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Cancer cell heterogeneity & plasticity in glioblastoma and brain tumors

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Abstract

Brain tumors remain one of the most difficult tumors to treat and, depending on the histology, have a poor prognosis. Of brain tumors, glioblastoma (GBM) is the most common malignant glioma and has a dismal prognosis, with only about 5% of patients alive five years after diagnosis. While advances in targeted therapies and immunotherapies are rapidly improving outcomes in a variety of other cancers, the standard of care for GBM has largely remained unaltered since 2005. There are many well-studied challenges that are either unique to brain tumors (i.e., blood-brain barrier and immunosuppressive environment) or amplified within GBM (i.e., tumor heterogeneity at the cellular and molecular levels, plasticity, and cancer stem cells) that make this disease particularly difficult to treat. While we touch on all these concepts, the focus of this review is to discuss the immense inter- and intra-tumoral heterogeneity and advances in our understanding of tumor cell plasticity and epigenetics in GBM. With each improvement in technology, our understanding of the complexity of tumoral heterogeneity and plasticity improves and we gain more clarity on the causes underlying previous therapeutic failures. However, these advances are unlocking new therapeutic opportunities that scientists and physicians are currently exploiting and have the potential for new breakthroughs.

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Conflict of Interest

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Introduction

A study published in 2017 by the National Cancer Institute found across-the-board improvements in 5-year survival for patients with cancer from the 1970s to 2013 (except for cervical and uterine cancer) [1]. However, even with this improvement in survival, patients with brain tumors still have only a 30% survival at 5 years after diagnosis, and this number is far worse for some tumors types, such as glioblastoma (GBM) in adults and diffuse intrinsic pontine glioma (DIPG) in children.

The brain and spinal cord are the most well-protected organs in the body and are evolutionarily designed to be protected from damage as much as possible throughout one's lifetime. In addition, each region of the brain has a specific function and can cause serious physical or mental disability when perturbed. These truths lead to various challenges that are not seen in other regions of the body when attempting to treat tumors within the central nervous system (CNS). An obvious example of this is that neurosurgeons must first maneuver through the skull and, when in the parenchyma, must resect the tumor with as narrow margins as possible to limit debilitating comorbidities.

This review will discuss the many ways by which brain tumors have evolved and prevented scientists and clinician from developing treatments that improve long-term survival. We will focus on the high levels of heterogeneity and plasticity in GBM and other brain tumors as well as discuss new therapeutic avenues that have been exposed due to the exponential growth in our understanding of these tumors.

Overview of brain tumors

Adult brain tumors can be generally classified into three categories: peripheral tumors that metastasize to the CNS, primary benign or low-grade neoplasms, and high-grade gliomas.

It is estimated that between 10-20% of patients with cancer will be diagnosed with a brain metastasis, and autopsy studies have reported a true incidence of 30-40% in all patients with cancer[2-5]. A total of 67-80% of brain metastases come from either lung, breast, or melanoma primary tumors. In all three of these tumors, the presence of brain metastases portends a poor survival. The median survival of patients with brain metastasis has been observed to be 13 months[6], and even in patients with the best prognostic factors, the most recent data demonstrate a median survival of only 33 months after the diagnosis of a brain metastasis[6]. While more recent studies have identified genetic differences between the primary tumor and brain metastases[7], the intratumoral heterogeneity of brain metastases has not been investigated in great detail.

Primary brain tumors are classified according to the World Health Organization (WHO) classification system. Meningioma is the most common primary brain tumor, with 90%

classified as grade 1 and benign. For most patients, complete surgical resection is curative, but a subset will have recurrent disease[8]. A recent study interrogated the genomic enhancer landscape of meningioma and identified novel disease subgroups[9]. Studies such as these are rapidly improving our understanding of meningiomas and our ability to determine which patients will have recurrent disease.

Gliomas are a group of brain tumors that were once thought to derive from glial cells. However, more recent evidence points to multiple cells of origin, including neural and glial progenitor cells within the brain [10-12]. Gliomas consist of astrocytomas, oligodendrogliomas and ependymomas, and until 2016, the WHO classification primarily relied on histologic features. However, the latest WHO classification system from 2016 integrates molecular data into the diagnostic strategy[13]. Grade I (rarely seen in adults) and II tumors are classified as low-grade gliomas and have a relatively favorable prognosis of around 10 years depending on the histological subtype. Most of these patients have a mutation to the *isocitrate dehydrogenase* (*IDH1)* gene, which leads to the generation of the oncometabolite 2-hydroxyglutarate. The natural course of some low-grade gliomas is to progress to high-grade gliomas, and therefore, maximal safe surgical resection of these tumors is the primary treatment. In some instances, these patients will also receive radiation or the chemotherapy temozolomide (TMZ). However, a recent retrospective study identified TMZ as a modifiable risk factor that contributes to malignant transformation along with wild-type *IDH* or mutated *IDH* with intact 1p/19q[14].

Pediatric brain tumors are now the number one cause of cancer-related death in children. These pediatric tumors include a wide range of benign and malignant tumors with various prognostic indications. These tumors include medulloblastoma, a primitive neuroectodermal tumor that accounts for approximately 20% of childhood brain tumors[15]. Medulloblastoma comprises a biologically heterogeneous group of embryonal tumors that have been subgrouped into the following four types: WNT, Sonic Hedgehog, Group 3 and Group 4[16-19]. As a result of its prevalence, the genetic profile of medulloblastoma has been well characterized, and the cell heterogeneity and plasticity of these tumors have been investigated. However, for the purposes of this review, we will focus on adult GBM as a paradigm for cellular heterogeneity and plasticity.

Finally, high-grade gliomas are made up of WHO grade III and IV tumors. Here, we will focus on WHO grade IV glioma, or GBM. Treatment for GBM includes maximal safe surgical resection, radiation, TMZ, and the recently approved tumor-treating fields (TTF), an antimitotic treatment that delivers alternating electric fields to the scalp[20,21]. Despite these therapies, the latest clinical trials have demonstrated a median survival of only 20.9 months, with a median survival outside clinical trials thought to be around 15 months[20]. There are a multitude of hypotheses for the poor prognosis of patients with GBM, including a failure of drugs to penetrate the blood-brain barrier[22], tumor cell invasion into the brain parenchyma, a hypoxic microenvironment[23], cancer stem cells (CSCs)[24] and tumor cell heterogeneity and plasticity[25].

Here, we will focus primarily on GBM and the heterogeneity observed within individual tumors and between tumors. We will investigate how advances in single-cell sequencing

and epigenetic profiling are providing new insights into the complexity of the disease and discuss how CSCs can interact with other cells in the tumor microenvironment and drive treatment resistance, tumor recurrence and tumor cell plasticity. Finally, we discuss the ways that immune cells, endothelial cells, astrocytes, and neurons support the tumor microenvironment and may provide novel therapeutic targets.

Molecular heterogeneity of glioblastoma

It has long been observed that GBM possesses extensive inter- and intra-tumoral heterogeneity. Early histological studies of GBM focused on the extent of necrosis, nuclear size, astrocytic differentiation, cell size, number of mitotic cells, distribution of cell density and vascularization[26]. Through this approach and the sampling of various locations within the tumor, scientists and pathologists were able to observe significant histological variations even within the same tumor. This phenomenon can be demonstrated ex vivo, as well. In one example of this, five subclones were isolated from a single tumor, and after orthotopic xenotransplantation, each subclone had a distinct histology and tumorgenicity[27].

One of the first technological advancements that furthered our understanding of GBM heterogeneity was the development of whole genome amplification (WGA) methods, which enable the identification of chromosomal imbalances. Nobusawa et al. utilized WGA on 14 different primary GBM samples from two to five locations within each tumor and identified some alterations that were common among all locations and others that were region specific[28]. In another study, single molecule molecular inversion probes targeting 33 cancer genes were used to assay point mutations and gene amplifications. The authors found evidence of regional mutational heterogeneity even within the same tumor[29]. Some commonly altered genes among GBM patients include epidermal growth factor receptor (*EGFR*), telomerase reverse transcriptase (*TERT*), platelet-derived growth factor receptor alpha (*PDGFRA*), cyclin-dependent kinase 4 (*CDK4*), murine double minute-2 (*MDM2), MDM4,* and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*)[30]. Other common mutations or deletions of tumor suppressors include phosphatase and tensin homolog (*PTEN*), ATRX chromatin remodeler (*ATRX*), *IDH1*, and *TP53*[31,32]. Amplification of the genes encoding receptor tyrosine kinases (RTKs), especially *EGFR* and *PDGFRA,* has been well documented in GBM. Snurderl et al. demonstrated that amplification of different RTKs were rarely found in the same region of the tumor, however, different RTKs were amplified in distinct subpopulations of cells[33]. Spatially distinct RTK amplifications were then verified by two additional studies, further emphasizing the intra-tumoral heterogeneity[34,35]. Studies such as these may help to explain the lack of efficacy thus far of EGFR targeted therapies in GBM[36-39]. This lack of efficacy is in contrast to the use of EGFR inhibitors in EGFR-mutant lung cancer with CNS metastases, where one study found a 91% CNS objective response rate in patients treated with the EGFR inhibitor osmertinib[40,41]. As a result of these studies demonstrating heterogeneity within individual tumors and the lack of efficacy of RTK inhibitor monotherapy, the field has largely moved away from the use of these drugs. However, there is ongoing research on the use of RTK inhibitors as a combination therapy as well as on developing immunotherapies that target cells with high RTK expression.

Advances in sequencing technology and the initiation of The Cancer Genome Atlas (TCGA) led to a major shift in our classification of GBM. Bioinformatics analysis of gene expression profiles of tumors identified four distinct molecular subtypes of GBM: proneural, mesenchymal, classical and neural (the neural subtype is excluded from most in follow-up classifications as the neural subtype was thought to be a contamination from normal neural tissue)[42,43]. While this characterization of bulk tumor RNA revolutionized the classification of GBM, heterogeneity and plasticity within these groups were quickly identified. In the earliest challenge to this classification scheme, Sottoriva et al. sampled spatially distinct tumor fragments from 11 patients with GBM and found that within the same patient, multiple molecular subtypes could be identified[44]. The authors also found heterogeneous driver aberrations and copy number alterations (CNA) within the same tumor. In addition, by comparing CNA data and measuring mitotic distances among cells of a tissue fragment, the investigators reconstructed the phylogeny and relationships among subclones. Through this method, the authors found that loss of cyclin-dependent kinase inhibitor 2A (*CDKN2A/B*) and amplification of *EGFR, CDK6* and *MET* occur early in tumor development, while alterations in *PDGFRA, PTEN* and *TP53* are later malignant events[44]. A later study utilizing 127 multi-sector or longitudinal specimens from 52 patients confirmed these findings[45]. These authors also conducted a chemical screen of patient-derived cells and found that targeting truncal events is more efficacious in reducing tumor burden[45]. Finally, viewing GBM from a temporal standpoint, Wang et al. found that 66% of patients undergo a subtype switch at recurrence, highlighting the plasticity of these expression states[46].

Gill et al. performed RNA-seq on biopsies taken from within the contrast-enhancing core and the non-enhancing margins of tumors. The contrast-enhancing regions resembled proneural, classical, or mesenchymal subtypes, whereas the non-enhancing margin resembled a neural subtype[47]. The authors also found that the expression pattern of the non-enhancing region was influenced by non-neoplastic brain cells, which will be discussed in greater detail in later sections. Finally, patterns of cell type alterations varied among the GBM subtypes, as the non-enhancing regions of proneural tumors were enriched with oligodendrocyte progenitor genes, whereas mesenchymal tumors were enriched for astrocytic and microglial genes[47]. The importance of molecular subtyping for laboratory studies cannot be overstated; however, almost ten years after its initial development, molecular subtyping has had almost no translational impact on the clinical setting.

Another attempt to further our understanding of the spatial heterogeneity of GBM was the development of the Ivy Glioblastoma Atlas Project (Ivy GAP)[48]. The Ivy GAP database includes a comprehensive pathology-molecular map of GBM that enables the unbiased study of molecular alterations of known anatomical features. The authors utilized laser microdissection and RNA-seq in 41 patients, dissecting out the leading edge, infiltrating tumor, cellular tumor, pseudopalisading cells around necrotic regions, and microvascular proliferation regions for each tumor. In general, samples from the same anatomical feature were more like each other than like other samples from the same tumor. However, even with this complex analysis, there was no mutation associated with a particular anatomic feature that predicted overall survival better than O6-methylguanine–DNA methyltransferase (*MGMT*) promotor methylation status of the bulk tumor, suggesting that this known

adversary remains a significant and predictive hurdle[48]. The creators of the Ivy GAP platform have deposited their data online (http://glioblastoma.alleninstitute.org/), and this data have already led to numerous additional publications[49-52].

The next technological leap that added to our understanding of intratumor heterogeneity was single-cell sequencing. In the first of these studies, Patel et al. performed single-cell RNA-seq on five different tumors. The authors confirmed previous finding by showing that GBM subtype classifiers are variably expressed across individual cells within a single tumor[53]. A more recent study combined single-cell RNA-seq, analysis of the TCGA, and experimental models to argue that malignant cells in GBM exist in four main cellular states that are reminiscent of canonical neurodevelopmental cell types. This includes astrocyte (AC)-like, oligodendrocyte progenitor cell (OPC)-like, neural progenitor cell (NPC)-like and mesenchymal (MES)-like states. Single-cell lineage tracing experiments found plasticity between these four states. The NPC-like, OPC-like, and AC-like transcriptional states correlated with copy number aberrations in specific loci: *PDGFRA, CDK4* and *EGFR,* respectively[54].

Another question that remains to be completely resolved is treatment-induced plasticity and temporal heterogeneity. In 2014, Johnson et al. sequenced the exomes of 23 initial low-grade *IDH* mutant gliomas and recurrent tumors from the same patient[55]. In 43% of cases, at least half of the mutations in the initial tumor were undetected at recurrence. Additionally, 6 of 10 patients treated with TMZ at recurrence were hypermutated and harbored driver mutations in retinoblastoma and Akt-mTOR pathway genes[55]. However, more recently, the Glass consortium assembled a database of initial and recurrence samples from 222 patients. In this study, 35 patients exhibited treatment-related hypermutation at recurrence, and 70% of the cohort had an increased mutational burden after recurrence. However, the authors found that driver genes detected at the initial disease were retained at recurrence and there was little evidence of recurrence-specific gene alterations. Only 16% of *IDH* wild-type patients exhibited treatment-related hypermutation, similar to the 17% estimate in Wang et al.[46]. Therefore, the authors concluded that the strongest selective pressures occur early in glioma development and that current therapies shape this evolution in a stochastic manner[56]. An important difference between the two studies is that in Johnson et al. all 23 patients were *IDH* mutant, while only 88/222 patients in Barthel et al. were *IDH* mutant. Additionally, within *IDH*-mutant tumors without 1p19q deletion, 47% of patients had treatment-associated hypermutation, suggesting that treatment-related mutations may be dependent on *IDH* mutation status[56].

Study after study in GBM consistently observe a high level of intratumoral heterogeneity. Early pathologists observed this heterogeneity through histology, and with each advance in genomic technology, the heterogeneity of GBM has been further clarified on multiple molecular levels (Figure 1). In the next section, we will discuss more recent studies on the epigenetics of GBM that are providing additional insights into the intra-tumoral heterogeneity and plasticity of these cells.

Epigenetic heterogeneity in glioblastoma

While advances in our understanding of the genetics and expression patterns of GBM have enabled vertical advancements in the field, these advances have yet to result in changes in the clinical management of GBM, and the five-year survival remains around 5%[57]. Additionally, the GLASS consortium finding that mutations at recurrence are largely random suggests that mutational evolution is not driving therapeutic resistance and plasticity in GBM. This has led many in the field to investigate potential epigenetic pathways that may be contributing to therapeutic resistance and recurrence.

The most established of these epigenetic changes is *MGMT* promotor methylation[58]. The *MGMT* gene encodes a DNA repair protein that removes alkyl groups from the O^6 position of guanine, an important site of DNA alkylation. Therefore, patients with a hypomethylated *MGMT* promotor have elevated levels of the MGMT protein and are more resistant to alkylating agents such as TMZ. *MGMT* methylation status remains one of the best predictors of survival in GBM.

In the first attempt at measuring global methylation patterns in GBM, researchers utilized TCGA data and observed a subset of patients with hypermethylation at a large number of loci. This study then classified a subset of patients as having a glioma-CpG island methylator phenotype (G-CIMP). These patients are generally younger and experience significantly improved outcomes[59]. To investigate intratumoral heterogeneity in methylation, Wenger et al. took three to four spatially separated biopsies from 12 GBM patients and performed genome-wide DNA methylation analysis[60]. As Sottoriva et al. found using transcriptional data, when GBM subtype was determined by methylation status, 5 of 12 patients had cells from multiple subtypes[44,60]. Ultimately, the authors concluded that GBM contains a significant variety of intratumoral DNA methylation patterns. In another study, methylation changes over time were investigated using formalin-fixed paraffin-embedded samples and bisulfate sequencing of 112 patient samples. DNA methylation was predictive of immune cell infiltration, extent of necrosis, and shape of tumor cell nuclei. Interestingly, DNA methylation erosion and subclonal heterogeneity were variable across patients but similar between primary and recurring tumors, which argues against a therapy-induced increase in epigenetic heterogeneity, as well as against a decrease in epigenetic heterogeneity due to strong selective sweeps driven by therapeutically resistant subclones[61].

Another epigenetic modification that has been identified as markedly upregulated in GBM is N6-methyladenine (N6-ma). N6-mA levels are dynamically regulated by ALKBH1, and depletion of ALKBH1 led to the transcriptional silencing of numerous oncogenic pathways by decreasing chromatin accessibility. In another study from this group, N6-methyladenosine (m6A) mRNA modifications were found to be upregulated in CSCs. The m6A reader YTHDF2 then stabilizes this modified mRNA, including the mRNA encoding *MYC* and *VEGFA*[62]. Another study investigated the super-enhancer landscape of 44 patient-derived GBM stem cells, 50 primary tumors and 10 neural stem cells. The authors found two highly distinct super-enhancer states that showed opposing patterns of H3K27ac. Group 2 was highly associated with mesenchymal features, while group 1 exhibited

proneural, classical and proliferative features[63]. Finally, extrachromosomal DNA can lead to oncogene amplification that drives cancer growth[64]. In GBM, this extrachromosomal DNA is unevenly inherited by offspring cells and impacts the oncogenic potential of the daughter cells[65]. Furthermore, *EGFR* amplification occurs in circular extrachromosomal DNA, and these circular pieces of DNA tend to harbor epigenetic enhancer regions that topologically interact with the *EGFR* locus to increase *EGFR* gene expression[66]. Together, these epigenetic observations bolster our understanding of intratumoral heterogeneity and provide a novel avenue for therapeutics targeting the epigenetic landscape[64].

As a whole, the field of GBM epigenetics is relatively young, and there is still much to learn about how epigenetic changes at all levels impact tumor cell behavior. The use of drugs targeting epigenetic modifications either by histone deacetylase (HDAC) inhibitors, bromodomain & extra-terminal domain (BET) inhibitors, or other mechanisms was recently reviewed[25]. However, to date, only slightly more than 20 clinical trials have been initiated in the epigenetics field, and this number pales in comparison to the number of trials using chemotherapies, anti-angiogenic agents and immunotherapies. Therefore, while there is still much to learn, targeting epigenetic changes in GBM is an exciting path moving forward.

Cancer stem cells

Any discussion of GBM heterogeneity and plasticity is not complete without a discussion of the CSC population. CSCs are a population of tumor cells that are defined by their functional ability to self-renew, initiate tumors, and undergo persistent proliferation[31,67]. There is no single marker of CSCs in GBM, however the glycoprotein CD133 was initially found to mark a CSC population and is widely used for sorting tumor cells today[68,69]. Furthermore, it was discovered that those with a higher proportion of $CD133^+$ cells present correlated with shorter survival[70,71]. CD133 has been shown to signal through AKT and Wnt to drive the CSC state[72]. However, it has been well documented that subgroups within the $CD133^-$ population can also exhibit CSC functional characteristics[73,74]. To date, various intracellular proteins (SOX2, MYC, and NESTIN) and cell surface markers (CD133, CD15, CD49f, L1CAM and CD44) have been identified as markers of CSCs in GBM[75-82]. The multiple, not necessarily overlapping, CSC markers begins to describe the vast amount of heterogeneity among the CSC population itself[74].

Drivers of the cancer stem cell state

While the CSC state has been widely studied and numerous factors have been shown to drive cells into a stem cell-like phenotype or to be necessary for CSC maintenance including microRNAs[83,84] and metabolism[85] here we will highlight two of these factors, hypoxia and treatment resistance. Hypoxia is a hallmark of the GBM microenvironment and leads to phenotypic changes[86]. Hypoxia promotes growth of CSCs and increases the expression of hypoxia-inducible factors (HIFs) and vascular endothelial growth factor (*VEGF*)[86-89]. In chronic hypoxic conditions, HIFs can upregulate stem cell transcription factors including KLF4, SOX2, and OCT4[88,90] that in turn affect downstream pathways. Furthermore, HIF1α can directly activate Notch and WNT signaling pathways[91]. Thus, the hypoxic

conditions present in GBM can induce a more stem cell-like phenotype that leads to further propagation of tumor growth.

Current standard of care for GBM follows what is known as the Stupp protocol. This includes a gross total surgical resection followed by radiotherapy and chemotherapy using the alkylating agent TMZ and, more recently, TTF[20,21,92,93]. However, both chemotherapy and radiotherapy can cause changes to CSC phenotypes, and CSCs have elevated levels of resistance to these therapies[94,95]. CSCs contribute to the radiation resistance through increased use of DNA repair mechanisms[94]. A recent study found that exposure to ionizing radiation leads to CSCs that were initially enriched for a $CD133^+$ proneural signature to transition into a $CD109^+$ mesenchymal subtype. This shift to CD109 positivity often leads to a mesenchymal recurrence[96].

As previously discussed, TMZ functions by adding alkyl groups to thymine and guanine, which causes DNA damage and initiates apoptosis. However, TMZ does not work on all CSC populations, leading to divergent CSC phenotypes[95]. Further studies have also shown that after exposure to TMZ, the CSC pool increases both *in vitro* and *in vivo*. Lineage-tracing analysis demonstrated that the CSC pool had shifted toward increased expression of stem markers such as CD133, SOX2, and OCT4[30,97]. These studies highlight how ionizing radiation and TMZ treatment can induce plasticity within the CSC population and drive post-treatment changes.

Models of glioblastoma heterogeneity

Three primary models have been formulated to explain the heterogeneity within GBM: the clonal evolution model, the CSC model, and the plasticity model. The clonal evolution model suggests that certain CSCs are selected over time based on factors such as their genetic, epigenetic, and tumor microenvironment (TME) interactions. The selection of these cellular traits drives progression and increases heterogeneity, as the selective pressures are temporally and spacially distinct[98-101]. Therefore, certain CSCs evolve to be more fit to survive in hypoxic environments, while others may grow in more nutrient-dense regions[99]. The second model is the CSC model, which suggests that a small subpopulation of cells is predisposed to drive tumor progression, invasiveness, and therapeutic resistance. Thus, the observed heterogeneity is a result of the differentiation of the CSC population, which gives rise to intermediate progenitor and terminally differentiated progeny[31,100,101]. Therefore, in this model, CSCs are the source of tumor initiation and heterogeneity. Lastly, the plasticity model builds upon the CSC model and states that CSCs can interconvert between stem cell and differentiated states. Unlike the CSC model, the plasticity model suggests that even upon differentiation, the differentiated cells can convert back into CSCs[49,54,86]. Two recently published studies in GBM provide strong evidence that the behavior of CSCs is best described by the plasticity model. Dirkse et al. demonstrated that the phenotypic heterogeneity observed in GBM arises from non-hierarchical, reversible state transitions that are driven by the microenvironment[86]. In another study, Neftel et al. utilized single-cell RNA-sequencing, bulk genetics and the TCGA and found that malignant cells exist in four main cellular states that each exhibit a high level of plasticity[54]. Together, these studies

and others are slowly shifting the focus of the field from identifying therapies that target CSCs to identifying therapies that target the plasticity of GBM.

Glioblastoma interaction with non-tumor cells

One fact that has been understood for many years is that the GBM microenvironment consists of more than merely tumor cells. Numerous other cell populations within the brain and the immune system contribute to the conditions that lead to tumor growth. These populations include endothelial cells, microglia, astrocytes, neurons, and immune cells. The failure of many tumor cell targeting therapies has led scientists and physicians to investigate the potential of targeting other cells within the tumor microenvironment (Figure 2).

Endothelial cells

It has long been understood that malignant cells require oxygen, nutrients, and the removal of waste to proliferate and survive. Brain tumors expand their vasculature through a process known as tumor angiogenesis, where endothelial cells proliferate or are recruited from the bone marrow. In addition, endothelial cells also play an essential role in what is known as the blood-brain barrier, which functions to closely monitor and control which chemicals and cells come into and out of the brain[102]. Tumor cells and endothelial cells can interact through three primary mechanisms. First, tumor cells secrete numerous growth factors including stromal-derived factor-1 (SDF-1), platelet-derived growth factor (PDGF), transforming growth factor (TGF-β), fibroblastic growth factor 2 (FGF2) and the most well studied, VEGF[103]. Secondly, the two cell types communicate via direct contact through gap junctions, in particular through connexin 43[104-106]. Finally, indirect interactions via intermediate cells such as pericytes, astrocytes and neurons also occur. The complex relationship between endothelial cells and tumor cells was recently reviewed in further detail[103].

Brain tumors are some of the most vascularized solid tumors found in humans, and the targeting of endothelial cells in GBM was a leading effort in clinical trials in the late 2000s[107]. In 2009, after a phase II trial that demonstrated a dramatic overall radiographic response rate, the FDA granted accelerated approval to bevacizumab, a monoclonal antibody against VEGF-A[108,109]. However, two phase III trials in patients with newly diagnosed GBM failed to demonstrate any improvement in overall survival[110,111]. Numerous follow-up studies that combined bevacizumab with various chemotherapies and targeted therapies have also failed to demonstrate any overall survival benefit[112-123]. Only one phase II study of a combination of bevacizumab and lomustine in patients with recurrent GBM demonstrated a survival benefit at 9 months; however, the subsequent phase III trial did not show any improvement in survival[124]. Additionally, a multitude of other studies has investigated additional anti-angiogenic agents, but none of these treatments have led to improved overall survival, and as a result, the field has slowly shifted away from anti-angiogenic treatments[125-135]. While the endothelial-tumor cell interaction remains an active area of research, clinical efforts to target angiogenesis in GBM have for the most part come to a halt. However, the use of bevacizumab as an alternative to steroids in the setting of immunotherapy has gained traction (NCT03452579).

Neurons

The first known observation of an interaction between GBM cells and neurons came in 1938 by the Belgian pathologist Dr. H. J. Scherer. He observed that glioma cells had a tendency to grow along and around normal neurons, and he described this observation as “precocious perineural growth”[136]. Since that time, determining whether invasive tumor cells were merely traveling along a path of least resistance or whether the two cell types are actively engaging in bidirectional signaling has been the goal of numerous neuroscientists and cancer biologists[137]. When considering the latest evidence for neuron-tumor cell relationships, it is worth remembering that there is ample evidence for a close, bidirectional relationship between glial cells and neurons in normal physiology.

The first molecule that was uncovered as a pro-tumorigenic neuronal molecule is the synaptic cell adhesion protein neuroligin-3 (NLGN3). NLGN3 is proteolytically cleaved at the cell surface, releasing the N-terminal ectodomain, which in turn leads to tumor cell proliferation[138]. In mice with *NLGN3* knocked out, intracranial injection of xenografts leads to failure of the tumor to progress. While this was true across numerous molecularly distinct gliomas, xenografts of breast cancer brain metastases were not impacted by *NLGN3* knockout[139].

The role of neurotransmitters in glioma progression has been of interest to clinicians and scientists since the observation that the use of tricyclic antidepressants reduces the odds of developing glioma in a dose- and time-dependent manner[140]. Because tricyclic antidepressants are classically thought to act via the inhibition of serotonin and norepinephrine re-uptake, the role of neurotransmitters in glioma development is of great interest. Glioma cells have been shown express dopamine, glutamate, GABA and serotonin receptors[141-145].

In 2019, three ground-breaking studies were published that further characterized the interaction between neurons and tumor cells within the brain and the importance of glutamate. Venkataramani et al. were able to demonstrate functional bona fide chemical synapses between presynaptic neurons and postsynaptic glioma tumor cells[146]. These synapses formed with typical synaptic ultrastructure and produced postsynaptic currents via glutamate receptors of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype. Furthermore, tumor growth and invasion were reduced by anesthesia or the AMPA receptor antagonist perampanel. Venkatesh et al. observed a similar finding mediated through AMPA receptor-dependent neuronglioma synapses. They also found that neuronal activity evokes non-synaptic activity-dependent potassium currents that are amplified by gap junction-mediated tumor interconnections[147]. Finally, Zeng et al. investigated breast-to-brain metastasis and identified activation of N-methyl-D-aspartate receptors (NMDARs) by glutamate ligands as essential. Pseudo-tripartite synapses between cancer cells and glutamatergic neurons were responsible for stimulating the NMDARs[148].

Uncovering the interactions between neurons and tumor cells has unlocked an entirely new avenue of therapeutic options. One method for targeting this interaction is the utilization of neurotransmitter-blocking drugs that may mimic the phenotype observed with

tricyclic antidepressants[140]. Another innovative approach that has been discussed is the implantation of electrodes into the resection cavity during craniotomy to disrupt neuron-tumor cell interactions. This would be an additional electromagnetic treatment option for GBM (in addition to TTF[20]).

Astrocytes

Tumor-associated astrocytes directly interact with GBM cells, leading to the formation of reactive astrocytes. These astrocytes facilitate tumor progression, proliferation and migration through multiple mechanisms[149]. The signaling between astrocytes and GBM tumor cells is bidirectional. Tumor cells have been shown to activate astrocytes through receptor activator of nuclear factor kappa-B ligand (RANKL), extracellular vesicles, and connexin 43 transmission of cGAMP, whereas astrocytes promote tumor growth through the secretion of IL-6, VEGF, TGF-β, growth/differentiation factor 15 (GDF-15), glutamine, tumor necrosis factor (TNF) and numerous other cytokines[149]. As evidenced by many of the anti-inflammatory molecules just mentioned, astrocytes play a significant role in driving an immunosuppressive microenvironment in GBM[150]. Astrocytes have also been shown to play a role in assisting tumor cell migration and infiltration. Connective tissue growth factor (CTGF) secreted by astrocytes mediates GBM cell infiltration and is a potential therapeutic target in GBM[151]. While less is known about astrocytes compared to other cell populations within the microenvironment, targeting astrocytes is an underexplored area and remains a viable option for GBM therapies due to their central role in regulating other cell populations.

Immune Cells

Two lines of thinking have long driven the dogma that the brain is an “immunologically privileged” organ: the existence of the blood-brain barrier and the lack of a conventional lymphatic system[152]. However, evidence is rapidly accumulating that emphasizes the importance of immune cells within the tumor microenvironment for tumor progression and the potential for immune-stimulating therapies to be the treatments of the future. However, it is worth remembering that the immune checkpoint (PD-1 and CTLA-4) inhibitors that have shown field-altering efficacy in tumors including melanoma[153] and lung cancer[154] have thus far failed to demonstrate any efficacy in GBM[155].

The different GBM subtypes outlined above have been shown to have heterogeneity within their immune populations. Utilizing the CIBERSORT method, which characterizes the cellular composition of a complex tissue from gene expression profiles[156], Wang et al. found that tumor-associated macrophages (TAMs), neutrophils and $CD4^+$ T cells are increased in tumors enriched in the mesenchymal signature, whereas an activated dendritic cell signature was found more in the “classical” tumors [51]. Another group utilized a tissue microarray to investigate the immune cell composition of 98 tumors. Microglia and TAMs were the most prevalent cells in all four subtypes, and $CD3^+$ cells made up only 1% of cells within the tumor, independent of the subtype[157]. Additionally, tumor-infiltrating lymphocytes (TILs) generally displayed an exhausted phenotype, and NK cells expressed reduced levels of activating receptors, highlighting the challenges of T cell- and NK cell-

targeted therapies in GBM[158,159]. Studies such as these highlight the low frequency of TILs, which may account for the lack of efficacy of immune checkpoint inhibitors thus far.

The exhausted phenotype of TILs in GBM is in part due to the highly immunosuppressive tumor microenvironment of GBM. The secretion of cytokines including TGF-β, IL-6, IL-10, macrophage migration inhibitory factor (MIF) and numerous others has been demonstrated to cause a local immunosuppressive phenotype[160-163]. In addition to soluble factors, direct cell-cell communication through PD-L1, tolerogenic HLA, the apoptosis-inducing receptor FAS, and changes in glycosylation can all cause immune suppression[164-167].

Another type of immune cell found in GBM is myeloid-derived suppressor cells (MDSCs). MDSCs are a population of immune cells that have a remarkable ability to suppress T cells through a variety of mechanisms and are subdivided into monocytic (mMDSC) and granulocytic (gMDSC) subsets. MIF secreted by CSCs within the tumor leads to increased MDSCs, and targeting this cell population with low-dose capecitabine has been demonstrated to be a potential therapeutic option in clinical trials[168,169]. Additionally, it was recently observed that there is a sex difference in MDSC populations in GBM, with gMDSCs elevated in the blood of females and mMDSCs enriched in the tumors of males[170].

Finally, microglia are the resident immune cells within the brain. Numerous studies have demonstrated that microglia within GBM predominately exhibit anti-inflammatory M2 polarization[171,172]. Microglia and tumor cells cross-talk though the secretion of EGF, colony-stimulating factor-1R (CSF-1R), TGF-β and IL-10[173]. Microglia can facilitate the invasion of GBM cells through the upregulation of matrix metallopeptidase 14[174]. GBM cells also upregulate CD47 to prevent phagocytosis by microglia, and work is being done to block CD47[175]. Finally, a recent study demonstrated that microglia function in a sex-specific manner, as female microglia that were deficient in junctional adhesion molecule-A (JAM-A) succumbed to GBM more rapidly than WT females, whereas the same phenotype was not observed in males[176]. This study combined with the MDSC data above highlights the heterogeneity between males and females and opens up the prospect of sex-specific treatments[177].

While the clinical data for therapies targeting the immune system thus far have shown little efficacy, our understanding of the immune compartment of the GBM tumor microenvironment has grown since the initiation of these trials. In addition to the studies mentioned above, the role of dendritic cells[178] and B cells is actively being researched[179]. Currently, there are at least 60 active immunotherapy clinical trials for GBM that are outlined in a recent review[180]. These trials rage from novel checkpoint inhibitors, vaccines targeting specific antigens or patient tumor lysates, chimeric antigen receptor (CAR)-T and CAR-NK cell therapies, oncolytic viruses, and macrophage-based immunotherapies. Studying the many ways immune cells interact with tumor cells is an active area of research and may be the foundation of future therapies.

Conclusion

Brain tumors are among the most heterogeneous tumors to have been characterized. From early histological studies that documented regions of necrosis to more recent single-cell RNA sequencing and methylation profiling, GBM is a tumor with resounding intra- and inter-tumoral heterogeneity. In addition, heterogeneity at the level of cell type, the sex and age of the patient, and plasticity over time all add to the complexity of the disease. Unfortunately, this lack of homogeneity or single driver mutations has left GBM patients without the targeted therapies that have revolutionized treatments for tumors such as breast cancer, melanoma, lung cancer and chronic myeloid leukemia.

With each new technological advancement, new research emerges, at a significant financial price, that verifies what pathologists have known for well over 50 years. This leaves physicians and scientists with the challenge of translating laboratory advancements into changes in clinical care. Our understanding of *MGMT* promotor methylation and *IDH* mutation status has revolutionized prognostic outlook for patients, and these characteristics are utilized in the design of clinical trials. In contrast, laboratory advancements such as tumor subtyping (classical, proneural and mesenchymal) and methylation profiles have for the most part remained an academic exercise. The goal moving forward must be the integration of these advancements into clinical trial designs that will match treatment approaches with the patients most likely to benefit.

With recent findings, three topics emerge as areas that require further research and are most likely to lead to future therapies. First, a move toward growing our understanding of the plasticity of CSCs and the epigenetics of GBM as a whole is shifting our understanding of the disease. To date, CSC targeted therapies have not panned out as viable treatment opportunities; however, focusing on the plasticity of GBM and the epigenetic changes that drive this plasticity may unlock unexpected therapeutic avenues. The second area is furthering our understanding of the communication between GBM cells and other cells within the tumor microenvironment. In particular, the interaction between neurons and tumor cells provides an opportunity for pharmacologic inhibitors, as well as surgical and electromagnetic techniques, that could revolutionize the treatment of brain tumors. Finally, it is impossible to ignore the success of immunotherapy in other cancers, and therefore, furthering our understanding of the immunosuppressive microenvironment in GBM has the potential to usher in the next generation of immune-stimulating therapies.

The efficacy of drugs such as osimertinib[40] (an EGFR inhibitor) and nivolumab/ipilimumab[181] in the setting of brain metastases has demonstrated that targeted therapies and immunotherapies have the potential to reach tumors within the brain. For the reasons outlined above, GBM heterogeneity adds additional layers of complexity that must be overcome for these and other drugs to improve survival in GBM patients. However, due to advances in our understanding of the genetics and epigenetics of tumor cells as well as of the cell populations that tumor cells rely on, the prospect of making the first new meaningful developments in clinical care for only the second time since 2005 remains high[20,21].

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Abbreviations.

CNS	Central nervous system
GBM	glioblastoma
DIPG	diffuse intrinsic pontine glioma
WHO	World Health Organization
TMZ	temozolomide
TTF	tumor-treating fields
CSCs	cancer stem cells
WGA	whole genome amplification
EGFR	epidermal growth factor receptor
IDH1	isocitrate dehydrogenase
MDM	murine double minute
TERT	telomerase reverse transcriptase
PDGFRA	platelet-derived growth factor receptor alpha
PTEN	phosphatase and tensin homolog
ATRX	ATRX chromatin remodeler
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
CDK4	cyclin-dependent kinase 4
TCGA	The Cancer Genome Atlas
CNA	copy number alteration
Ivy GAP	IVY Glioblastoma Atlas Project
NPC	neural-progenitor cell
OPC	oligodendrocyte progenitor cell

AC	astrocyte
G-CIMP	glioma-CpG island methylator phenotype
N6-ma	N6-methyladenine
m6A	N6-methyladenosine
ALKBH1	AlkB homolog 1, histone H2A dioxygenase
HIFs	hypoxia-inducible factors
VEGF	vascular endothelial growth factor
TME	tumor microenvironment
SDF-1	stromal derived factor-1
TGF-β	transforming growth factor-β
FGF2	fibroblastic growth factor 2
NLGN3	neuroligin-3
NMDARs	N-methyl-D-aspartate receptors
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
RANKL	receptor activator of nuclear factor kappa-B ligand
GDF-15	growth/differentiation factor 15
CTGF	connective tissue growth factor
TAMs	tumor-associated macrophages
TILs	tumor-infiltrating lymphocytes
MIF	macrophage migration inhibitory factor
MDSCs	myeloid-derived suppressor cells
mMDSCs	monocytic MDSCs
mMDSCs	granulocytic MDSCs
CSF-1R	colony-stimulating factor-1R
JAM-A	junctional adhesion molecule-A

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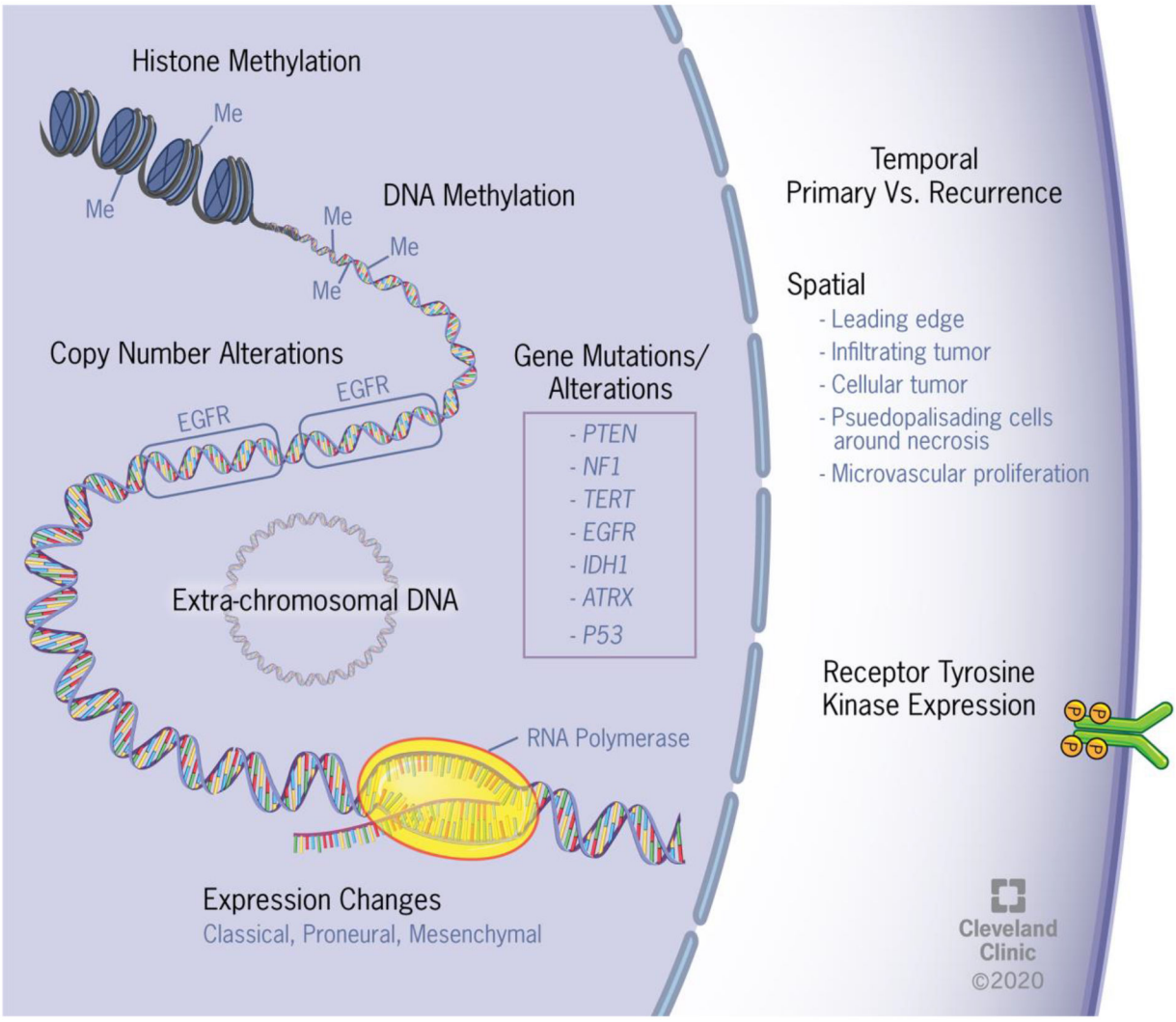

Figure 1. Glioblastoma exhibits inter- and intratumoral heterogeneity at multiple levels.

Schematic depicting inter- and intratumoral heterogeneity ranging from epigenetic changes to the enhancer landscape to amplification of receptor tyrosine kinases.

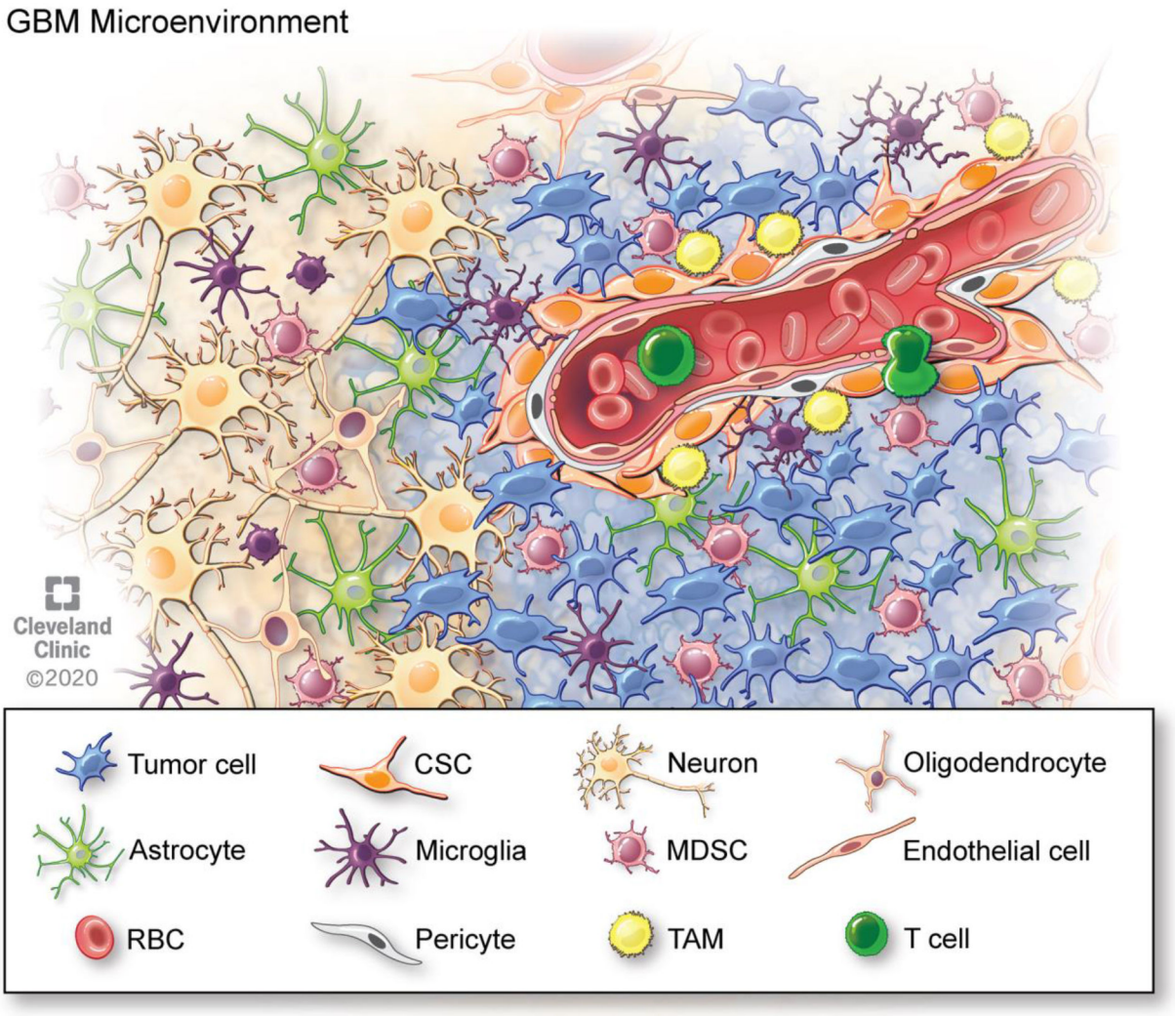

Figure 2. The tumor microenvironment of glioblastoma includes various other cells types in addition to tumor cells.

Schematic depicting the crosstalk between cell types in the tumor microenvironment that drive glioblastoma progression and recurrence.