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Molecular, histopathological, and genomic variants of glioblastoma

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1. ABSTRACT

Glioblastoma (GBM), the most common primary brain tumor, has a poor median prognosis despite modern surgical, chemotherapeutic, and radiation modalities, which have shown little clinical efficacy. Initially categorized by clinicopathological classification into de novo primary GBM and secondary GBM, which arises from lower-grade glioma, genomic studies have elucidated several distinct genotypes. In addition, distinct patterns of dysregulated epidermal growth factor receptor, platelet-derived growth factor receptor, p53, phosphatase and tensin homolog, cell cycle proteins, and isocitrate dehydrogenase 1, as well as loss of heterozygosity in multiple chromosomes complicate the GBM mutational landscape. Even with the many approaches in targeting these mutations, a long-standing clinical cure remains limited because of the tremendous heterogeneity and challenges in developing targeted treatments. Furthermore, this cancer utilizes ingenious approaches to subvert targeted agents and pathological variants of GBM demonstrate distinct molecular signatures, which may impact prognosis. This review discusses the collective understanding of GBM heterogeneity, including molecular, histopathological, and genomic features; why treatments have failed in the past; and how future clinical trials and therapies can be devised.

2. INTRODUCTION

Glioblastoma (GBM) is the most common primary brain tumor, designated a World Health Organization (WHO) grade IV astrocytoma, with approximate survival of 15 months (1,2). Historically, GBM has been categorized as a primary or secondary type, with primary GBM arising de novo without signs and symptoms of previous disease and secondary GBM ariseing from dedifferentiation of lower-grade gliomas (3). In addition, the pathological features of GBM vary widely. Recent molecular studies have revealed the underlying differences between established GBM variants, such as gliosarcoma and giant cell GBM (gcGBM), as well as emerging variants, such as fibrillary/epithelial glioblastoma, small cell astrocytoma (SCA), glioblastoma with primitive neuroectodermal features (GBM-PNET), gemistocytic astrocytoma (GA), granular cell astrocytoma (GCA), glioblastoma with oligodendroglioma component (GBMO), and pediatric glioblastoma. Recent studies have suggested several well-defined driver mutations in primary and secondary GBM; however, heterogeneous genetic expression plays a role in modulating individual tumors (4). Regional variation in chromosomal abnormalities, gene expression, and mutation can be found within a tumor mass and may account for mixed clinical responses to therapy (5,6). A comprehensive understanding of which GBM mutations indeed drive initiation and progression of this disease remains limited. Furthermore, genomic studies of GBM have added further complexity to traditional understanding by supporting four genetic subtypes of GBM, namely classical, mesenchymal, proneural, and neural types (7). The aim of this review is to discuss the variants of GBM molecular biology, pathology, and genomics as well as how this heterogeneity impacts therapeutic treatments.

An improved median overall survival has been shown for modern treatments in GBM. Primary GBM presents in patients with a mean age of 62 years, whereas secondary GBM shows a mean age of 45 years. Approximately 5% of all presenting GBM cases were secondary types in several studies (4). Traditionally, the median overall survival of patients with primary and secondary GBM was reported as 4.7 and 7.8 months, respectively, reflecting the different ages of presentation (4). More recent studies suggest upwards of 14 months of median overall survival can be achieved with current treatment modalities (1). The currently best available standard-of-care includes gross-total resection of the tumor as safely possible, followed by radiotherapy as well as concomitant adjuvant temozolomide (Temodar, Merck and Company, Whitehouse Station, NJ, USA), a blood–brain barrier–permeable alkylating agent, as well as fractionated whole brain radiation (8). Treatment of recurrent GBM with further tumor resection and the vascular endothelial growth factor (VEGF) targeted agent bevacizumab (Avastin, Genentech, San Francisco, CA, USA) have also been cited as improving survival (9,10); however, significant variability in recurrence rates, treatment, and follow-up occurs during the individualization of patient care for GBM. In addition, a remarkable number of clinical trials are currently investigating novel therapeutic agents in patients with GBM (clinicaltrials.gov).

3. THE MOLECULAR UNDERSTANDING OF GLIOBLASTOMA

Crucial to the modern understanding of glioblastoma pathogenesis are the many genetic alterations implicated in disease formation and progression (Figure 1). Although primary and secondary GBM as well as many GBM variants have been categorized by specific mutations, often these mutation patterns are overlapping. Several critical genes and their pathways have been extensively investigated in GBM and many other cancers owing to their high number of identified mutations (11). Alterations in critical genes are referred to as driver mutations in the thought that they promote tumorigenesis, whereas so-called passenger mutations, which are also mutated during the rapid turnover of tumor cells, do not induce tumor formation. Each driver mutation involved in tumor formation confers an estimated 0.4% increased growth advantage to individual cells, which can accumulate over a long period of time (12). In fact, cancers, such as GBM, that are more difficult to treat with molecularly targeted agents can often utilize multiple signaling pathways for growth, recurrence, and avoidance of therapeutic agents. Some studies have supported a cancer stem cell that shows features of self-renewal, resistance to modern therapy, and potential for differentiation and tumor recurrence, which may account for the difficulty in targeting GBM (13-15). It must also be noted that intertumoral heterogeneity can play an important part in identifying the defining mutations of GBM. Comparison of GBM tumors shows unique patterns of mutation, where not all cells in a tumor mass may contain the same mutational pattern. How these sets of mutations and their complex related signaling pathway alterations account for the clinical and pathological variation in GBM remains to be fully explained. Throughout this article, the names of individual genes will be italicized while their corresponding proteins will not (Table 1).

3.1. Epidermal growth factor receptor (EGFR)

The receptor tyrosine kinase epidermal growth factor receptor (EGFR) is a potent mitogenic signaling molecule implicated in a variety of signaling pathways. EGFR is a cell-surface bound receptor activated by EGF and the most commonly amplified gene in GBM, with mutation in approximately 50% of primary GBMs and <10% of secondary GBMs (16-18). Amplification of EGFR commonly results in the upregulation of downstream mitogenic signaling including the AKT and mitogen-activated protein kinase (MAPK) pathways. Approximately 10–60% of primary GBM with EGFR amplification contain the EGFRvIII mutation, which has a deletion of the regulator N-terminal domain (?6-273) resulting in constitutive upregulation of mitogenic signaling pathways as well as a loss of down-modulation by EGFR-targeted agents (19-22). EGFRvIII expression correlates with worse clinical prognosis, enhanced tumorigenicity, increased cell proliferation, and resistance to apoptosis (20,23,24); however, mutations other than EGFRvIII have also been identified in GBM, including loss of the C-terminal domain (C-958), intergenic deletions (?521-603), duplication-insertion mutations (664-1030 and 664-1014), and multiple mutations (16,21). The biological significance of these

Table 1. Key genes and potential targets involved in gliomagenesis

Gene	Abbreviation	Function	Mutation % in GBM	Targeted agent
Epidermal growth factor receptor	EGFR	Cell-surface bound receptor activated by EGF and involved in growth and proliferation signaling	50% primary, <10% secondary,EGFRvIII is a constitutively active mutation in 10-60% of primary GBM	Erlotinib
Platelet derived growth factor receptor	PDGFR	A membrane bound receptor that modulates a variety of growth and proliferation signaling pathways	40%	Imatinib
Tumor protein p53	p53	DNA-damage induced tumor suppressor involved in cell cycle arrest, DNA repair and apoptosis	50% overall, 28% primary, 65% secondary	PRIMA-1RITANutilin
Mouse double minute 2 homolog	MDM2, MDM4	E3 ubiquitin-protein ligase that targets and inhibits p53	50% primary	
Phosphatase and tensin homolog	PTEN	Tumor suppressor involved in converting Phosphatidylinositol (3,4,5)-triphosphate (PIP3) to Phosphatidylinositol 4,5-bisphosphate (PIP2) and reducing activity of the AKT/mTOR signaling pathway	25% primary, neg secondary	
Phosphatidylinositol 3-kinase, PI3K regulatory subunit, PI3K catalytic subunit alpha	PI3K, PI3KR1, PI3KCA	Phosphorylates PIP2 to PIP3 activating the AKT/mTOR signaling pathway	PIK3CA: 10%PI3KR1: 8%	BEZ235
Mammalian target of Rapamycin	mTOR	A major metabolic regulator in cells Forms the mTOR complex 1 (mTORC1) involved in protein translation, and mTORC2 involved in migration, cell cycle, and cell proliferation	Rapamycin/ sirolimus, BEZ235	
Retinoblastoma	RB1	Regulator of cell cycle progression, RB1 binds and inhibits E2F normally	14% primary, 43% secondary	
Cyclin-dependent kinase 4 and 6	CDK4, CDK6	Kinases involved in cell cycle progression through specific phases of the cell cycle	14% overall	
Cyclin-dependent kinase inhibitor 2A	(CDKN2A) p16, p14	Proteins that phosphorylated CDKs to regulate cell cycle progression p14 is generated from the alternative reading frame of the CDKN2A locus 30-60% overall, 3% primary, 19% secondary		
Isocitrate dehydrogenase 1	IDH1	Protein involved in Krebs cycle converting isocitrate to a-ketoglutarate, when mutated results in 2-hydroxyglutarate and stabilizes hypoxia inducible factor 1 (HIF-1)	3-11% overall	
Neurofibromin 1	NF1	Negative regulator of Ras signaling Mutation results in neurofibromatosis type 1 disease 15% overall		
O-6-methylguanine-DNA methyltransferase	MGMT	Repairs O6-methylguanine back to guanine in order to preserve genomic stability	45% overall	
Vascular endothelial growth factor	VEGF	Signaling protein that binds to tyrosine kinase receptor stimulating angiogenesis	Bevacizumab	
Promonin-1	CD133	Marker of some cancer-like stem cells of GBM		
Hypoxia inducible factor 1	HIF1	Protein involved in inducing expression in hypoxic cell states		

alternative EGFR mutations is thus far unknown. EGFRvIII expression histopathologically is only found in a minority of cells within the tumor, which are thought to act by an autocrine manner to drive proliferation (25). Interestingly, when tumor cells containing EGFRvIII are passaged, this alteration is carried through, suggesting it plays an important role in gliomagenesis (26). Unfortunately, despite the wide abundance of dysregulated EGFR

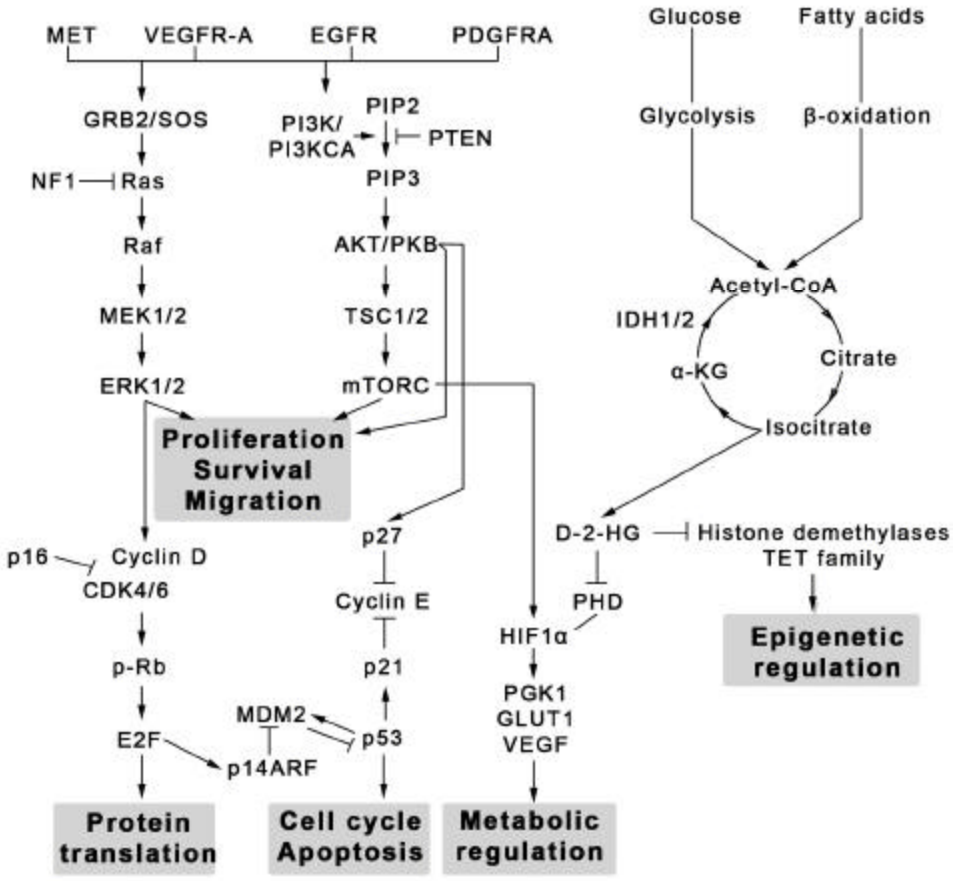

Figure 1. Altered signaling pathways in glioblastoma and potential targets Key signaling pathways in GBM are shown and their effects on proliferation, survival, migration, protein translation, cell cycle, apoptosis, metabolic regulation and epigenetic regulation In addition, some of the many-targeted therapies being investigated in GBM are presented a -KG: a-ketoglutarate, CDK4/6: cyclin-dependent kinase 4/6, D-2-HG: D-2-hydroxyglutarate, EGFR: epidermal growth factor receptor, ERK1/2: extracellular signal-regulated kinase 1/2, GLUT1: glucose transporter 1, GRB2: growth factor receptor-bound protein 2, HIF1a: hypoxia-inducible factor 1a, IDH1/2: isocitrate dehydrogenase 1/2, MEK1/2: mitogen-activated protein kinase kinase, mTORC: mammalian target of rapamycin complex, PDGFRA: platelet-derived growth factor receptor A, PGK1: phosphoglycerate kinase 1, PHD: prolyl hydroxylase domain-containing protein 2, PI3K: phosphoinositide 3-kinase, PIP2: phosphatidylinositol 4,5-bisphosphate, PIP3: phosphatidylinositol (3,4,5)-triphosphate, PKB: protein kinase B, PTEN: phosphatase and tensin homolog, Rb: retinoblastoma, TSC1/2: tuberous sclerosis proteins 1 and 2, VEGFR-A: vascular endothelial growth factor receptor A

signaling in GBM, the use of EGFR-targeted agents in clinical practice has failed thus far (27,28). Although various treatments target overexpressed EGFR in other tumors, such as erlotinib in non-small-cell lung cancer, targeting of EGFR in GBM has not been successful. Among the possible reasons for this failure, EGFR amplification in glioma occurs at much higher levels (>20 copies) and shows extracellular mutation (e.g., vIII) as opposed to mutations of intracellular domains often seen in other tumor types (29,30). Variation in EGFRvIII (31) and other growth factor receptors (32) can occur within a single GBM tumor. It is also not clear whether EGFRvIII drives the same downstream signaling pathways as EGFR activation in other tumor types (33). The understanding of EGFRvIII in gliomagenesis and targeting EGFR highlights many of the recurrent difficulties with targeted molecular therapy in GBM. Namely, variation in mutational patterns, tumor expression heterogeneity, and signaling pathway crosstalk generate a resilient disease.

3.2. Platelet-derived growth factor receptor (PDGFR)

Increased expression or mutation of platelet-derived growth factor receptor (PDGFR) is seen in a subset of up to 30% of GBM (34). Similar to EGFR, PDGFR is a membrane-bound receptor that modulates a variety of growth and proliferation signaling pathways. Findings from The Cancer Genome Atlas (TCGA) group suggested that PDGF signaling alterations were predominant in the proneural subtype of GBM (29,35). The PDGFRA?8,9 isoform is found in 40% of GBMs with PDGFRA amplification, resulting in a 243-base-pair deletion of exons 8 and 9, containing the extracellular domain, which results in constitutive protein activation (36). Furthermore, coexpression of PDGFR and its ligand indicates an autocrine or paracrine positive feedback loop upregulating

the PDGF network (32). Concomitant upregulation of PDGFRA and EGFR has been seen in GBM, with marked heterogeneity of expression within individual tumors and the necessity for simultaneous targeting of both receptors to reduce phosphoinositide 3-kinase (PI3K) signaling activity (37). In some GBMs with PDGFR gene arrangements, gene fusion between the kinase insert domain receptor of VEGF receptor II (VEGFRII) and PDGFR, as well as intragenic deletions, have been demonstrated (36). These novel gene fusions can further increase the complexity of signaling networks in GBM. Despite the importance of PDGFR in GBM, targeting this molecule has not proven successful in treatment.

Mutations in tumor suppressor p53 are the most common abnormality in GBM and play a complex role in promoting tumor formation. The p53 signaling pathway controls a major decision between cell cycle progression and apoptosis during DNA damage, along with its many other roles (38). Being the most common mutation in cancer generally, p53 is mutated in approximately 50% of GBMs overall, 28% of primary GBMs, and 65% of secondary GBMs, but mutations have also been reported at even higher rates (4). Inactivation of p53 protein can also occur by mutation of its antagonists MDM2 and MDM3, as well as deletion of p14ARF, which was shown to be present in 78% of GBMs in one study (29). Overexpression of MDM2 is observed in 50% of primary GBM tumors while gene amplification is seen in 8–10% of GBM, making MDM2 the second most amplified gene in GBM after EGFR (39,40). Furthermore, loss of p14, an MDM2 inhibitory protein, because of homozygous deletion or promoter methylation is found in approximately 60% of GBMs. Promoter methylation of the p14ARF-inducing gene silencing can be seen in 6% of primary GBM and 30% of secondary GBM (41). Despite the variety of mechanisms altering p53 signaling in GBM, therapeutic approaches to normalize p53 signaling or reverse p53 mutation remain limited (38). Indeed, the use of compounds like PRIMA-1 or nutlins have had limited success likely because of complex signaling pathways independent of p53.

The most common type of p53 mutation includes codon-specific mutations that occur within the DNA binding domain (38). These mutations generate a constitutively active form of p53, which can induce loss of wild-type p53 function, as well as gain-of-function and dominant-negative effects by upregulation of other pathways. Recent findings suggest that loss of transactivation and dominant-negative effects, where p53 tetramerizes with wild-type p53 and downregulates anti-tumorigenic wild-type p53 activity, are the predominant alterations in cancers (42). Thus, it is likely that therapies that will prove to be effective in targeting the p53 signaling pathway will need to disrupt mutant p53. Despite the numerous studies on p53 in GBM, the impact of mutations on prognosis remains unclear, with some studies supporting improved survival (43) and others the opposite (44-46). Different types of p53 mutation can also alter clinical progression. Dominant-negative mutations show a younger onset of sporadic GBM compared with recessive mutations or wild-type p53 (47). Distinctions in p53 mutation can be found within a GBM tumor mass and can select for certain cells during treatment, which can account for tumor recurrence (48-50)

3.4. Phosphatase and tensin homolog (PTEN)

Phosphatase and tensin homolog (PTEN) is the second most common mutated tumor suppressor seen in GBM after p53. PTEN is involved in regulating the PI3K/mammalian target of rapamycin (mTOR)/AKT signaling pathway (51,52). The mTOR signaling pathway plays a major role in cell metabolism, shifting a cell between protein synthesis (mTOR complex 1) and cell cycle, as well as in migration (mTOR complex 2) (53). Approximately 25% of primary GBMs and a negligible percentage of secondary GBMs show direct PTEN mutation (18). Mutation or epigenetic silencing of PTEN occurs in 40–50% of gliomas (52), and other mechanisms of PTEN alteration, such as mutation of Na(+)/H(+) exchanger regulatory factor 1 (NHERF1) and pleckstrin-homology domain leucine-rich repeat protein phosphatases 1 (PHLPP1) domains, are important in GBM (54). PTEN loss increases PI3K-mediated activation of the downstream AKT and MAPK signaling pathways, which influence cellular proliferation and migration, as well as resistance to chemotherapy (55-57). Alternatively, mutations in the catalytic p110a subunit of PI3K (PIK3CA) are also able to induce constitutive activation of the PI3K/AKT signaling pathway but are seen in only 5% of primary GBMs and 13% of secondary GBMs (58). PTEN is located on chromosome 10 (10q23-34), where loss of heterozygosity (LOH) of chromosome 10 can be found in 50–90% of primary GBMs and 50–70% of secondary GBMs but is rare in lower-grade gliomas (18,59). Mouse models of GBM (GFAP-GRE-p53lox/loxPTENlox/+) support the role of PTEN in combination with p53 in generating tumors that mimic human glioma (60). Furthermore, mutation of PTEN in a neurofibromin 1 (NF1) mutant background, $NF1^{-/-}p53^{-/-}$ model also supported the formation of more dedifferentiated glial tumors (61). Clinical trials targeting the PTEN/AKT/mTOR pathway with mTOR-specific agents, such as rapamycin/sirolimus, everolimus, and tacrolimus, have mostly failed (53,62). The impressive diversity of the PTEN signaling pathway and methods of circumventing mTOR blockade such as through upregulating the mTORC2 pathway or the MAPK pathway support mechanisms of cancer recurrence (51,57). Newer investigational approaches aim to target multiple proteins within the PTEN/AKT/mTOR signaling pathway to inhibit tumors (53). Dual inhibition of mTORC1/mTORC2 and mTOR/PI3K, as well as mTOR/AKT, is a promising approach but has yet to be thoroughly studied in clinical trials.

3.5. Cell cycle proteins

Dysregulation of cell cycle proteins is also common in GBM. These many molecules aid in the progression of the cell cycle through growth (G1), DNA synthesis (S), growth (G2), and cell synthesis (M). Disruption of the cell cycle in normal cells prevents proliferation in order to prevent cancer formation; however, these normal regulatory mechanisms are lost in GBM. Homozygous loss of retinoblastoma 1 (RB1), an important regulator of the cell cycle, has been reported in

approximately 25% of GBMs, while promoter methylation of RB1 has been seen in 14% of primary GBMs and 43% of secondary GBMs (63). RB1 downregulation has also been seen by amplification of its negative regulators cyclin-dependent kinase 4 (CDK4) and CDK6 (64,65). CDK4/cyclin D phosphorylation of RB1 normally results in release of E2F, allowing for G1/S transition and cell cycle progression. LOH of 13q, where RB1 is located, has been observed in 12% of primary GBMs and 38% of secondary GBMs (66). Additionally, homozygous deletion or promoter methylation of p16, regulating the G1/S checkpoint, has been seen in approximately 30% of GBMs, with promoter methylation seen in 3% of primary GBMs and 19% of secondary GBMs (41). p16 and p14 mutations have been shown to correlate with poor prognosis and treatment resistance (67-69). Unfortunately, these very important molecules have not been significantly investigated as targets in GBM, likely because of their importance in normal cells and difficulty in targeting.

3.6. Chromosomal alterations

LOH is defined as the mutation of a gene or chromosomal segment after only 1 copy has been transmitted in the germline; it results in a "double-hit" mutation. LOH has been evaluated in GBM but requires understanding of how these chromosomal changes alter cancer growth through further investigation. LOH of 22q is seen in 41% of primary GBMs and 82% of secondary GBMs (70) as well as a lower percentage of anaplastic astrocytomas (20-30%) (3). Loss of chromosomes 1p and 19q has frequently been associated with improved survival in oligodendroglioma and has been evaluated in GBM because of rare progression of oligodendroglioma to GBM (71-74). LOH of 1p has been seen in 12% of primary GBMs and 15% of secondary GBMs, while LOH of 19q has been seen in 6% of primary GBMs and 54% of secondary GBMs (66). The association of 1p/19q LOH in GBM correlated with improved survival in some studies but not across all studies (75,76). Although chromosomal losses are common in GBM, whether they are key drivers of gliomagenesis or passengers of the rapid, disorganized growth of the tumor remains uncertain.

3.7. Isocitrate dehydrogenase 1 (IDH1)

Isocitrate dehydrogenase 1 (IDH1) has recently been shown in several genomic studies to play an important role in regulating GBM metabolism and improving prognosis when mutated (29,77). IDH1 is involved in an NADPH-dependent reduction of isocitrate to a-ketoglutarate during oxidative phosphorylation (77-79). IDH1 and its closely related family member IDH2 play many roles including hypoxia sensing, histone and DNA demethylation, fatty acid metabolism, and collagen modification (80). Mutated IDH1 catalyzes conversion of a-ketoglutarate to R-2-hydroxyglutarate at high efficiency (81). Formation of 2-hydroxyglutarate may stabilize hypoxia inducible factor 1 (HIF-1) and stimulate proliferation in hypoxic environments (82). Mutations of IDH1 in GBM generate a hypermethylated state similar to early stages of embryogenesis and may result in a persistently dedifferentiated state (83-85). How this hypermethylated state impacts clinical formation and progression remains uncertain. Nonetheless, IDH1 was shown to represent an important prognostic indicator and is widely used in clinical practice. Patients with anaplastic astrocytomas (WHO grade II, III) and GBM with mutated IDH1 have a better overall survival and present at younger ages than patients possessing tumors expressing wild-type IDH1 (77,78,86,87). IDH1 mutant tumors also show less contrast enhancement and peritumoral edema but greater initial size and cystic components on presentation (88). The use of magnetic resonance spectroscopy has been suggested as a method of detecting 2-hydroxyglutarate and thereby predicting improved prognosis prior to obtaining tumor tissue (89). The ability to offer preoperative evaluation of IDH1 may truly revolutionize the care of patients with GBM as there are few clinically relevant prognostic markers other than patient age, tumor size, and patient Karnofsky performance score. Should patients be preoperatively selected and be able to receive an IDH1-specific therapy, this would represent a remarkable advance in patient care using a molecular target in GBM; however, thus far, no targeted IDH1 therapy exists and no prospective analysis of survival using the 2-hydroxyglutarate marker has been performed.

3.8. Summary

Primary and secondary GBM show distinct genetic alterations yet such mutations are not unique to either disease (4). Primary GBM commonly shows mutations in EGFR and PTEN whereas secondary GBM shows mutations of p53, p16, and RB1. Similarly, chromosomal aberrations and IDH1 mutation are common among GBM types. LOH of chromosome 10 is more frequent in primary GBM while LOH of 19q and 22q are more common in secondary GBM. Mutation of p53, LOH of chromosome 10 and 17, as well as alteration of EGFR and PDGF in low-grade astrocytomas correlate with malignant progression and suggest that these are important driver mutations (90). Even within traditional GBMs, however, significant pathological and molecular heterogeneity exists. Microdissection of GBM specimens has shown distinct areas of mutation (91-93) as well as expression of, angiogenic factors (94). A personalized approach towards a patient's tumor is the eventual goal of the remarkable research completed on GBM, and tailored treatments may represent the long-sought remedy. Nonetheless, the many underlying molecular changes in GBM create tremendous complexity in understanding this tumor and designing rational therapy.

4. THE COMPLEXITY OF GBM PATHOLOGY

Heterogeneity defines both the clinical and the pathological features of GBM (3). Although the disease favors the larger cerebral cortex, it can present nearly anywhere in the brain and with a diversity of clinical symptoms. In addition, tumors can show remarkable differences in the degree of progression prior to presentation as well as after treatment. Common features of GBMs during surgery include expansion of gyri, recruitment of blood vessels through dilated superficial vessels, and zones of intratumoral necrosis and peritumoral edema. Tumors

Table 2. Pathological variation of glioblastoma

Subtype	Description	Median overall survival	Mean overall survival	Reference
Traditional glioblastoma (GBM)	Infiltrating, hyperchromatic cells with pseudopalisading necrosis, neovascularization and high mitotic activity	127-217 months	8 months	(1,4,7,77,171,175,176)
Gliosarcoma	GBM along with features of sarcoma/ mesenchymal differentiation	9 months	4-116 months	(91,100,177-180)
Giant cell GBM (gcGBM)	GBM with multinucleated giant cells and lymphocyte infiltration	12 months	11 months	(113,115,117,181-184)
Fibrillary/epithelial GBM	GBM with epithelial differentiation	7 months		(120,121,185-187)
Small cell astrocytoma (SCA)	GBM with monomorphic small nuclei cells, mild hyperchromasia, limited stroma, scant mitotic index	6-143 months		(99,125,175,188)
GBM with oligodendroglioma component (GBMO)	GBM with cytoplasmic inclusions (fried egg), significant necrosis	26 months	143-26 months	(34,126,131,132,189-192)
GBM with primitive neuroectodermal tumor (GBM-PNET)	GBM with PNET-like areas containing hypercellularity, small undifferentiated cells, oval-round nuclei and Homer Wright neuroblastic rosettes	91-17 months	44 months	(134,193,194)
Gemistocytic astrocytoma (GA)	GBM with glassy, non-fibrillary cytoplasm	34 months	64 months	(138,139,142-144,195,196)
Granular cell astrocytoma (GCA)	GBM with large, round granular cells with eosinophilic cytoplasm	76 months		(150,151)
Pediatric high-grade glioma (pHGG)	GBM in pediatric patients			(152-154,156,158,159,161,162,164,197)

commonly infiltrate normal tissues, which can make resection difficult, and may demonstrate cysts, which mimic lower-grade tumors; however, both macroscopic and microscopic features of GBM show significant mutability (Figure 2, Table 2). Tumor masses can contain different cell architectures, genetic expression patterns, and degrees of vascularization and necrosis. WHO-established variants of GBM include gliosarcoma and gcGBM. In addition, several emerging variants have been described, including fibrillary/epithelial GBM, SCA, GBMO, GBM-PNET, GA, and GCA (95). Morphologies such as GA, SCA, and GCA, which are often described as lower WHO grade tumors, have many features similar to GBM in addition to their poor prognosis. Studies evaluating these new variants are limited, and further work is necessary for better characterization.

4.1. Traditional GBM

GBM traditionally shows infiltrative, pleomorphic, hyperchromatic cells with aggressive pathological features including pseudopalisading necrosis, microvascular proliferation, and neural invasion (3). Histopathological features include hyperproliferating nuclei with variable glassy cytoplasm, focal pseudopalisading necrosis, perivascular pseudorosettes, and microvascular proliferation including glomeruloid formation. Staining with glial fibrillary acidic protein (GFAP) is typically used to identify the astrocytic nature of the tumor, while staining for Ki-67, a key nuclear protein, can identify its high proliferative capacity. Primary vs. secondary GBM classification guiding treatment and prognosis has been traditionally organized around the presentation of the disorder. Secondary GBM arises from lower-grade astrocytomas (WHO grade II and III) and accounts for 5% of GBM cases; it is unclear how many cases eventually progress (4)

Multiple classification schemes have been designed to organize the heterogeneity of gliomas. The first grading system by Bailey and Cushing (96) was followed by the Kernohan (97), St. Anne-Mayo (3,98), and WHO (3) grading systems. Gliomas are currently classified under the WHO system, a modification of the St. Anne-Mayo grading scheme, where grades are based on four key histomorphological features including 1) nuclear atypia, 2) mitotic figures, 3) microvascular proliferation, and 4) necrosis (3). Lesions with 3–4 variables are grade IV tumors (GBM), those with 2 variables are grade III tumors (anaplastic/malignant astrocytoma), and those with 1 characteristic are grade II tumors (diffuse astrocytoma). Although higher WHO grade correlated with poor prognosis, multivariate analysis of these various tumor grades shows significant variability in prognosis within each tumor grade, supporting the use of additional molecular means to allow better risk stratification (99). Overall, it will

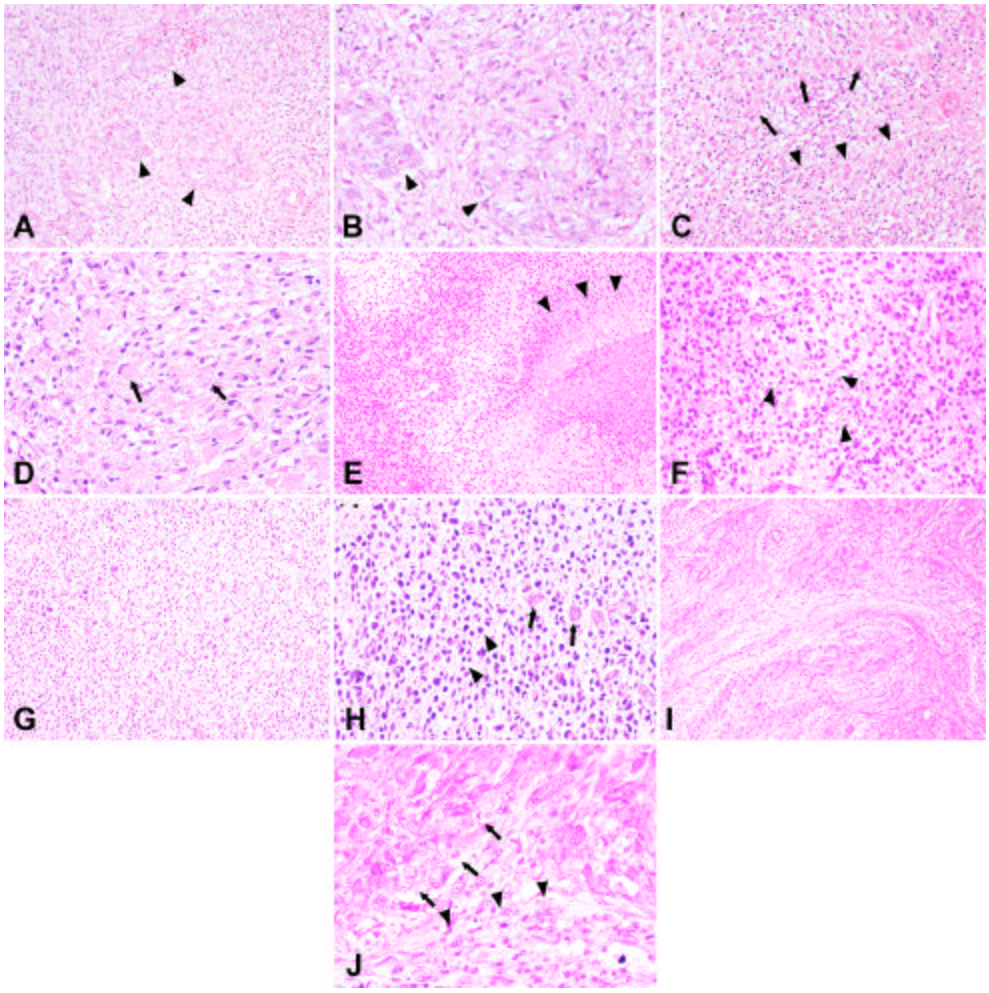

Figure 2. Pathological heterogeneity in the subtypes of GBM Various histopathological features of GBM heterogeneity are demonstrated A) Low and B) high power views of traditional GBM show neoepithelialization and glomeruloid formation (arrowheads) Marked hypercellularity is also seen C) Low and D) high power views of gemistocytic features are shown (arrows) along with an area of pseudopalisading necrosis (arrowheads) E) An area of clear pseudopalisading necrosis (arrowheads) is shown in a low power view of GBM with oligodendrocytic features F) A high power view shows marked oligodendrocytic-like cells with perinuclear halos (arrowheads) G) A low power view of monotonous small cell in GBM H) A high power view demonstrating significant small cell infiltration (arrowheads) along with some giant cell features (arrows) I) A low power view of a gliosarcoma is shown with glial component abutting a sarcomatous/mesenchymal area Several distinct areas of glomeruloid formation are seen (arrowheads) J) A high power view distinguishes the glial (arrowheads) vs sarcomatous (arrows) areas

be crucial to understand the molecular mechanisms accounting for these differences in tumor aggressiveness in order to design targeted treatment strategies.

4.2. Gliosarcoma

Gliosarcoma is a WHO-defined tumor expressing a biphasic pattern of glial and sarcomatous/mesenchymal cells (100). This variant accounts for 1–5% of GBM diagnoses, and patients present between 50 and 70 years of age (3,91). As with GBM, gliosarcoma has a poor prognosis, with a mean overall survival of 4–11.5 months (3,91). Simplistically, the additional sarcomatous component to GBM yields a slightly lower survival of 1 to 3 months, a significant amount for a disease with a median 15-month survival (1). Gliosarcomas typically occur in the temporal lobe and have the potential to metastasize to the lungs, liver, and spinal cord (91,101). This is a unique property different from GBM, which almost never metastasizes outside of the central nervous system. In addition, gliosarcomas show expression of various mesenchymal (laminin, collagen type IV, etc.) and glial markers (GFAP, S-100), highlighting the distinct areas of this tumor (102-104). Mutational patterns of gliosarcoma are somewhat unique from those of GBM despite a remarkable overlap. Gliosarcomas show infrequent EGFR mutations unlike GBM (105), as well as less common mutations of O-6-methylguanine-DNA methyltransferase (MGMT) (12%) and IDH1 (8%) (106); however, mutations in p53 (26%), PTEN (37%), and Rb (53%) pathways are found at similar rates to GBM (107). Gains of chromosomes

7, 9q, 20q, and X as well as losses of 1p, 9p, 10, 13q, 17p, and 19q are also seen in gliosarcoma (108-111). Interestingly, these mutations are concordant between gliomatous and sarcomatous tumor regions, suggesting a common origin for this tumor.

4.3. Giant cell glioblastoma (gcGBM)

gcGBM is another WHO-defined tumor, encompasses 2–5% of GBM diagnoses, and is characterized by giant, multinucleated cells (>500 μm) in addition to the traditional features of GBM (3). gcGBM variably stains for neuronal (S-100, class III-ß tubulin) and mesenchymal markers (vimentin) (3,111,112). Multiple studies have shown improved survival for gcGBM compared with GBM (111,113,114). gcGBM often show distinct surgical borders, and patients present at younger ages than with traditional GBM, thus affording more aggressive surgical resections (115). A multivariate analysis from the Surveillance, Epidemiology, and End Results (SEER) showed a significantly younger age of diagnosis (51 vs. 62 years), a greater likelihood for gross total surgical resections, and a reduced hazard ratio of 0.7.6 for gcGBMs (95% CI: 0.5.9–0.9.7) (113). Mutations in gcGBM are also unique from those associated with GBM, with 90% of gcGBMs showing p53 mutations along with infrequent EGFR and p16 alteration (116). Interestingly, the giant cell population of gcGBM shows marked levels of polyploidy (72–84% cells) compared with traditional GBM (11–49%) (117). Comparison of pediatric gcGBM and GBM in the HIT-GBM trial showed no difference in median age, male:female ratio, or clinical history (118). In addition, median survival (1.18 vs. 1.08 years) and event-free survival (0.54 vs. 0.53 years) were also not significantly different despite greater gross total resection in gcGBM (44 vs. 25%). Thus, while gcGBM suggests a better prognosis in adult patients, no difference is seen in pediatric patients.

4.4. Fibrillary/epithelial glioblastoma

Evidence of a fibrillary/epithelial GBM distinct from traditional GBM with components of epithelial differentiation continues to emerge. Some studies evaluating tumors with extensive epithelial differentiation showed concordant losses of 1p36, 9p21, 10q23, 17p13, and 10q22-26 among glial as well as epithelial components of these tumors, suggesting that epithelial cells were derived from a common progenitor as glial cells (111,119). A recent study of 3500 GBM samples identified 20 samples with predominant epithelial features and 10 with epithelioid features (120). Furthermore, epithelial GBMs stained for epithelial membrane antigen and cytokeratin CAM5.2, as well as a variety of other markers. Significant differences in mutations of p21 (93%), p53 (41%), and EGFR (19%) were seen compared with traditional GBM. Despite these findings, median overall survival remained 7 months, similar to traditional GBM used as controls for the study. Some similarity between epithelial GBM and metastatic cancer is evident by the expression of E-cadherin, an important marker of epithelial–mesenchymal transition during metastasis. In a clinicopathological study examining samples from 27 GBM patients and several GBM cell lines, E-cadherin expression correlated with worse patient prognosis and greater tumor dissemination (121). Thus, treatment modalities used in the treatment of metastatic carcinoma may be useful in this variant as well. Despite these findings, further clarification of true epithelial GBM is required to differentiate it from traditional GBM with epithelial features.

4.5. Small cell astrocytoma (SCA)

SCAs are histologically characterized by monomorphic, round nuclei and scant cytoplasm, although these features can be found in 10% of traditional GBM as well (122). Several studies have shown predominant EGFR amplification in SCA compared with GBM and an aggressive phenotype despite the bland histological appearance (123-125). One study showed a median survival of 11 months for grade IV SCA where deletion of chromosome 10q, polysomy of chromosome 7, and EGFR amplification were predominant features (124). In another study where SCA tumors were defined by small cell morphology in >80% of samples, SCA tumors uniformly lacked codeletion of 1p/19q and showed greater amplification of EGFR (83%) and EGFRvIII (50%) than traditional GBM. Because this and other studies have shown similar mortality compared with traditional GBM, however, it is possible that that there are distinct genetic underpinnings without implications for the clinical course of this disease.

4.6. Glioblastoma with oligodendroglioma component (GBMO)

Among the many emerging variants of GBM, none has a greater possibility of being recognized as a truly distinct variant than GBMO. Discussed in the 2007 WHO classification guidelines, these tumors resemble oligodendroglioma with the typical fried-egg appearance microscopically, but contain significant necrosis and features of astrocytoma and are truly aggressive tumors (3). GBMO tumors may show improved survival compared with traditional GBM. In a retrospective study, patients with GBMO treated using chemotherapy (nimustine and teniposide) and radiotherapy had improved median overall survival of 26 months and a 2-year overall survival rate of 60% compared with traditional GBM (126). In another retrospective study including 450 patients with GBM, of which 36 patients had GBMO, median age of onset was lower in GBMO (52.1. vs. 62.2.4 years) and LOH of 1p/19q and 10q was greater, but comparable rates of altered EGFR and p53 were observed in GBMO and traditional GBM patients (127). The interest in 1p/19q extends from findings in oligodendroglioma showing improved survival with this alteration; however, other studies have not shown a consistent difference in 1p/19q between GBMOs and GBMs (128,129). Another interesting study using microdissection of GBMO showed subclassification by chromosomal gains and losses into astrocytic (+7/-10), oligodendroglial (-1p/19q), intermediate (-1p/+7), and non-specific cell types, suggesting distinct subtypes even within this already subdivided variant (130). This study also reiterated that patients with GBMOs were younger, had improved median overall survival (13 months), and responded better to radiotherapy than those with traditional GBM. Another recent database study showed that 18.3% of 219 consecutive GBM samples were GBMOs, and these were associated

with a greater frequency of clinical seizures, IDH1 mutations (31 vs. <5%), reduced MGMT expression, and prolonged median overall survival (13.5. vs. 19.0. months) (131). Furthermore, presence of an oligodendroglioma component predicted improved survival despite rare codeletion of 1p/19q (<5% samples). An analysis of the EORTC 26981/NCIC CE.3 trial showed GBMO in 15% of 339 samples, with higher IDH1 mutation (19 vs. 3%) and EGFR amplification (71 vs. 48%) than GBM but no difference in 1p/19q deletion or MGMT methylation (132). Moreover, a recent study confirmed GBMOs contain a higher percentage of IDH1 mutation (24%) compared with matched traditional GBM controls (4%), which predicted a better prognosis for patients with GBMOs (133). Further prospective studies and meta-analyses are warranted to discern these survival differences.

4.7. Glioblastoma with primitive neuroectodermal features (GBM-PNET)

Primitive neuroectodermal tumors (PNET) are rare, neural crest-derived tumors in children and commonly show poor prognosis. GBM with PNET features are a potential variant with distinct PNET-containing areas showing oval-round hyperchromatic nuclei with Homer-Wright rosettes, lower GFAP expression, increased S-100, synaptophysin, NeuN, and neurofilament protein (NFP) expression (3). A clinicopathological study including tumor samples from 53 patients with GBM-PNETs showed a median age of 54, distinct areas of PNET staining with synaptophysin and NeuN, p53 expression (83%), and amplification of n-myc or c-myc (43%) (134). This study also showed significant rates of 10q deletion (50%) in both glial and PNET components but limited alterations in PTEN and EGFR. Furthermore, as with GBM, median survival was poor (9.1. months). Another clinicopathological study of 40 patients with GBM-PNET in whom GFAP and NFP coexpression were required for diagnosis showed a high rate of recurrence (36%) but better median overall survival (44 months) in these patients compared with matched GBM controls (135). In a recent study of 86 cases of GBM, PNET-like features were seen in 27% of the samples but did not correlate with prognosis (136). Interestingly, GBM-PNET tumors in children showed lower p53 and PTEN expression (8%), lack of mutation in EGFR, CDK4, and MDM2, and absent LOH of 17p compared with GBM, suggesting distinct a molecular architecture between the two tumor types (137).

4.8. Gemistocytic astrocytoma (GA)

GA, evidenced by gemistocytes with glassy, non-fibrillary cytoplasm, is defined as a WHO grade II tumor; however, this tumor behaves more aggressively and resembles GBM (138,139). Despite its low-grade designation, some studies of GA suggest that an increased percentage of gemistocytes correlates with poor prognosis (138), although others have not found a similar correlation (140,141). In a study of 40 patients with low-grade gliomas that progressed to GBM, those patients whose tumors had >5% gemistocytes showed worse survival than tumors with fewer gemistocytes (35 vs. 64 months) (142). This study also showed that GAs had greater p53 mutation, Bcl-2 expression, and Ki-67 expression. Several studies of microdissected gemistocytes and non-gemistocytes showed concordant p53 mutation, as well as p27 and cyclin D1 immunoreactivity, along with rare PTEN and EGFR alteration (143,144). Similarly, chromosomes 7 and 10 showed concordant alteration (143). These studies support a common progenitor for the different pathological architecture seen.

4.9. Granular cell astrocytoma (GCA)

Gliomas characterized by abundant granular cells, with pronounced cell borders, round shapes, and eosinophilic granular cytoplasm, are termed granular cell astrocytomas (GCAs). GCAs stain for periodic acid-Schiff, GFAP, epithelial membrane antigen, and S100, among other proteins. Several reports have suggested that GCA tumors may be more aggressive than granular cells at other sites of the body with granular cell features being present in a variety of tumors, including GBM, meningioma, and ganglioglioma (145,146). This may suggest that granular cells are characteristic of tumor degeneration. GCAs demonstrate poor patient survival despite low Ki-67 indices (146). Only 50 cases of GCA have been reported in the literature (147). One study showed a one-year survival rate of 12% for high-grade GCA and 40% for low-grade GCA (148). Another study showed that transition to infiltrating astrocytoma could be seen in 72% of the patients and recurrence could be seen in 83% of the patients, with a mean overall survival of 7.6 months (149). A recent study of the molecular features of GCA showed LOH at chromosome 1p, 9p, 10q, 17p, and 19q along with mutations of p53, p16, p14, and EGFR (150). This study also showed that loss of 9p and 10q was a defining feature, as were higher frequencies of chromosomal aberrations compared with astrocytomas at corresponding WHO grade. However, despite these many studies, distinct molecular patterns of GCAs have not been identified.

4.10. Pediatric glioblastoma

Recent studies have begun to elucidate the difference in clinical course and molecular features between adult and pediatric glioma. Pediatric high-grade gliomas (pHGGs) account for 2.8% of central nervous system tumors (3,152). Commonly related diffuse intrinsic pontine glioma (DIPG) is thought to be a subset of pHGG in children (152). Two-year survival is 10–30% for patients with pHGG and <10% for those with DIPG (153). A study of 231 children with pHGG showed mutations in p53 in 33% of patients, which correlated with poorer 5-year progression free survival (154). Furthermore, progression of pHGG from lower-grade glioma was not a usual course for this disease. In contrast to adult GBM, pHGG shows less frequent dysregulation of MGMT, IDH1/IDH2, PTEN, or EGFR (155-157) but a much higher rate of AKT and Ras activation (158-160). Mutations in BRAFV600E, not commonly shown to play a role in adult GBM, are often seen in pHGG (161,162). Recent genomic studies of pHGG have shown alterations of PDGFRA, along with distinct changes in chromosomes 1q, 7, and 10q when comparing pHGG and adult GBM (163). Interestingly, mutations of IDH1 were not seen in pHGG in this study, and subtyping of pHGG into proneural, neural, mesenchymal, and classical categories showed a molecular profile distinct from adult

GBM. Mutations in histone H3.3 protein variant, ATP-dependent helicase, and death-associated protein 6 have also been seen uniquely in HCC (164); however, the significance of these alterations remains to be further evaluated.

4.11 Summary

A resurgence of interest in the pathological features has emerged from the molecular understanding of GBM along with interest in understanding mechanisms of therapeutic resistance. Emerging variants of GBM, such as fibrillary/epithelial GBM, SCA, GBMO, GBM-PNET, GA, and GCA, are gaining interest in addition to established variants of GBM, including gliosarcoma and gcGBM. Despite the significant similarity in mortality among all the established and emerging variants, some differences in molecular underpinnings do support targeted approaches in treatment. It may be feasible that unique approaches in treatment will be required because of their molecular differences. Additional studies are needed to identify other molecular drivers of these potential variants beyond the common mutated genes. The difficulty in identifying tumor-driving mutations and molecular models in parent diseases becomes much more difficult in the study of closely related variants that are far more rare diseases. Whole genome expression, copy number analysis, and comparison to known GBM genomic data remain to be pursued. Already, distinctions between pediatric and adult GBM have become more pronounced, with these two entities likely representing distinct diseases and mechanisms of pathogenesis. Ultimately, information obtained from the study of these variants may be useful to better shape treatments for GBM.

5. HETEROGENEITY OF THE GLIOBLASTOMA GENOMIC LANDSCAPE

5.1. Genomic analysis

Recent landmark studies involving the use of microarray and DNA sequencing technology have allowed for the rapid characterization of large numbers of GBM genomes and demonstrated that clinicopathological classification does not encompass all the variation in gliomas (7). Microarray analysis of GBM has been utilized for subtyping as well as prognostic classification in a wide variety of studies (35,165-167). Initial GBM gene expression profiles showed marked variability in sample size and type of platform used, resulting in distinct clusters of categorization depending on the study (168). The cumulative effect of theses studies has resulted in determining distinct genomic subtypes of GBM, including the proneural, neural, mesenchymal, and classical genotypes, which have important effects on prognosis and potential therapeutic targeting (7).

A recent genomic analysis using gene sequencing, copy number analysis, and transcriptome analysis has illuminated key features regarding molecular abnormalities in GBM (77). Next-generation parallel sequencing methods (e.g., Serial Analysis of Gene Expression) were used to identify the DNA sequences of GBM samples at a substantially faster rate and lower cost than the Human Genome Project. Copy number analysis utilized expression arrays of single-nucleotide polymorphisms (SNPs) where repeated SNPs in close proximity compared with a normal set indicated gene duplication, and vice versa. Transcription analysis used expression arrays of mRNA to identify actively transcribed genes. Sequencing of 20,661 genes indicated mutations in 685 genes (3.3% of total), with genes showing the highest level of mutation including p53 (35%), PTEN (26%), NF1 (15%), EGFR (14%), IDH1 (11%), PIK3CA (10%), PI3K regulator subunit (PI3KR1; 8%), and RB1 (8%). Analysis of copy number indicated 147 amplifications, equating to >12 copies per nucleus, and 134 homozygous deletions. The highest levels of amplification were seen in EGFR (23%) and CDK4 (14%), while the highest levels of deletion were seen in p16 (50%), p53 (5%), PTEN (5%), and RB1 (5%). The combination of sequencing, copy number, and transcription data highlighted mutually exclusive driver mutations important in gliomagenesis within the p53, RB1, and PI3K/PTEN pathways. Namely, tumors predominantly utilized one of three potential pathways for pathogenesis. This landmark study helped identify the IDH1 mutation, a key player in the Krebs cycle, which was associated with younger age, secondary GBM, p53 mutation, and improved prognosis; however, criticisms of this study highlighted the contamination of normal tissue and tumor heterogeneity during genomic analysis and the presence of hyper-mutated profiles from patients treated with temozolomide. Nonetheless, this study helped to elucidate that GBM favored an exclusive pathway for growth and presented a method for designing future targeted therapies.

At the time of the Parsons *et al.* 2008 publication (77), a national initiative by TCGA also published a cross-platform analysis of GBM performed by analysis of copy number, expression profiling, methylation patterns, and whole-genome sequencing (29). Similarly, significantly amplified genes included EGFR, CDK4, PDGFRA, MDM2, MDM4, MET, CDK6, N-Myc, Cyclin D2, PIK3CA, and AKT3, while homozygous deletion was commonly seen in p16, p14, PTEN, CDKN2C, RB1, PARK2, and NF1. Whole-genome sequencing of 91 tumor-normal pairs (72 untreated, 19 treated) detected 453 non-silent somatic mutations, with significant alterations in p53, PTEN, NF1, EGFR, ERBB2, RB1, PIK3R1, and PIK3CA. Combined analysis of somatic mutations and copy number alterations demonstrated abnormalities in 3 major signaling pathways (and their respective proteins): the receptor tyrosine kinase pathway (PTEN, EGFR, ERBB2, PDGFRA, MET), the p53 pathway (p16, MDM2, MDM4, p53), and the RB pathway (p16, p14, RB1). Additional study of gene methylation was performed using a customized expression array of 2305 gene promoters containing repeat GC-nucleotides (e.g., CpG dinucleotides)

Methylation of these nucleotides can result in histone binding and aggregation of chromatin, thus silencing gene expression. Methylation of CpG dinucleotides in 2305 genes was correlated with expression patterns and demonstrated that 21% of tumors showed methylation of MGMT with a slightly higher number in treated versus untreated patients. MGMT methylation results in gene silencing and increases GBM sensitivity to alkylating agents by preventing DNA repair (2,169). Treatment with

Table 3. Genomic subtyping of glioblastoma

Subtype	Molecular features	Defining features	Clinical impact
Classical	EGFR amplification, EGFRvIII mutation, rare p53 mutation, PTEN mutation, 9p213 deletion (encoding p16 and p14), gains chromosome 7, losses chromosome 9p	Nestin, notch3, JAG1, LFNG, SMO, GAS1, GLI2, HES1	Improved survival with intensive therapy
Mesenchymal	17q11 deletion (encoding NF1),, p53 and PTEN mutation, comutations of NF1 and PTEN,	IRS-1, CHI3L1/YKL40, MET, CD44, MERTK, TRADD, RELB, TNSFRSF1, NF-kB	Improved survival with intensive therapy, most similar to immortalized cell line signature Proneural
4q12 amplification (encoding PDGF), PDGF, IDH1/IDH2, p53, PI3KCA/PI3KR1 mutation	PDGFA, NKX2-2, OLIG2, SOX genes, DCX, DLL3, ASCL1, TCF4	Younger age, improved survival not affected by intensive therapy, suspected predominant subtype of hypermutated and secondary GBM	
Neural	EGFR amplification, poorly defined molecular features	NEFL, GABRA1, SYT1, SLC12A5	Improved survival with intensive therapy, resembles normal brain expression profile

ASCL1: Achaete-scute homolog 1; CHI3L1: Chitinase-3-like protein 1; DCX: doublcortin; DLL3: delta-like 3; EGFR: epidermal growth factor receptor; GABRA1: gamma-aminobutyric acid (GABA) A receptor, alpha 1; GAS1: growth arrest-specific protein 1; HES1: hairy and enhancer of split-1; IDH1/2: isocitrate dehydrogenase 1/2; IRS-1: Insulin receptor substrate 1; JAG1: jagged 1; LFNG: N-acetylglucosaminyltransferase; NEFL: neurofilament; NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells; PDGF: platelet derived growth factor; PI3K: phosphoinositide 3-kinase; OLIG2: oligodendrocyte transcription factor 2; SLC12A5: potassium-chloride transporter member 5; SMO: smoothened; SYT1: synaptotagmin-1; TCF4: transcription factor 4; TNFRSF1: tumor necrosis factor receptor superfamily, member 1A; TRADD: tumor necrosis factor receptor type 1-associated DEATH domain

alkylating agents typically causes spontaneous nucleotide deamination (GC > AT) within CpG islands, including those at MGMT and mismatch repair genes (e.g., MLH1, MSH2, MSH6, PMS2). Deamination of methylated C nucleotides can result in T residues, which induces gene activation and DNA repair. The overall effect is that treatment of GBM with alkylating agents results in unregulated DNA activity if cell death is not achieved. In concordance with these results, background mutation was higher in treated GBM patients as expected and correlated with increased mutations in mismatch repair genes. Recent studies have strived to better integrate somatic mutations by whole-exome sequencing and copy-number variation analysis to improve resolution of significant mutations from background changes (170). In this study, whole-exome sequencing was performed for 139 GBM and matched normal brain samples from the TCGA while copy number analysis was performed for 469 GBM samples. Elucidation of genetic alterations in LZTR1, CTNND2, and recurrent EGFR gene fusions were discovered as promising candidates of novel driver mutations in GBM. Future studies are needed to verify to which extent these findings translate into relevant clinical features of GBM.

5.2. Glioblastoma molecular subtypes

Recent genomic studies have evaluated GBM subtypes and have shown that heterogeneity can be categorized into distinct groups (Table 3). In earlier studies, profiling of GBM genomic expression clustered tumors into three groups, including proneural, proliferative, and mesenchymal genotypes, based on a 35-gene signature (35), while later studies further separated these categories into proneural, mesenchymal, classical, and neural subtypes (7,171) The original mesenchymal group was later divided into the mesenchymal and classical groups, and the proliferative group was similar to the neural group. These signatures reflect normal tissue gene profiles of brain (proneural), hematopoietic stem cells (proliferative), as well as bone, synovium, smooth muscle, endothelium and dendritic cells (mesenchymal) (35). Proneural subtypes are common in grade III tumors and correlate with younger age and prolonged survival. Proliferative subtypes correlated with expression of proliferation markers (Ki-67, proliferating cell nuclear antigen, topoisomerase IIa), while mesenchymal and proneural subtypes correlated with expression of angiogenesis markers (VEGF, VEGFR, and platelet endothelial cell adhesion molecule 1). Expression of neural stem cell markers (vimentin, nestin, human homolog of the Drosophila tailless gene, CD133, and maternal embryonic leucine zipper kinase) was predominantly present in proliferative and mesenchymal subtypes. Neuroblastic markers (oligodendrocyte transcription factor 2, microtubule-associated protein 2, doublecortin, ectoderm-neural cortex protein 1, v-erb-b2 erythroblastic leukemia viral oncogene homolog 4, and glutamate decarboxylase 2) and notch pathway proteins (Delta-like 1/3 (DLL1/3), Hairy/enhancer-of-split related with YRPW motif protein 2, and Achaete-scute homolog 1 (ASCL1)) were mainly expressed in proneural tumors. Furthermore, tumor samples with greater similarity to the proliferative subtype were capable of forming neurospheres when grown with EGF and fibroblast growth factor (FGF), while tumors with expression patterns similar to mesenchymal subtypes were less likely to form neurospheres (35). Matched primary and recurrent astrocytomas of treated patients showed a loss in proneural expression patterns and similarity to mesenchymal expression patterns indicating these genotypes could change as well as affect therapeutic resistance. Recent studies have shown that the mesenchymal expression profile correlates with CD133 expression (172) and resistance to radiation (173). The transition of subtype may depend on NF-?B (173), Bcl-w (174), and other as-yet-undefined factors. Thus, distinct subtypes of GBM, expression patterns that define each type, and some differences in clinical outcomes can be shown from genomic analysis. Interestingly, these tumors would have all been categorized

as primary GBM should molecular analysis not have uncovered further complexities in their genetic backgrounds.

A recent study utilized hierarchical factor analysis to characterize and analyze the GBM subtypes (171). The classical subtype demonstrated significant EGFR amplification/expression (97% samples) and EGFRvIII alteration (50% samples) and rare p53 mutation, the presence of 9p21.3 deletion (encoding p16 and p14), high expression of neural precursor and stem cell markers (nestin, notch3, JAG1, O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase, smoothened, growth arrest-specific 1, GLI family zinc finger 2), gains on chromosome 7, and losses of chromosome 9p. The mesenchymal subtype demonstrated significant hemizygous deletion of 17q11.2. (encoding NF1), low expression of NF1 and/or NF1 mutation, comutations of NF1 and PTEN, as well as expression of mesenchymal markers (CHI3L1, MET), astrocytic markers (CD44, c-mer proto-oncogene tyrosine kinase), tumor necrosis super family (tumor necrosis factor receptor superfamily member 1A-associated via death domain (TRADD), v-rel reticuloendotheliosis viral oncogene homolog B), and nuclear factor kappa B (NF-?B) pathway proteins. The proneural subtype correlated with 4q12 amplification (encoding PDGF), mutation of IDH1 and p53, younger age, improved outcome, as well as high expression of oligodendrocytic development genes (PDGFA, NK2 homeobox 2, OLIG2) and proneural genes (SOX genes, DCX, DLL3, ASCL1, transcription factor 4). Secondary GBM and the hypermutated phenotype were hypothesized to belong to the proneural subtype, cultured GBM cells resembled the mesenchymal expression pattern, and recurrent tumors were found in all subtypes. A nonspecific neural subtype was also identified that expressed neuron markers (neurofilament, light polypeptide, gamma-aminobutyric acid A receptor alpha 1, synaptotagmin I, solute carrier family 12 member 5) and resembled normal brain. Interestingly, more aggressive treatment regimens containing concurrent chemotherapy and radiotherapy or more than 3 cycles of chemotherapy yielded improved patient survival in classical, mesenchymal, and neural subtypes but not in the proneural subtype.

This study suggested that classical GBM may be more responsive to radiation and chemotherapy because of intact p53, mesenchymal tumors may be responsive to Ras, PI3K, and angiogenesis inhibitors, and proneural tumors may sensitive to HIF, PI3K, and PDGFRA pathway inhibitors. Moreover, another study suggested that proneural, classical, and mesenchymal subtypes are enriched for in GBMO, scGBM, and gliosarcoma variants, respectively (99), thus further elucidating how molecular variation may account for pathological differences. Important questions regarding how these subtypes affect outcomes remain to be answered. Much of the data for this analysis were retrospectively analyzed without uniform sample collection methods and targeted treatment in mind.

While many of the subtyping studies used microarrays involving mRNA, expression of protein may not always correlate with mRNA levels. In this light, a proteomic hierarchical analysis of GBM was also performed using quantitative protein levels obtained by Western blot to confirm GBM subtypes (34). A proteomic analysis of GBM tumor protein expression levels categorized tumors along key signaling pathways involved in glioma formation. This study showed the presence of 3 statistically significant core clusters (PDGFR, EGFR, NF1). Copy number analysis showed EGFR amplification for EGFR class tumors and gain of chromosome 7 for NF1 class tumors. Retrospective analysis of archived TCGA data in this study indicated that PDGFR, EGFR, and NF1 showed mutually exclusive clusters while an indeterminate genotype cocluster was also identified. Furthermore, the PDGFR cocluster showed expression of proneural markers, the NF1 cocluster showed mesenchymal markers, and the EGFR cocluster showed a mix of proneural and mesenchymal markers, thereby correlating proteomic and genomic expression patterns. The EGFR cluster overexpressed EGFR, JAG1, and HES1, while the NF1 cluster overexpressed insulin receptor substrate 1 and chitinase 3-like 1; however, genomic expression data from TCGA revealed that the PDGFR cluster overexpressed PDGFR mRNA despite a lack of correlation with protein levels. This finding emphasized that both gene expression analysis and protein analysis should be performed to identify true driver mutations that result in aberrant protein signaling and expression.

5.3. Summary

The accumulation of these various genomic studies has resulted in the delineation of 4 molecular subtypes of primary GBM, with unique genetic features and clinical impact. These findings have led to new understanding about the ability of genetic expression patterns in GBM to change. Further work is required to identify other driver mutations within each subtype and potential therapeutic targets. A major point of criticism of all genomic studies on GBM has been the contamination of normal tissue or various parts of tumor stroma, which can greatly alter which genes are significantly detected by expression analysis. The use of tumor laser microdissection, in-situ expression analysis, and cheaper, more rapid sequencing tools may be useful methods to overcome these limitations. Additional statistical methods will also be necessary to analyze the immense amounts of expression data to find driver mutations. Individualized therapeutic approaches based on tumor subtype are promising avenues of investigation. The use of aggressive chemotherapy and radiation in classical GBM with intact p53 signaling, inhibitors of PI3K and angiogenesis in mesenchymal tumors, and inhibitors of HIF, PI3K, and PDGF signaling in proneural tumors has yet to be fully validated in clinical trials. Current studies aim to identify the unique signature of each subtype to allow for rapid, personalized therapy.

6. CONCLUSION

Although GBMs were originally delineated by clinical means into primary and secondary types, molecular research has better elucidated the underlying genomic changes in the many pathological variants of GBM and has shed light into the complex subdivisions of GBM (e.g., proneural, neural, mesenchymal, and classical genotypes)

that were once imperceptible. The genomic literature has further supported the clinicopathological heterogeneity of this disease that has long been suspected. Initial findings of key mutated genes in GBM led to the hypothesis that agents used towards aberrantly upregulated pathways may serve as effective therapies; however, single molecular targets in GBM have mostly failed to achieve a meaningful improvement in survival. It is likely that these approaches have failed to address the many molecular pathways that this tumor uses for proliferation. Newer approaches are geared towards understanding the many pathways of cancer resistance and utilizing multiple treatment agents (53). Molecular treatments in addition to current standards of care are also being pursued, including antiangiogenic therapies and radiosensitizing agents. Treatments to target the cell of origin for GBM, namely the cancer stem cell, are also potential approaches (14). With the realization that GBM is more a complex entity than one single disease, new investigation into the genetic basis of the disease and mechanism for the remarkable clinicopathological heterogeneity has arisen. The use of more modern, sophisticated expression tools and statistical modeling have aided in the identification of multiple genetic subtypes and novel drivers of GBM (e.g., IDH1). Understanding the effects of these molecular subtypes remains an ongoing area of investigation. Future studies on GBM will surely utilize these findings in the design of clinical trials and targeted treatments. It is hoped that the development of novel therapeutic strategies based on the identification of individual genetic and/or proteomic tumor signatures will lead to a more efficient treatment of GBM.

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