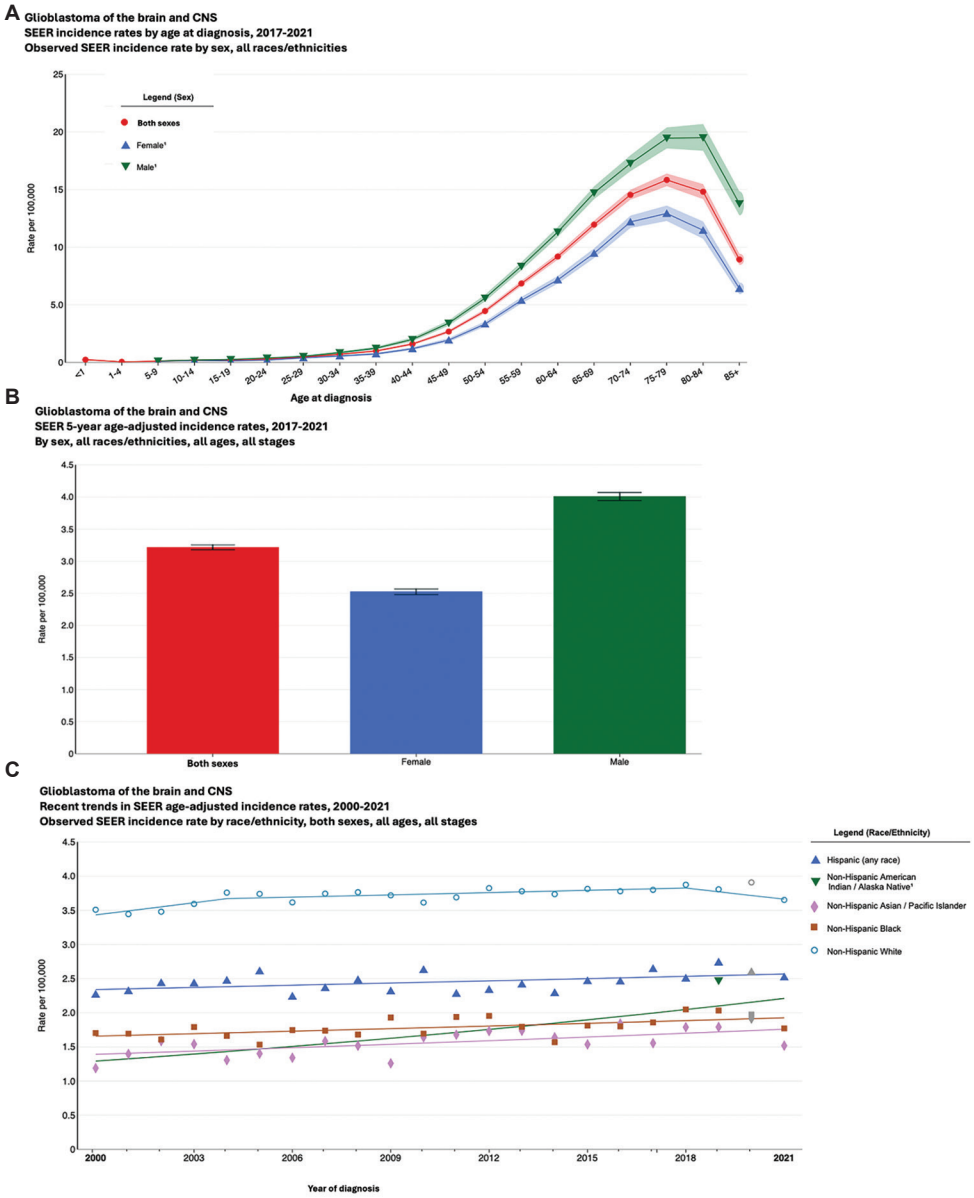


Figure 1. Overview of current GBM SEER incidence rates. (A) GBM SEER incidence rates (2017 – 2021) by age at diagnosis. (B) GBM SEER 5-year age-adjusted incidence rates (2017 – 2021) by sex. (C) GBM trends in SEER age-adjusted incidence rates (2000 – 2021) by race. Graphs generated with SEER*Explorer (Surveillance Research Program) on January 2, 2025.
Abbreviations: CNS: Central nervous system; GBM: Glioblastoma; SEER: Surveillance, Epidemiology, and End Results Program.

but they do not commonly spread into adjacent tissue types, such as the meninges or bone.[20] The CNS lymphatic system also lacks nodes and differs radically from the lymphatic organization found in systemic bodily tissues.[21] It is also extraordinarily rare for primary CNS tumors to metastasize to distant organs or tissues.[22] Collectively, these realities make TNM assessment an impractical and unhelpful tool for characterizing primary CNS cancer.

Primary CNS malignancies are assessed using the World Health Organization (WHO) Classification of Tumors of the CNS.[23] The WHO system uses a standardized grading scale (1 – 4) to characterize the behavior of these tumors from slow-growing and benign (Grade 1) to highly proliferative and aggressively malignant (Grade 4) (Table 1). GBM is classified as a WHO Grade 4 tumor.

4. GBM diagnosis

Final diagnosis and tumor grade assignment for CNS malignancies still rely on the physical evaluation of tumor tissue. Neurosurgical intervention, consisting

of a stereotactic tumor biopsy, is required to diagnose GBM definitively. Tissue is most commonly collected during standard-of-care tumor resection operations. The assignment of a GBM diagnosis was historically based on histopathological and gross tissue evaluation. Grossly, GBM is a poorly demarcated, vascularized, and friable tumor that commonly exhibits a grayish rim of rubbery tissue with a central area of yellow-tan necrosis (Figure 2A and B).[24,25] Tumors may demonstrate foci of hemorrhage and/or thrombosed vessels.[25]

Microscopically, GBM is composed of cells that resemble a spectrum of normal and reactive astrocytes that demonstrate nuclear atypia, cellular pleomorphism, mitotic activity, microvascular proliferation, and/or tissue necrosis (Figure 2C and D) (often, but not necessarily pseudopalisading necrosis).[25] Immunohistochemical evaluation for markers of astrocytic cells, such as glial fibrillary acidic protein (GFAP), or cellular proliferation, such as Ki-67, may help distinguish gliomas from other primary CNS malignancies.[24]

Molecular pathology was first incorporated into the diagnostic and grading algorithm for diffuse glioma in 2016 when the WHO released the 4th edition of CNS tumor classification guidelines.[27] In 2021, the WHO released the 5th edition of these guidelines, which strongly emphasizes integrated histomolecular diagnostics and redefines the diagnostic criteria for several categories of diffuse glioma-based molecular features.[23] Increased incorporation of diagnostic molecular markers is critically important for researchers and clinicians to better identify and stratify subgroups within the glioma and GBM diagnostic spectrum.[28] Distinct molecular subgroups of GBM have already been identified and reflect the rapidly increasing body of GBM multi-omic molecular knowledge.[29] A comprehensive summary of the histomolecular diagnostic algorithm is provided in Figure 3.

One noteworthy change featured in the 2021 WHO Guidelines is the diagnostic separation of WHO Grade 4 adult-type diffuse gliomas. Before 2021, the diagnoses "Glioblastoma, *IDH*-wild type, WHO grade 4" and "Astrocytoma, *IDH*-mutant, WHO grade 4" were both classified as GBM and referred to as primary GBM and secondary GBM, respectively. While these tumors are grossly and histologically indistinguishable, the new diagnostic algorithm reflects evidence that they are molecularly and clinically distinct malignancies.[31] They are distinguished by the mutational status of isocitrate dehydrogenases (*IDH1* and/or *IDH2*), and this is now indicated by the new diagnostic category names.[8] In gliomas, *IDH1/2* mutations cause both a loss of function and a gain of function in the IDH enzymes.[32] Distinctly,

Table 1. World Health Organization central nervous system tumor grading system

Grade	Description
Grade 1	• Slow-growing, benign
Grade 2	• Relatively slow growing • May be malignant or benign • Commonly recur as higher-grade tumors
Grade 3	• Rapid growing • Very likely to recur as higher-grade tumors • Malignant
Grade 4	• Highly proliferative • Rapid growing • Aggressively malignant

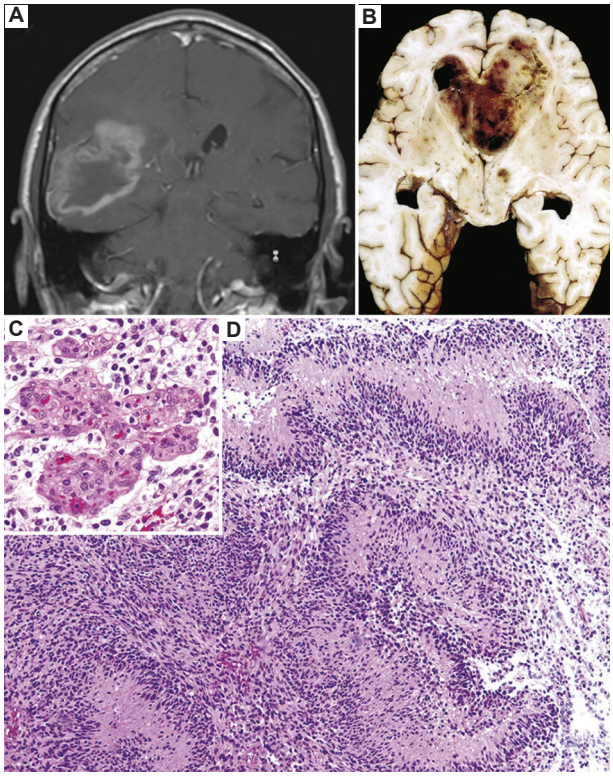


Figure 2. Glioblastoma diagnosis and histopathology. (A) Contrast T1-weighted coronal magnetic resonance image shows a large mass in the right temporal lobe with "ring" enhancement. (B) Glioblastoma appears as a necrotic, hemorrhagic, infiltrating mass. (C and D) Microscopic images of glioblastoma. Serpiginous foci of palisading necrosis (tumor nuclei lined up around the red anucleate zones of necrosis). (C) Microvascular proliferation. Image reproduced from Kumar *et al.*[26]

"Astrocytoma, *IDH*-mutant, WHO grade 4" typically (d) evolves from a pre-existing grade 2 or grade 3 astrocytoma and represents a progression of prior disease.

"Astrocytoma, *IDH*-mutant, WHO grade 4" is also associated with different clinical demographics and

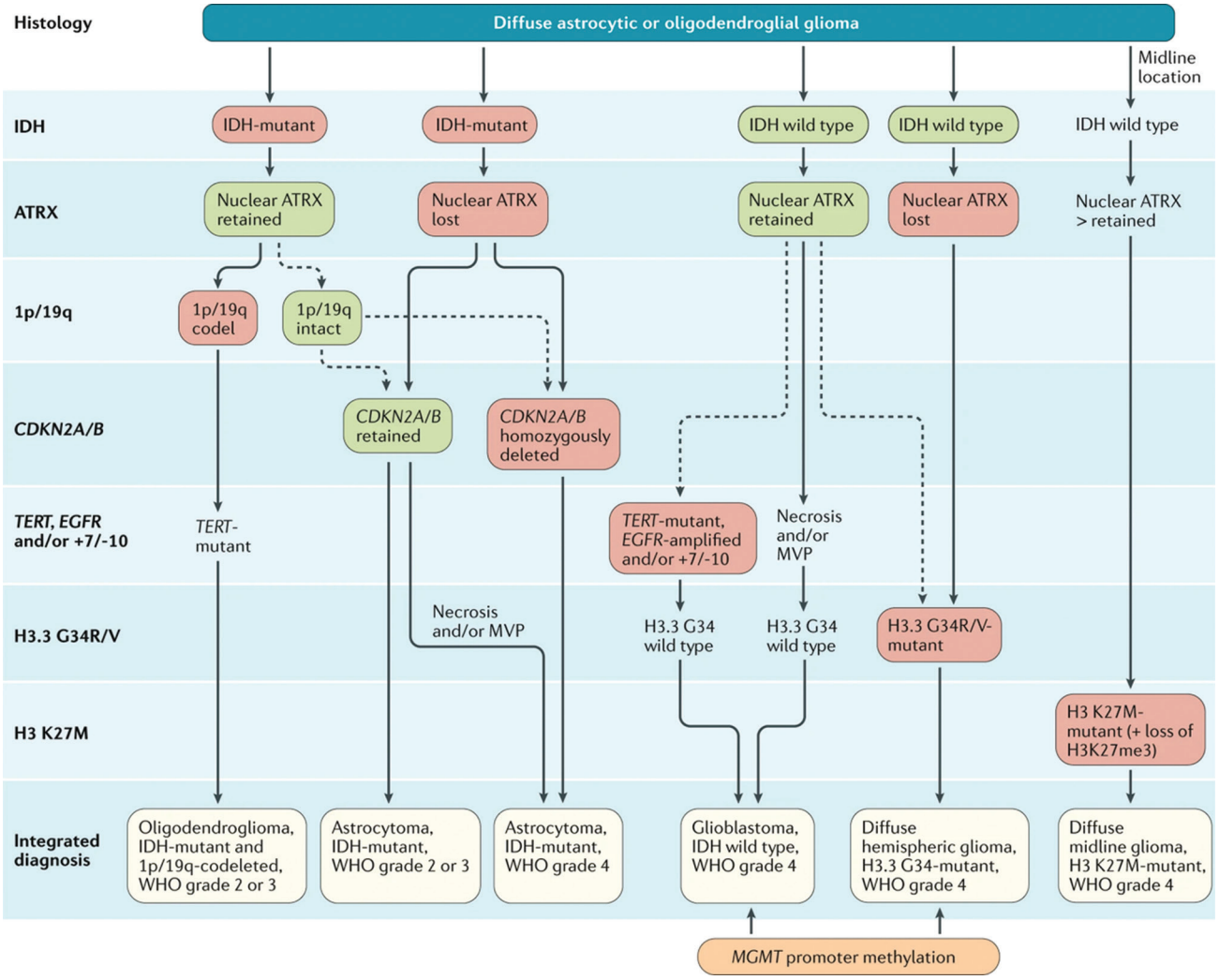


Figure 3. Molecular diagnostics of diffuse glioma in 2021. The molecular diagnostic algorithm was originally published by the European Association of Neuro-Oncology (EANO) and was subsequently adopted in the 2021 WHO central nervous system tumor guidelines. Reprinted from Weller *et al.*[30]
Abbreviations: ATRX: Alpha-thalassemia mental retardation X-linked; EGFR: Epidermal growth factor receptor; IDH: Isocitrate dehydrogenase; MGMT: O6-methylguanine-DNA methyltransferase; MVP: Microvascular proliferation; TERT: Telomerase reverse transcriptase; WHO: World Health Organization.

treatment responses. These tumors are more commonly found in females, generally affect younger adults, and are more responsive to standard-of-care chemotherapy treatment.[33] *IDH*-mutant patients also live significantly longer than *IDH*-wild type GBM patients.[8,12] Because *IDH*-wild type GBMs previously accounted for the great majority of cases in the GBM diagnostic category, the overall disease demographics were significantly skewed to reflect the primary GBM cohort. The 2021 change is intended to enable more precise epidemiological characterization of each tumor cohort, inform more accurate clinical prognostics, and facilitate efforts to develop specific therapeutic targets for each disease.

5. The clinical disease course of GBM

5.1. Clinical presentation

Adult GBMs most commonly arise above the tentorium cerebelli in the subcortical white matter of the cerebrum (frontal, temporal, parietal, and occipital lobe).[25] GBMs arising below the tentorium (cerebellum, brainstem, or spinal cord) are rare, molecularly distinct, and most prevalent in children. Initial presentation occurs with focal neurological deficits that are significantly dictated by the location in which the tumor arises. Tumors tend to be smaller upon discovery if the affected brain region causes obvious symptoms, such as vision loss, language

disruption, weakness, numbness, or pain.[34] Tumors arising in brain regions associated with more subtle symptoms, such as memory disruption, executive dysfunction, mood disturbances, or fatigue, are more frequently larger upon discovery.[34] Approximately 24% of GBM patients will present with seizures.[35] Symptoms associated with increased intracranial pressure (ICP), such as headache, nausea, sleepiness, decreased alertness, atypical pupillary response to light, and confusion, are common.[34]

Although GBM is frequently first detected with computed tomography scans in the emergent care setting, it is best visualized using magnetic resonance imaging (MRI). On MRI, GBM consistently appears as an irregularly shaped ring-enhancing mass associated with substantial peritumoral edema (Figure 2A).[36]

5.2. Standard-of-care treatment for GBM

Newly diagnosed GBMs are treated with maximally safe surgical resection, ionizing radiation, and temozolomide (TMZ) chemotherapy.[5] Among these three interventions, surgery provides the greatest survival benefit to GBM patients and resection is usually performed as soon as clinically feasible.[37] The extent of resection and residual tumor volume are both directly correlated to overall survival time.[38] The primary goal of neurosurgical GBM care is to balance aggressive tumor resection with the maintenance of patient function. Subtotal resection of GBM puts patients at considerable risk for potentially fatal intratumoral hemorrhage and cerebral edema (wounded glioma syndrome).[39] GBM resection should be pursued only when there is reasonable confidence that most of the mass can be removed. An estimated 17% of GBMs are unresectable due to their location, extent of spread, or the existence of comorbid clinical features at the time of presentation (Karnosky performance score <70).[40,41]

Once surgical wounds are healed, GBM patients complete the Stupp protocol chemoradiation. Targeted external beam radiation is typically delivered in daily 2 Gy fractions, 5 days a week, for 6 weeks (total 60 Gy) in combination with daily TMZ (75 mg/m^2).[5] Cycles of maintenance TMZ (150 – 200 mg/m^2) consisting of 5 consecutive days of treatment per 28-day cycle are then continued for approximately 6 – 12 cycles.[5] TMZ is an oral alkylating agent that creates DNA adducts at the O^6-guanine to form O^6-methylguanine.[42] O^6-methylguanine-DNA-methyltransferase (MGMT) is a DNA-damage repair response enzyme that repairs the TMZ-induced DNA adduct by removing the methyl in a degenerative reaction that depletes the MGMT enzyme pool.[42] GBMs that possess a hypermethylated *MGMT* promoter region are unable to upregulate *MGMT* transcription to regenerate the pool of functional MGMT and are thus significantly more sensitive to the effects of TMZ.[33] In 2005, Stupp *et al.* published the results of a phase III randomized multicenter clinical trial demonstrating that TMZ + radiation therapy (RT) (Stupp protocol) extends overall survival in GBM by 2.5 months in comparison to RT alone.[5,43] However, stratifying the patients enrolled in the Stupp protocol trial demonstrated that the median overall survival benefit for GBM patients with a hypermethylated *MGMT* promoter was 6.4 months. In contrast, the benefit for patients without a hypermethylated *MGMT* promoter was less than a month.[33] The Stupp protocol remains the standard-of-care for all GBM patients, regardless of MGMT methylation status, because more effective treatment alternatives do not currently exist.

Some patients additionally elect to treat their GBM with tumor-treating field (TTF) technology. TTFs are anti-mitotic medical devices that use adhesive arrays of external transducers to produce alternating electric fields of low-intensity and intermediate-frequency.[44] These alternating electric fields disrupt cell polarization and microtubule dynamics.[44] TTF clinical trials showed that the use of TTFs in combination with Stupp protocol maintenance TMZ significantly extended both the median progression-free survival and median overall survival by 2.7 and 4.9 months, respectively.[45] However, these results required that patients use the device at least 18 h/day.[45] TTF uses currently requires patients to shave their heads to adhere to transducer arrays accurately. However, adhesive-related itching is a common complaint (approximately 42%).[46]

Standard clinical care beyond the Stupp protocol for newly diagnosed GBM consists of palliative management of tumor-associated symptoms, including seizure control, pain control, and depression treatment. Some patients also pursue experimental therapy through enrollment in clinical trials. However, most clinical trials limit enrollment to cases of recurrent GBM.

5.3. Patterns of recurrence

GBM is an aggressive malignancy that exhibits significant resistance to therapeutic intervention and will recur in nearly all cases despite aggressive treatment efforts. The median progression-free survival time in GBM is 6.9 months.[5] Approximately 90% of GBMs recur within 2 cm of the surgical resection bed margin,[47,48] and this is particularly interesting considering that this is the area most heavily targeted by RT. There is no evidence indicating that tumor location, initial presentation, or other clinical factors influence the progression pattern or that progression pattern is linked to differences in overall survival time.

There is currently no standard-of-care treatment protocol for recurrent GBM. Repeat surgical resection is sometimes appropriate, though patient quality of life must be considered alongside the potential survival benefits provided by a second operation. Patients commonly pursue treatment with bevacizumab – an anti-vascular endothelial growth factor monoclonal antibody – but it has not been shown to increase overall survival and may instead increase invasive infiltration of the brain.[49,50] Other commonly pursued treatment options at the time of recurrence include carmustine and experimental therapies through clinical trial enrollment.

5.4. Cause of death

The end-of-life phase of GBM is characterized by a significant progression of neurological deficits. The majority of GBM patients exhibit decreased consciousness, dysphagia, and fever within the last days of life.[51,52] Seizures, headaches, and incontinence are also common.[53,54] Terminal-phase GBM patients report significantly lower rates of bodily pain than other cancer patients,[51] but much higher rates of depression[55] and cognitive dysfunction.[56] Causes of death routinely reported in GBM patients are associated with increased ICP and diffuse tumor infiltration of the brain and brainstem and include brain herniation, seizure, tumor-related brain hemorrhage, and dyspnea or respiratory arrest.[57,58]

6. Roles of the cytoskeleton in GBM

The cytoskeleton is an organized and dynamic meshwork of protein filaments that reinforce cell membranes, provide cells with shape/structural integrity, and facilitate essential cellular functions. In eukaryotic cells, the cytoskeleton is composed of three classes of filamentous fibers: actin, intermediate filaments, and microtubules.

Actin is a ubiquitous and evolutionarily conserved protein that makes up approximately 15% of the total protein in human cells.[59] The actin cytoskeletal system is composed of monomeric or globular actin (G-actin) and filamentous actin (F-actin). F-actin forms through the polymerization of G-actin monomers into a polarized, double-stranded helical fiber.[60] Actin filaments are highly concentrated at the cell periphery and form organized arrays of bundles and networks that underlie and give structure to the plasma membrane.[61] Manipulation of the balance between F-actin polymerization/depolymerization underlies the generation of different types of membrane-deforming forces required for cellular motility and many other essential homeostatic processes, such as endocytosis and cytokinesis.

Microtubules are long polarized fibers.[62] Like all cytoskeletal filaments, microtubules are composed of arrays of monomeric protein building blocks that organize into higher-order filamentous polymers.[63] The fundamental protein unit of microtubules is tubulin. Microtubules also cycle through phases of gradual polymerization and rapid depolymerization at the plus end.[63] A large cohort of microtubule-binding proteins regulates microtubule dynamics through a diverse set of functions, such as nucleation, capping, trafficking, stabilizing, destabilizing, bundling, cross-linking, and integration with other cytoskeletal elements.[63]

Microtubules participate in a wide variety of cellular processes. They form portions of the mitotic spindle apparatus that separate sister chromosomes.[64] They are scaffolds for motor-driven cellular trafficking, and their ultrastructural organization within the cell plays a large role in determining the distribution of other intracellular organelles.[65] This makes microtubule organization extremely important for cells that require polarity to execute physiologic functions (i.e., neurons and brush border epithelia), as a loss of microtubule organization leads to a profound disruption in internal cellular organization.[65] Microtubules are important mediators of cellular motility, and this is especially true in neurons and astrocytes where lamellipodial extension is limited, and microtubules closely underlie the leading edge membranes of migrating cells and developing neuritis.[66]

The most genetically diverse class of cytoskeletal fibers is the intermediate filaments, which are a family of cytoskeletal fibers derived from 73 different genes.[67] Despite substantial genetic diversity, intermediate filaments are remarkably consistent in size.[68,69] Intermediate filaments exhibit a high degree of homology in their sequences and structures.[70]

Intermediate filament expression profile varies greatly by cell and tissue type. For example, keratins are highly expressed in epithelial cells, whereas mesenchymal cells more commonly express vimentin.[67] The intermediate filaments GFAP and Nestin are particularly relevant to GBM biology. GFAP is used as a glial cell marker in GBM diagnostics,[24] and Nestin is a well-established marker of CNS neural progenitor cells that are also detected in GBM stem-like cells (GBMSC).[71]

7. GBM invasion and the cytoskeleton

7.1. Invasion patterns in GBM

Macroscopically, GBM favors invasion along pre-existing CNS structures, such as the white matter tracts (i.e., corpus callosum) and blood vessels (i.e., perivascular space/Virchow Robin Space).[72] Migration within the subarachnoid/leptomeningeal space, which is continuous

with the perivascular space, occurs but is less common.[72] GBM does not invade across sulcal margins.[72]

Both white-matter tracts and blood vessel scaffolds are invaded by GBM cells.[72] Signaling pathways underlying white-matter tract invasion remain largely unknown,[73] while molecular drivers of perivascular GBM invasion are well-characterized.[74] Endothelial basement membranes are enriched with collagen and fibronectin,[75] providing valuable substrates for integrin engagement that facilitate coordinated movement.[76] Blood vessels offer a large supply of oxygen/nutrients that support malignant proliferation and attract invasive cells. In contrast, the parenchymal extracellular matrix (ECM) lacks stiffening molecules such as collagen and fibronectin and is instead composed of hygroscopic molecules such as proteoglycans, hyaluronans, tenascins, and link proteins.[77] These molecules retain water, creating a gelatinous ECM that densely fills the extracellular compartment and leaves a limited amount of unoccupied extracellular space available to accommodate invasive movement.[78] Intraparenchymal invasion requires GBM cells to erode their way through the existing tissue architecture.[79] Still, GBM extensively invades cerebral gray matter, using proteolytic-guided mesenchymal and collective invasion strategies.[80-82]

7.2. Molecular drivers of GBM invasion: Rho proteins and their effectors

Dynamic remodeling of both the actin and microtubule cytoskeletal systems is required for microstructural and macrostructural patterns of GBM invasion.[83] Actin polymerization drives leading-edge protrusions, and genetic or pharmaceutical targeting of actin nucleator proteins, such as formins or Arp2/3, have been shown to reduce GBM invasion.[84-89] Microtubules are also required to support elongating cell protrusions and to transport ECM-degrading enzymes to leading-edge structures.[90,91] Microtubule targeting agents also reduce GBM invasion.[91,92]

7.2.1. Rho guanosine triphosphatases (GTPases) in GBM invasion

In GBM, differential activation of molecular switches called Rho GTPases coordinates cytoskeletal remodeling required for invasive motility[93] and dictates specific invasion programs.[94] Rho GTPases cell division control protein 42 homolog (Cdc42) and Ras-related C3 botulinum toxin substrate 1 (Rac1) are upregulated in gliomas relative to normal brain tissue.[95] Cdc42 and Rac1 activation is associated with pseudopodial extension into the brain parenchyma and guidance of other tumor cells with lower Cdc42 and Rac1 activation toward the invasive front.[94] In U87 GBM spheroid invasion, activated Cdc42 increased migration and invasion, whereas Cdc42 depletion reduced invasion.[96] Rac1 inhibition suppressed GBM cell invasion.[97]

RhoA's role in the GBM invasion is less well-defined. RhoA expression decreases with increasing grade of glial malignancy.[98] RhoA and Rac1 are known to be functionally antagonistic, with Rac1 activation predominating in mesenchymal migration and RhoA mediating the contractility required for so-called amoeboid motility.[99] Some RhoA activity is required for early adhesion and trailing edge contraction in mesenchymal motility, and RhoA regulates the expression of transmembrane matrix metalloproteases (MMPs) that remodel the ECM for mesenchymal invasion.[100] *In vivo*, RhoA activation is predominantly associated with perivascular invasion.[94] Importantly, directed invasion in GBM can be triggered in response to ECM composition (stiffness, porosity, and topography),[101] autocrine or paracrine signaling factors (pleiotrophin, CXCL12, glutamate),[102-104] or ion flux (Ca^{2+}, Cl^-, K^+).[105-107]

7.2.2. Roles for GTPases effector proteins in GBM invasion: The formins

The formin family of proteins is an evolutionarily conserved group of Rho GTPase effector proteins that make essential contributions to the organization and maintenance of the actin and microtubule cytoskeletal systems.[108] Human cells express at least 15 different formin family proteins. The diaphanous-related formin subfamily, also referred to as mammalian diaphanous-related formins (mDias), is the most extensively characterized subgroup of formins and includes mDia1, mDia2, and mDia3 (Figure 4).

The mDia formins are well known for their roles in facilitating actin polymerization. The exposed formin homology (FH2) domains of active mDia formins homodimerize to form a ring structure that stabilizes G-actin trimers and catalyzes energetically unfavorable nucleation of actin filaments. mDia formins are among the few Rho GTPase effector proteins that directly influence both actin and microtubule dynamics.[110] In addition to their roles as mediators of actin nucleation, polymerization, and bundling, mDias directly bind to and stabilize microtubules.[111,112]

Temporal regulation of mDias is controlled by Rho GTPase activity, which is, in turn, controlled by external signaling cues. Thus, Rho GTPases link mDia activation with the environmentally directed cellular demand for cytoskeletal modification. Several different Rho GTPases activate mDia1, mDia2, and mDia3, with each isoform exhibiting different GTPase specificities that facilitate a widely diverse set of cellular processes and macrostructural modifications.

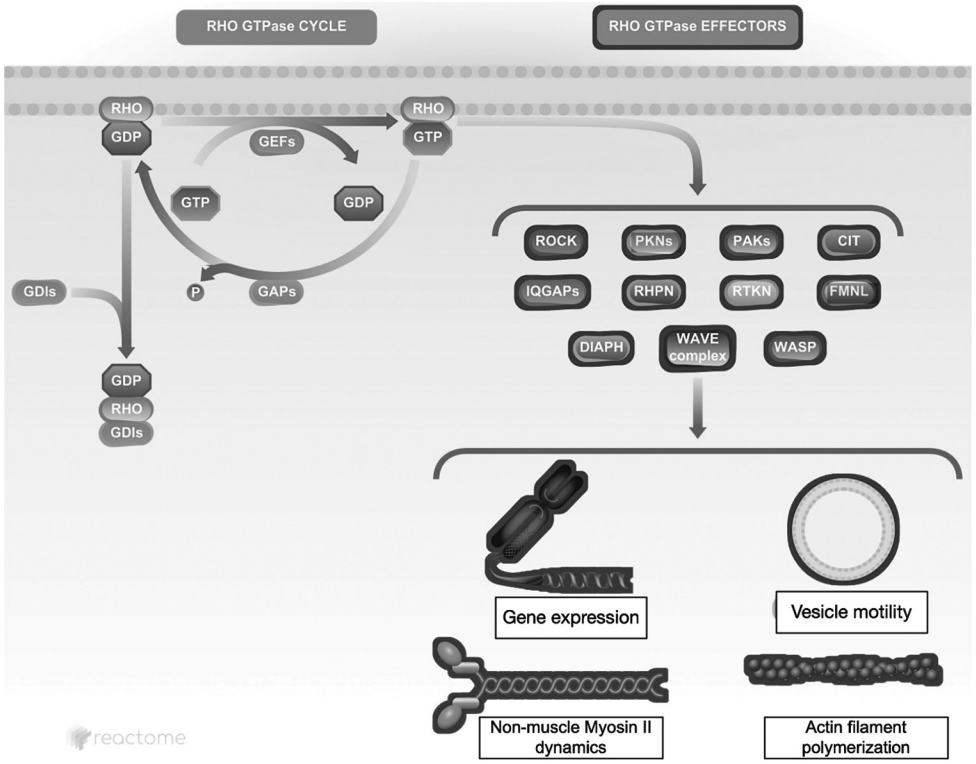


Figure 4. Overview of Rho guanosine triphosphatases (GTPases) and mammalian diaphanous (mDia) formins interplay. The Rho GTPases are molecular switches that influence gene expression, vesicle motility, non-muscle myosin II dynamics, and actin filament polymerization through association with effector proteins, including mDia proteins, which are encoded by the DIAPH genes. Reproduced from Reactome.[109]

The distinct morphology and complex physiology of CNS cells critically rely on the organization and adaptability of the cytoskeleton. Thus, mDia formins directly participate in multiple neurodevelopmental processes, and their mutations are associated with neurodevelopmental defects. Within the mDia subfamily, mDia1's contributions to neurodevelopment are well understood. mDia1 is found in the perinuclear region and co-localizes with centrosomes and mitotic spindles in cortical neural progenitor cells.[113] Multiple brain regions continue to express high levels of *DIAPH1* (the gene encoding mDia1) throughout development and adulthood.[114-116] mDia1 also supports stromal cell-derived factor-α chemoattractant-mediated axon formation in entorhinal cortical neurons[117] and cerebellar granule cells,[114] and is required for normal dendrite formation[118,119] and adherens junction formation in the ventricular zone.[115]

The mDia formins contribute significantly to GBM pathophysiology. In C6 rat glioma cells, mDia1 knockdown blocked directed migration, prevented focal adhesion turnover, and impaired the localization of Cdc42 and Rac1 at the leading edge of invasive cells.[85] Gliomas express higher levels of mDia1 relative to normal brain tissue.[86,120] In U87 glioma cells, mDia1 knockdown impaired tumor cell proliferation both *in vitro* and *in vivo* and increased tumor cell apoptosis.[86] Decreased mDia1 expression also reduced invasion and invadopodia formation[86] and downregulated the expression and activity of ECM-degrading MMPs (MMP2 and MMP9).[120] It was also demonstrated that mDia formins can be targeted with small-molecule compounds in GBM and that both broadly activating and inhibiting mDias decrease GBM invasion.[84] Agonism of mDia formins disrupts the structural viability of pro-invasive GBM structures called tumor microtubes (TMs),[91] and this phenotype was mirrored in mDia2 knockdown experiments.[87] mDia2 contributes to the maintenance of the GBM stem cell phenotype by critically participating in the Wiskott–Aldrich syndrome protein-driven stabilization of YAP/TAZ signaling.[121] Other studies have also shown that several subfamilies of formins may participate in GBM invasion.[87-89,122]

Many essential cellular functions require the activity of mDia formins to be finely tuned through both time and cellular space. Correspondingly, any disruption to mDia function or regulation threatens cellular homeostasis and multiple mDia-targeting strategies cause cytotoxicity that could be therapeutically exploited.

7.3. Therapy resistance and other clinical consequences of GBM invasion

Aggressive and diffuse invasion is a significant contributor to poor survival in GBM. Diffuse invasion creates tumors with poorly defined margins that are impossible to resect completely with surgery. Early drastic attempts to remove the entire affected brain hemisphere failed to prevent tumor recurrence on the contralateral side.[123] Thus, while GBM initially presents as a distinguishable mass, diffuse invasion fundamentally makes GBM a whole-brain disease.[124] GBM cells invading the blood vessels also strip the astrocytic end feet from the endothelial basement membrane and secrete enzymes that damage endothelial tight junctions and degrade the basal lamina.[82,125]

This invasive denuding and degrading of the endothelium triggers reactive gliosis and disrupts the neurovascular unit, thereby initiating a pathological cascade that includes the loss of blood-brain barrier (BBB) integrity, loss of activity-dependent blood flow regulation, serum leakage, and uncontrolled CNS access for ions, toxic and inflammatory molecules, and immune cells, impaired CNS uptake of glucose and oxygen, hypoxia, necrosis, and edema.[82,125,126]

In contrast, intraparenchymal invasion carries cells far beyond the margin of the radiation target and into areas of the brain with a robustly intact BBB, where they are largely inaccessible to immune cells and therapeutic drugs.[127] Some evidence additionally suggests that invasive astrocytes may limit cell cycle progression and could, therefore, be less sensitive to conventional therapies that generally target proliferating cells.[128-130] Proteolysis and glutamate-mediated intraparenchymal invasion also physically erode the normal neural architecture, thereby disrupting circuit control, triggering seizures, and ultimately leading to functional deterioration.[131,132]

In addition, many glioma therapies are known to exacerbate the invasive motility that drives tumor recurrence and neurological decline.[49,50,133,134] Most GBMs exhibit invasive behavior and significant therapy resistance at the time of tumor recurrence. Thus, invasive motility remains a primary obstacle to the successful treatment of GBM.[135]

8. GBM TMs

8.1. TM structure and morphology

TMs are invasive neurite-like protrusions that extend from the cell bodies of diffuse astrocytoma cells into the surrounding brain parenchyma.[136] TMs are structurally enriched with actin and microtubules, but they locally express myosin IIa, protein disulfide isomerase, β-catenin, β-parvin, GFAP, Nestin, connexin43 (Cx43), growth-associated-protein 43 (GAP-43), and tweety-homolog 1 (TTYH1).[136,137] Although they share many features with other protrusive cellular structures, such as invadopodia and tunneling nanotubes (which are also found in GBM),[138] TMs are morphologically distinguished by their remarkable length and capacity for long-term stability.[136] Bona fide TMs are at least 50 μm long and have an average cross-sectional area of approximately 1.5 μm^2.[136] TMs have been observed to exceed 500 μm *in vivo*[136] and commonly surpass 1000 μm in *in vitro* models (Figure 5). TMs also exhibit significant plasticity in their temporal stability. They may be stable for weeks to months or dynamically remodeled to drive invasion at the tumor-brain interface.[136]

8.2. TM networks

TMs frequently arborize and interconnect into a multicellular network. Cx43-mediated gap junctions are evident at TM cross points and enable the TM network to function as a syncytium.[136] TM networks bi-directionally propagate intercellular calcium waves (ICWs) similar to those observed in the neurodevelopmental migration of neural progenitor cells[136,139] (Figure 6). TM networks also exchange signaling molecules[140] and traffic organelles, including mitochondria.[136] TM length and quantity increase with increasing astrocytoma grade, but they are not regularly observed in 1p/19q co-deleted oligodendrogliomas,[136] which may partially explain the therapeutic response and survival difference between the astrocytoma and oligodendroglioma cohorts.

8.3. Molecular understanding of TMs

Molecular drivers of TM formation have thus far been predominantly identified through *in silico* comparison

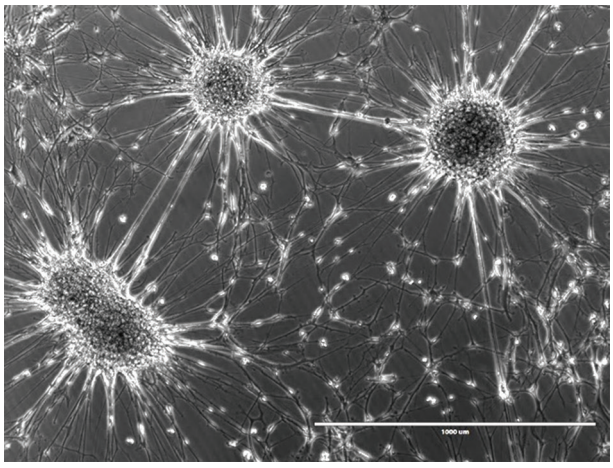


Figure 5. Tumor microtubes *in vitro*. Phase contrast image of three-dimensional networked patient-derived cell cultures demonstrates neurospheres and individually invasive cells highly connected through tumor microtubes. Scale bar = 1000 μm. Image created by the author(s).

of The Cancer Genome Atlas (*TCGA*) gene sets from 1p/19q co-deleted gliomas (i.e., oligodendrogliomas/TM-poor) and 1p/19q non-co-deleted gliomas (i.e., astrocytomas/TM-rich).[136,137] These efforts implicated pathways associated with normal neurodevelopmental processes. Pathways associated with stemness and underlying neurite outgrowth, such as signaling of the Rho family of GTPases, integrins, phospholipase C, and neurotrophin and tropomyosin/tyrosine receptor kinase signaling, are upregulated in TM-connected cells compared to unconnected controls.[71,136] Knockdown studies subsequently confirmed the roles of GAP-43,[136] TTYH1,[137] and thrombospondin 1 in TM formation.[141]

8.4. TMs in GBM invasion

At the invasive front, TMs act as dynamic probes that extend and retract in response to environmental cues. Established TMs act as scaffolds facilitating the movement of GBM nuclei into the brain parenchyma.[136] TMs display at least two phenotypes, wherein one, they are highly dynamic and associated with GBM cells exhibiting one to two minimally arborized TMs that drive invasive motility.[137] In the other phenotype, TMs are remarkably stable and associated with stationary GBM cells exhibiting four or more TMs that extensively arborize and connect to an extensive TM network.[137] Experimental knockdown of TTYH1 strongly inhibited the formation of invasive TMs, whereas networked TMs were largely unaffected.[137] Despite this, TTYH1 knockdown was associated with a significant survival advantage in a patient-derived xenograft (PDX) model of GBMSCs,[137] suggesting that targeting TM-associated invasion is a promising therapeutic strategy in GBM.[91,92]

8.5. TMs and the neuron-glioma synapse (NGS)

TMs receive synaptic input from normal neurons via a one-way, glutamatergic/alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)-mediated transmission. These NGSs generate spontaneous excitatory post-synaptic currents and slow inward currents that induce ICWs within TM networks that support the growth, invasion, and excitotoxic impact of GBM cells in the brain.[106,107] Treatment with the Food and Drug Administration-approved AMPAR antagonist perampanel reduced the invasion and growth of tumor cells and increased overall survival in PDX models of both adult and pediatric high-grade glioma.[106,143] Perampanel is well-tolerated, safe, and effective at reducing seizures in glioma-associated epilepsy.[144] In a 2009 trial, the pre-clinical AMPAR antagonist talampanel extended median overall survival from 14.6 to 20.3 months in patients with newly diagnosed GBM.[145] Tumor volume was also reduced in 8/10 glioma patients treated with six months of perampanel for glioma-associated epilepsy.[146] Targeting NGS activity with AMPAR antagonists is, therefore, a viable and promising therapeutic strategy in GBM. However, the use of perampanel has not yet been evaluated in clinical trials.

8.6. TMs in GBM therapy resistance

Tumor microtubule networks facilitate resistance to all three components of the GBM standard-of-care therapy (Figure 7). The cellular cohort that survives radiation and TMZ treatment is overwhelmingly comprised of TM-connected cells.[136,147] The TM network's ability to use ICWs to maintain calcium homeostasis within connected

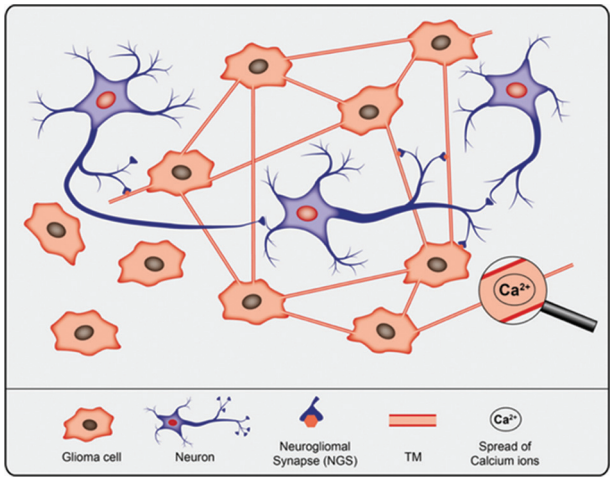


Figure 6. Schematic illustration of the gliomal network, the neuronal network, and their interconnectivity. Reproduced from Roehlecke *et al.*[142]

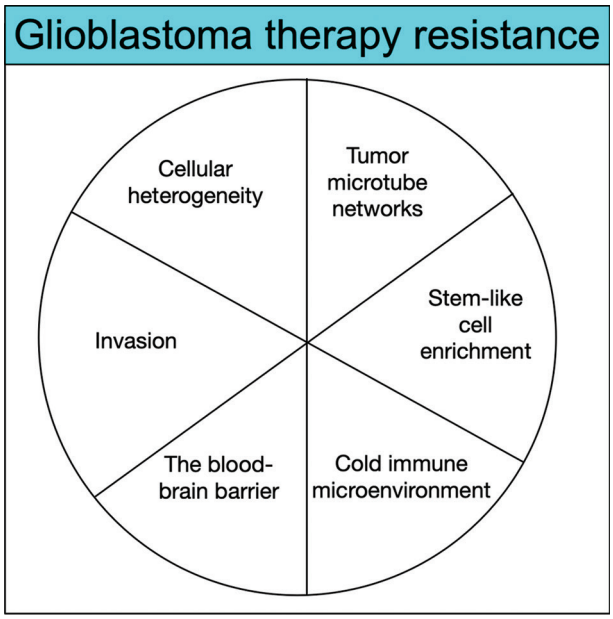


Figure 7. Multiple components of glioblastoma resistance

cells likely contributes to the observed survival advantage, as radiotherapy-induced cellular toxicity requires increases in intracellular calcium levels.[148] TM-connected cells extend new TMs toward the surgical resection lesions and into the resection bed within 3 days of tumor debulking surgery.[147] Following resection, the increase in tumor cell density within the lesion significantly outpaced the increase in tumor cell density in perilesional and distant brain tissue, and the invasive velocity was significantly directed toward the lesion.[147] GAP-43 deficient tumors did not robustly polymerize TMs toward the resection cavity and exhibited similar lesional density increases, suggesting that TMs may facilitate the coordinated repopulation of surgical lesions. This may explain why GBMs so commonly recur within 2 cm of the resection cavity margin despite aggressive regional radiation targeting.[147] Genetic targeting of TM networks in GBM PDX models decreased tumor burden, tumor invasion, and repopulation of surgical resection cavities while significantly increasing overall survival.[136,147] TMs serve as an excellent example of the ways in which the characterization of GBM pathophysiology is increasingly intersecting with developmental neuroscience.

9. Additional mechanisms of GBM therapy resistance

9.1. The BBB

The BBB is a physical and molecular feature of the CNS microvasculature that significantly prevents circulating ions, molecules, and cells from entering CNS tissue. Physically, CNS capillary beds are non-fenestrated, with endothelial cells that form continuous tight junctions.[149] This blocks paracellular solute diffusion and forces all agents to cross through endothelial cells to gain access to the CNS.[150] However, passage through CNS endothelial cells is also biochemically limited. CNS endothelial cells do not readily pinocytose molecules, and transcellular solute movement is, therefore, very difficult.[151] CNS endothelial cells additionally express luminal efflux pumps that actively export the majority of molecules that successfully pass through the endothelial membrane.[152] The barrier functions of CNS endothelial cells are further reinforced by (i) the inner vascular basement membrane and the outer parenchymal basement membrane (glial limitans),[151] (ii) pericytes, which are embedded in the vascular basement membrane and control the diameter of capillary vessels, regulate angiogenesis, deposit ECM, and control CNS immune cell trafficking,[153,154] and (iii) astrocytic end feet that link neuronal activity with blood vessel diameter and flow.[155] In the normal brain, these features collectively make up the neurovascular unit and block approximately 98% of blood-soluble molecules from accessing the CNS.[156]

The selectivity of the BBB significantly limits the number and diversity of therapeutic agents that can be used to treat CNS pathology, including GBM. While the GBM disease process notoriously disrupts the BBB, this breakdown is far from being homogenous or complete.[150] Imaging is regularly used in GBM care to identify areas of BBB breakdown, which enhances upon administration of a gadolinium contrast agent. However, many studies demonstrate that the GBM tumor burden extends far beyond this arbitrary imaging margin and into areas that remain sheltered by an intact BBB.[150] In addition, GBM causes BBB to be permeable by destroying the integrity of the neuro vasculature, not by enhancing controlled diffusion mechanisms.[82] The resultant indiscriminate leakage of fluid and ions through compromised vessels elevates the tissue oncotic pressure and causes edema that physically resists intratumoral drug penetration.[157] The BBB remains a significant hindrance to effective GBM treatment. Hence, developing strategies to circumvent this barrier is an area of concentrated research effort.

9.2. The immune-cold GBM microenvironment

The physical confines of the cranial vault significantly limit the amount of inflammation (and associated swelling) that the CNS can accommodate while still maintaining physiologic ICP. This mechanism underlies the evolution of existing anatomic and cellular mechanisms that tightly control immune access and function within the CNS.[158] Anatomically, the BBB significantly restricts circulating immune cells from entering the CNS tissue. While a limited number of peripheral immune cells are capable of entering the CNS under healthy conditions, baseline immune surveillance within the CNS is predominantly executed by resident microglia, which account for approximately 10% of CNS cells.[159] This reality notably limits not only inherent anti-GBM immune activity but also anti-GBM immunotherapeutics.[158]

CNS cells also use molecular mechanisms to limit intra-axial inflammation. The normal brain basally expresses and secretes high levels of the anti-inflammatory cytokines transforming growth factor-β and interleukin-10, which are further upregulated in the context of intracranial tumor development.[160] Microglia and infiltrating macrophages make up the majority of brain tumor-associated immune cells.[161] In GBM, tumor-associated macrophages predominantly exhibit an M1/pro-tumor phenotype that poorly activates the T-cell response.[162,163] Glioma cells also actively recruit regulatory T-cells and suppress T-cell activity by secreting indolamine 2,3-dioxygenase.[164,165] Thus, the population of tumor-infiltrating lymphocytes that do successfully infiltrate the tumor commonly exhibit an exhausted phenotype.[166]

Furthermore, intracranial tumors can cause T-cells to be sequestered within the bone marrow, and significant portions of GBM patients exhibit profound systemic baseline lymphopenia that can be further exacerbated by treatment.[167,168] Thus far, attempts to target GBM's intrinsic immunosuppressive mechanisms therapeutically have been largely unsuccessful.[158] Importantly, efforts to develop new immunotherapies in GBM must carefully balance increases in tumor-associated immune activity with the need to maintain physiologic ICP.[158]

9.3. Cellular heterogeneity

GBM also exhibits significant intratumoral heterogeneity that is attributable to genetic, epigenetic, developmental, and microenvironmental variation. Single-cell RNA sequencing analyses demonstrate that mixed populations of classical, mesenchymal, and pro-neural cells routinely co-exist within individual GBM tumors and that predominating TCGA subtype can vary significantly between different regions of a single tumor.[169] Each TCGA subtype is associated with a different tumor microenvironment immune signature.[170] Individual GBMs also harbor at least four different highly plastic neural-progenitor-like cellular states that interconvert in response to microenvironmental changes.[10] Remarkably, xenografts exclusively seeded from any single progenitor cell state consistently generate tumors comprised of all four signatures.[10]

The clinical targetability of identified GBM diversity remains unrealized, but it is well-recognized that clonal diversity and plasticity contribute significantly to GBM therapy resistance. Cancer therapies provide selective pressure that ultimately enhances the outgrowth of treatment-resistant clones.[171] In addition, both TMZ and RT are mutagenic treatments that can cause mutations that further drive GBM clonal diversity.[171] For example, hypermutation in GBM recurrence is only observed after TMZ treatment.[171,172] Recurrent tumors can exhibit both divergent evolution – in which the recurrent tumor is comprised of clones absent from the primary mass – and linear evolution, in which the predominant clones at recurrence existed in the original mass.[173] The predominating TCGA bulk expression subtype also commonly differs between primary and recurrent tumors,[174] with an observable predominance of the mesenchymal subtype at recurrence, suggesting that therapy induces genetic changes that converge on common pathways.

9.4. Stem-like cell enrichment

GBM stem-like cells are a slow-cycling population of GBM cells that represent a small percentage of the overall tumor mass but function as the cornerstone of tumorigenesis and therapeutic resistance in GBM.[175-178] GBMSCs are fundamentally defined by the capacity to self-renew and the ability to differentiate into a heterogeneous complement of progeny cells.[179] Thus, GBMSC clones occupy the multipotent apex of the GBM cellular hierarchy and exhibit substantial epigenetic and transcriptional plasticity that allows them to evade apoptosis.[180,181] Notably, GBMSCs can robustly activate DNA damage response pathways (178), autophagy,[182] and high expression of drug efflux transporters.[183]

It remains unclear whether GBMSCs arise from acquired mutations in normal neural stem cells (NSCs)[184] or develop through dedifferentiation of more terminally differentiated mutant glia,[170] but GBMSCs and NSCs share many biological features that underlie the clinical difficulties posed by GBMSCs. For example, GBMSCs reside in specific oncogenic niches that closely mirror neurogenic niches.[139] Interestingly, adult gliomas predominantly develop adjacent to or in direct contact with the lateral ventricular system, where the subventricular zone of adult neurogenesis also resides.[185] GBMSCs and NSCs express similar markers, such as CD133, Nestin, oligodendrocyte transcription factor 2 (OLIG2), and GFAP. They also rely on the activation of intersecting growth pathways such as Notch, epidermal growth factor, and fibroblast growth factor.[186] The counterbalancing pathways that restrain growth in NSCs are notably absent or dysregulated in GBMSCs.[186] Both GBMSCs and NSCs also form a three-dimensional neurosphere *in vitro* and give rise to fast-cycling and highly migratory progenitors.[139] GBMSCs also exhibit activity-dependent growth that includes significant downregulation of inhibitory γ-aminobutyric acid receptors and upregulation of glutamate signaling.[187,188] Understanding normal NSC function may highlight targetable dysfunction of similar pathways in GBMSCs.

10. Concluding remarks

The glioma research community has increasingly acknowledged that a lack of integration between cancer biology and neuroscience investigations has hindered progress in understanding glioma pathobiology. Correspondingly, the glioma research framework is steadily evolving toward a collective understanding that meaningful progress in treating diffuse glioma will require coordinated collaboration between cancer biologists and neuroscientists.[189] These emerging collaborative efforts aim to understand how the unique physiologic properties of the CNS fundamentally shape glioma development and aggression. Such studies now serve as the foundation for the burgeoning field of cancer neuroscience that has rapidly expanded beyond gliomas to additionally ignite diverse

investigations into the ways human cancers are shaped by the unique features of both central and peripheral nervous tissue.[190]

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Conflict of interest

The authors declare they have no competing interests.

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