

Signaling pathways and therapeutic approaches in glioblastoma multiforme (Review)

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Abstract. Glioblastoma multiforme (GBM) is the most aggressive type of primary brain tumor and is associated with a poor clinical prognosis. Despite the progress in the understanding of the molecular and genetic changes that promote tumorigenesis, effective treatment options are limited. The present review intended to identify and summarize major signaling pathways and genetic abnormalities involved in the pathogenesis of GBM, as well as therapies that target these pathways. Glioblastoma remains a difficult to treat tumor; however, in the last two decades, significant improvements in the understanding of GBM biology have enabled advances in available therapeutics. Significant genomic events and signaling pathway disruptions (NF- κ B, Wnt, PI3K/AKT/mTOR) involved in the formation of GBM were discussed. Current therapeutic options may only marginally prolong survival and the

current standard of therapy cures only a small fraction of patients. As a result, there is an unmet requirement for further study into the processes of glioblastoma pathogenesis and the discovery of novel therapeutic targets in novel signaling pathways implicated in the evolution of glioblastoma.

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1. GBM: Introduction

Glioblastoma multiforme (GBM) is the most prevalent type of malignant brain tumor in adults. It is considered the most aggressive form of primary intracranial tumor and is associated with a dismal prognosis (1,2). Survival of patients with GBM remains poor: The overall 5-year relative survival rate is one of the lowest among all cancer types (4-5%). Despite aggressive treatment, the median overall survival (OS) is ~15 months (2). There have been only modest improvements in survival rates for patients with GBM in the last 30 years (3).

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According to the 2021 World Health Organization classification of central nervous system (CNS) tumors, glioblastomas are defined as isocitrate dehydrogenase (IDH)-wild-type (wt) diffuse astrocytic tumors. Astrocytoma, IDH-mutant (mut) grade 2, 3 or 4 tumors, are now considered separate entities (4). The histopathological features of GBM include diffuse neoplastic infiltration of the nervous tissue with a necrotic core and cells resembling astroglia ('angular' nucleus, euchromatin) and vascular proliferation and/or pseudopalisading necrosis with mitoses (5). The structure of the blood-brain barrier (BBB), the suppressive tumor microenvironment and tumor heterogeneity provide an advantage to glioblastoma cells, leading to decreased efficacy of chemotherapeutic agents, targeted therapy and immunotherapy (6,7).

Despite multimodal approaches to treatment, most patients with GBM have an aggressive course of the disease. Glioblastomas frequently recur (in 75-90%) within 2-3 cm from the borders of the initial lesion and with multiple lesions observed in 5% of cases after treatment (2). Treatment has historically consisted of maximal surgical resection with adjuvant radiation therapy (RT) or primary RT for inoperable tumors. Within the last two decades, temozolomide (TMZ) and a non-invasive device called the tumor-treating field (TTF; Optune[®]; Novocure GmbH) have demonstrated clinical efficacy and achieved improved outcomes (8-10). Additional treatment options that demonstrated activity include bevacizumab, lomustine, carmustine, PCV (combination of procarbazine, lomustine and vincristine), and, more recently, multikinase inhibitor regorafenib, which demonstrated superior outcomes over lomustine in a recent phase 2 trial (11-14).

According to the 2020 National Comprehensive Cancer Network (NCCN) clinical practice guidelines in oncology (12), GBM treatment options depend on patient age, performance status (PS) and O⁶-methylguanine DNA methyltransferase (MGMT) promoter methylation status (methylated vs. unmethylated). Patients aged 70 years or younger with a good PS, regardless of the tumor's MGMT methylation status, should receive standard brain RT plus concurrent and adjuvant TMZ with alternating electric field therapy (9,10). Patients older than 70 years with good PS should receive hypofractionated or standard brain RT plus concurrent and adjuvant TMZ and alternating electric field therapy.

2. GBM: Epidemiology

According to The Central Brain Tumor Registry of the United States statistical report, the annual average age-adjusted incidence rate of GBM between 2012 and 2016 was 3.22 per 100,000 individuals in the US (15). GBM accounts for ~15% of all brain tumors and is mainly found in adults aged 45-70 years (16). Seminal clinical study results indicated that the median survival was 14.6 months for RT plus TMZ and 12.1 months for RT alone (9). The 5-year OS outcome with RT plus TMZ was 9.8 and 1.9% with RT alone (17). To date, there are no identified risk factors or underlying carcinogenic causes for the development of GBM and the only confirmed risk factor is exposure to high levels of ionizing radiation (18). Of note, patients with asthma and other allergic conditions have been described to have a lower risk to develop GBM. In addition, genotypes that increase the risk of asthma are

associated with a decreased GBM risk (3). Several studies have suggested a possible inverse relationship between GBM development and non-steroidal anti-inflammatory drug (NSAID) use. The Glioma International Case-Control Study reported that daily aspirin use for ≥ 6 months was associated with a 38% lower glioma risk (19). Another study assessed the risk of glioma among 325 glioma cases and 600 frequency-matched controls in the Houston metropolitan region (2001-2006) and it indicated that regular use of NSAIDs was related to a 33% reduction in the risk of glioma (20).

It has been proposed that human cytomegalovirus modulates the malignant phenotype in glioblastomas (21). In a limited study at Karolinska University Hospital, 50 patients with GBM received valganciclovir as adjuvant treatment. The rate of survival at 2 years was 62% compared with 18% of contemporary controls with a similar disease stage, surgical resection grade and baseline treatment ($P < 0.001$) (22). While these results sound promising, they should be validated in larger randomized studies in the future.

3. Current treatment options

Chemotherapy agents. The mechanisms of action of effective systemic standard of care treatments for GBM are summarized in Fig. 1. The most frequently used drug in the first-line setting is TMZ. TMZ is a prodrug that works by being converted to the monomethyl triazene 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (23). The biological actions of TMZ appear to be mediated by methylation at the O⁶ position of guanine (24), leading to mutations that ultimately escape the mismatch repair system (MMR). The MMR promotes a signaling cascade that activates cell cycle checkpoints and causes G2-M cell cycle arrest and apoptosis through single- and double-strand breaks in DNA (25). Tumor cells with methylated MGMT are more susceptible to the cytotoxic effects of TMZ than cells with functioning MGMT (26).

Carmustine (also known as BCNU) is a small alkylating agent and nitrogen mustard compound. It causes guanine and cytosine bases in DNA to form interstrand crosslinks. Lomustine is an oral alkylating anti-tumor therapy, which has anti-GBM efficacy due to its high lipophilicity and small size, which facilitates BBB crossover (27).

Role of angiogenesis in GBM. A high degree of tumor vascularization as a result of increased production of proangiogenic growth factors, including VEGF, is observed in GBM, which has led to the development of treatment methods aimed at targeting proangiogenic signaling pathways. The randomized, multicenter, open-label phase II BRAIN trial comparing the efficacy of bevacizumab in combination with irinotecan vs. bevacizumab alone contributed to the accelerated Food and Drug Administration (FDA) approval of bevacizumab for the treatment of recurrent glioblastoma in 2009 (28). The primary endpoints in this trial were 6-month progression-free survival (PFS) and objective response rate (ORR). The rate of PFS at 6 months was 42.6 and 50.3% for the bevacizumab-alone and the bevacizumab-plus-irinotecan groups, respectively. The ORR was 28.2 and 37.8%, respectively. The median OS was 9.2 and 8.7 months, respectively. Grade 3 adverse events occurred in 46.4% of individuals treated with bevacizumab

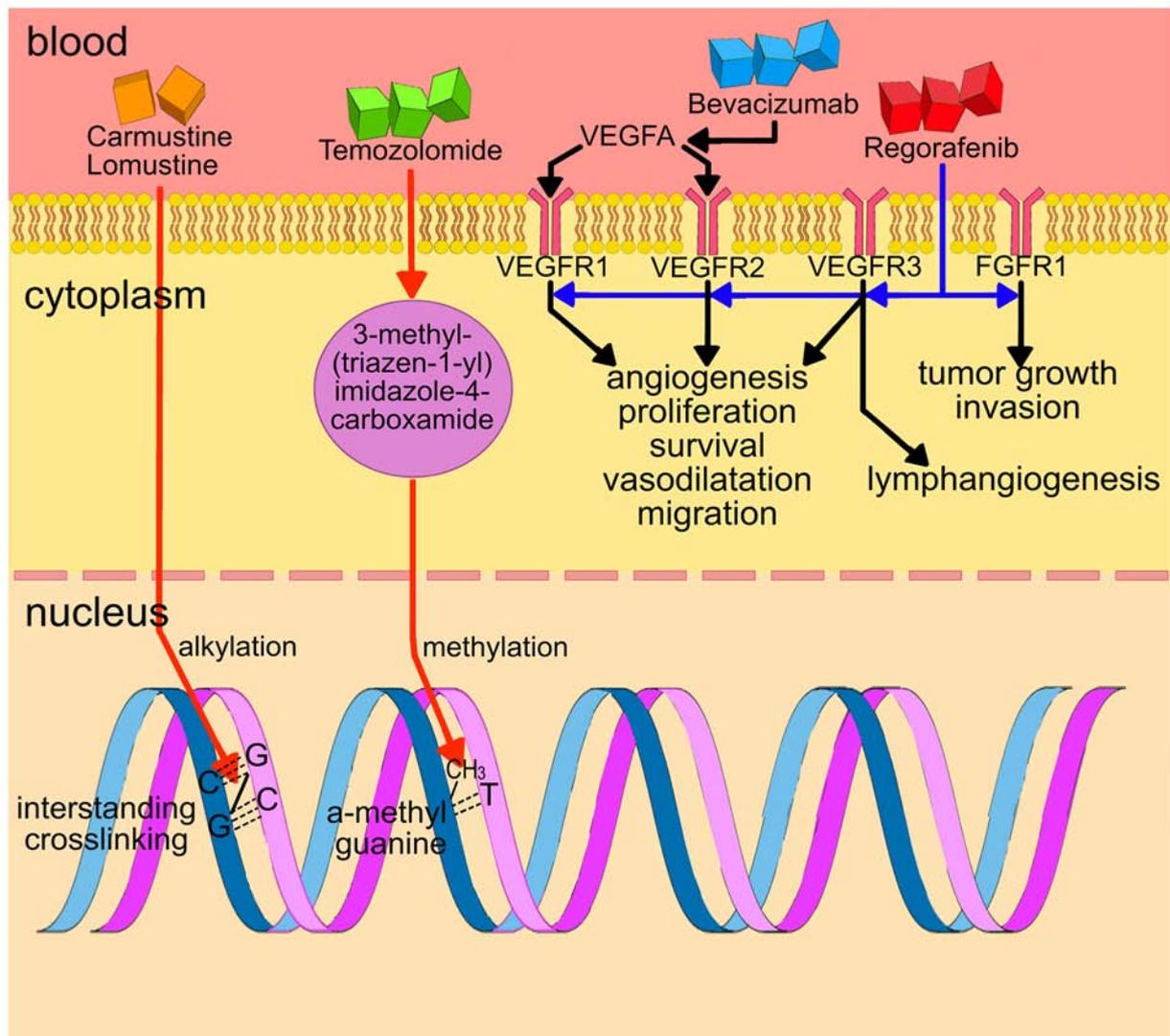


Figure 1. Mechanism of action of standard of care systemic therapeutic agents in glioblastoma multiforme. Temozolomide is converted to the short-lived active compound, MTIC. The cytotoxicity of MTIC methylase results in the methylation of guanine-rich areas of DNA, leading to inhibition of DNA replication and apoptosis. If the MGMT promoter is unmethylated, the alkyl group is removed from the DNA base guanine by MGMT protein. If the MGMT promoter is methylated, there is no active MGMT protein to repair it. Carmustine leads to inter-strand crosslinking of DNA and RNA. The mechanism of action of bevacizumab is to bind to VEGF-A and prevent its interaction with VEGFR tyrosine kinases VEGFR1 and VEGFR2 on the surface of endothelial cells. This leads to the inhibition of angiogenesis, proliferation, survival and migration of cells. Regorafenib is a multikinase inhibitor targeting several kinases (VEGFR1-3, TIE2, FGFR1 and 2, PDGFR, KIT, RAF and RET). MTIC, monomethyl triazeno imidazole carboxamide; FGFR fibroblast growth factor receptor; PDGFR, platelet-derived growth factor receptor.

alone, with hypertension (8.3%) and convulsions (6.0%) being the most common. In comparison, 65.8% of patients in the bevacizumab-plus-irinotecan group experienced grade 3 adverse events, including convulsions (13.9%), neutropenia (8.9%) and fatigue (8.9%) (29).

In patients treated with bevacizumab and standard therapy, PFS was better than with standard therapy in two studies at 4.4 months ($P < 0.0001$) in the AVAglio and 3.4 months ($P = 0.004$) in the RTOG0825 trial (30,31). However, the median OS was not different between treatment groups in both trials. In recurrent GBM, studies failed to achieve an improvement in OS and PFS in patients treated with combination therapy with bevacizumab compared to the use of bevacizumab alone (32). The combination of bevacizumab and lomustine was also studied. OS at 9 months was chosen as the primary endpoint. This combination increased the median OS to 12 months

compared to bevacizumab or lomustine alone with 8 months each. The combination also increased 6-month PFS to 42%. Several patients in the study had grade 4 or grade 3 thrombocytopenia (33). However, the increase in PFS may be related to the phenomenon of 'pseudo-response'. This phenomenon consists of improved contrast enhancement due to the normalization of vascular permeability (34). However, the usage of FLAIR or T2-weighted images revealed an increase in the nonenhancing part of the tumor. The Response Assessment in Neuro-Oncology criteria consider FLAIR/T2 hyperintensity as a surrogate for the nonenhancing component of the tumor (35).

Cediranib, a multi-kinase inhibitor targeting VEGFR, failed to achieve a significant improvement in PFS in a phase III randomized study of either cediranib alone or cediranib in combination with lomustine vs. lomustine based on independent or local review of postcontrast T1-weighted MRI (36).

Aflibercept is a recombinantly generated fusion protein that scavenges both VEGF and placental growth factor. In a phase II study, it had minimal evidence of single-agent activity in unselected patients with recurrent malignant glioma (37).

Finally, regorafenib is a recently approved oral multikinase agent that is now endorsed by the NCCN for the treatment of relapsed GBM post-radiation and TMZ (12). This drug demonstrated significant clinical activity and superiority to lomustine in a recent phase 2 trial (13).

TTF. TTF is a non-invasive antimitotic therapy, delivered by an alternating electric field by the Optune[®] system. Preclinical studies have indicated that TTF is able to alter microtubule formation, causing mitotic arrest and death. During cytokinesis, it also induces the dielectrophoretic migration of polar molecules. The phase III EF-14 study demonstrated a significant improvement in PFS and OS of patients with newly diagnosed GBM when Optune[®] was used with TMZ compared to chemotherapy alone. Compliance was associated with a better clinical outcome. The use of TTF with TMZ significantly improved median OS compared to chemotherapy alone (20.9 vs. 16.0 months, respectively; $P < 0.001$) (38). The clinical efficacy and tolerability of TTF in glioblastoma have been established in 2 large phase III studies and have been validated in real-world settings. TTF is associated with minimal adverse events (local or systemic). A limitation of TTF is that it must be worn continuously with minimal interruption. This inevitably leads to major lifestyle modifications. Furthermore, the total monthly therapy cost is ~\$21,000 (39). TTF has proven its clinical efficacy, but its usage is associated with certain disadvantages and side effects in patients with GBM, including significantly higher rates of localized skin toxicity (38). Following regulatory approval, NCCN guidelines included TTF in conjunction with TMZ for the treatment of patients with both newly diagnosed (Category 1) and recurrent glioblastoma (Category 2B) (12). Treatment strategies in conjunction with TTF provide important clinical benefits in limiting additional toxicity in patients with brain cancer and may be extended to patients with other types of solid tumor.

Surgical treatment. Despite the absence of randomized trials, surgery appears effective and the main principle of glioblastoma surgery is currently gross-total resection (40). Maximal resection improves survival irrespective of the age of the patient or the molecular status of the tumor (41). When resection is contraindicated, stereotactic biopsy is the method of choice for both histological verification and molecular evaluation of the tumor (42). The use of fluorescence-guided surgery with 5-aminolevulinic acid assessed in a prospective study provided an improvement (46 vs. 28.3%) of 6-month PFS (43). To perform safer interventions and reduce postoperative complications, functional MRI and assessment of diffusion-tensor fibers should be routinely used. As a mandatory requirement, postoperative contrast-enhanced MRI should be performed within 48 h of resection to determine the extent of the intervention (44). In case of recurrence, the most radical resection of the focus is performed, particularly if >6 months have passed since the intervention or in patients with a good functional status (high Karnofsky performance score) or a young age (45). Currently, there are no data from ongoing

randomized clinical trials regarding the surgical treatment of recurrent glioblastomas.

4. Key signaling pathways and molecular mechanisms in GBM

In GBM, alteration and/or upregulation of the Wnt, transforming growth factor β (TGF- β), VEGF, epidermal growth factor receptor (EGFR), cyclin-dependent kinase 2A (CDKN2A), nuclear factor- κ B (NF- κ B), phosphatidylinositol-3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) may be associated with pathogenesis of the disease and aggressive tumor behavior.

Wnt is responsible for the development, regeneration and homeostasis, where it mediates cellular proliferation, polarity, differentiation, motility and activity of stem cells (46). The increased activity of the canonical Wnt pathway may be responsible for the resistance to chemotherapy and RT, as well as growth, aggressiveness and invasive potential of GBM (46). EGFR (also known as ErbB1/HER1) is a type of receptor tyrosine kinase (RTK) that has an important role in the division, migration, adhesion, differentiation and apoptosis of cells (47). Under normal conditions, TGF- β is an inflammatory pathway responsible for the expression of p21 and other tumor suppressors. In cancer cells, however, TGF- β disrupts the cell cycle and mediates malignant characteristics (48). VEGF is a potent stimulator of endothelial cell growth and a key regulator of normal and pathologic growth of blood vessels and angiogenesis (49).

CDKN2A acts as a tumor suppressor gene. It encodes the p16^{ink4a} and p14^{ARF} proteins. The latter inhibits murine double minute 2 (MDM2), thus blocking MDM2-induced degradation of p53 and enhancing p53-dependent transactivation and apoptosis (50). NF- κ B is a protein transcription factor. Not only does NF- κ B have a role in immunity, but it also involved in inflammation, cancer and nervous system function (51). The active form of the NF- κ B protein dimer is the heterodimer of p65-p50. This dimer binds to specific κ B-sites regulating a wide range of cellular processes (52). Alterations of NF- κ B are frequently oncogenic due to the stimulation of tumor growth and invasion, apoptosis suppression and development of resistance to therapy (53). The PI3K/AKT/mTOR pathway regulates cellular quiescence, proliferation, cancer and longevity (54). PI3K is activated by several growth factors [e.g., human EGFR family and platelet-derived growth factor receptor (PDGFR) family growth factors]. PI3K participates in the phosphorylation of AKT. A phosphate group is also added to AKT by the mTOR complex 2 (mTORC2). Both events are required for complete AKT activation. AKT stimulates protein synthesis and cell growth; it impacts cellular proliferation by inactivating cell cycle inhibitors and promoting cell cycle proteins. AKT promotes cell survival as well (55). The PI3K/AKT/mTOR pathway is hyperactivated in several cancer types, including glioblastoma. The number of alternated signaling pathways reflects the potential targets, which may prove effective in improving clinical outcomes in patients with glioblastoma; these are summarized in Table I.

5. Biology of GBM and therapeutic targets of key pathways

Genomic analysis of glioblastoma revealed several signaling pathways and gene alterations that are critical for its

Table I. Potential molecular targets in glioblastoma.

Molecular mechanism	Function	Prevalence in glioblastoma multiforme	Current and possible treatment strategies	(Refs.)
VEGF-A	Potent stimulator of endothelial cell growth and a key regulator of normal and pathologic growth of blood vessels	Overexpression in 26.97%	Bevacizumab-humanized monoclonal IgG1 antibodies to VEGF-A	(49,29)
EGFR	A type of receptor tyrosine kinase that has an important role in the division, migration, adhesion, differentiation and apoptosis of cells	Overexpression and/or mutation in 40%	Therapies target EGFR or its mutant constitutively active form, Δ EGFR, including tyrosine kinase inhibitors, monoclonal antibodies, vaccines and RNA-based agents	(47,71,132)
PI3K/AKT/mTOR	Directly related to cellular quiescence, proliferation, cancer and longevity	Overexpression in 90%	BKM120 and PX-866-PI3K inhibitors. Perifosine-Akt inhibitor. Rapamycin (sirolimus) and its analogues, such as RAD001 (everolimus), CCL-779 (temsirolimus) and AP23573 (ridaforolimus)-mTORC1 inhibitors	(54)
p53	p53 suppresses cell transformation, causing cell cycle termination, repair of damaged DNA, cell aging or apoptosis	Mutated in 28,3% ^a	The nutlin analogs RG7112 and RG7388, MI77301, CGM097, MK8242 and AMG232-inhibitors of the MDM2/p53 interaction. PRIMA-1 alters mutant protein folding to restore wt-p53 conformation and p53 function	(64, 184)
NF- κ B	NF- κ B in neurons maintains neuronal health, synapse growth and plasticity-related functions and regulates the cell activity	Overexpressed in 81%	NF- κ B inhibitor parthenolide NF- κ B inhibitor CBL0137 NF- κ B inhibitor BAY 11-7082 Amentofavone	(52,89,95,185)
Wnt	The processes of development, regeneration and homeostasis, where it mediates cellular proliferation, polarity, differentiation, motility and activity of stem cells	Adenomatous polyposis coli mutations in 13%	While numerous molecular targeted drugs have entered early-stage clinical trials, none of them have been released into the market to date	(46,98)
TERT	TERT enables cells to avoid chromosome shortening during repeated replication by maintaining telomere length. Function of TERT in tumor formation and progression	TERTp mutations in 51%	A study indicated that in IDH-wt patients, pTERT mutation identified those individuals who would experience a survival benefit from adjuvant chemotherapy or radiotherapy	(186-188)

Table I. Continued.

Molecular mechanism	Function	Prevalence in glioblastoma multiforme	Current and possible treatment strategies	(Refs.)
CDKN2A	A gene that encodes two proteins, including the INK4 family member p16 (or p16 ^{INK4a}) and p14 ^{arf} . Both act as tumor suppressors by regulating the cell cycle		None	(69)

^ap53 signaling pathway that includes CDKN2A, MDM2 and TP53 is disrupted in ~5% of glioblastoma cases. CDKN2A, cyclin-dependent kinase 2A; IDH, isocitrate dehydrogenase; wt, wild-type; tert, telomerase reverse transcriptase.

development: Cell cycle checkpoints, apoptosis, TGF- β , NF- κ B, the notch signaling pathway, also signaling pathways associated with growth factors and RAS, such as the PI3K/AKT/mTOR pathway, EGFR, tensin/AKT homologs and the CDKN2A pathway (56,57).

It is worth noting that human malignant gliomas are rarely dependent on a single oncogene or tumor suppressor gene, which may explain the lack of efficacy of drugs targeting only one molecular alteration in clinical trials (58). Furthermore, the BBB restricts the entry of chemotherapeutic agents into the tumor. Intra-tumoral presence of cancer stem cells (CSCs) that are characterized by chemo- and radioresistance may also contribute to the aggressiveness and high recurrence rate of GBM (6). Another problem is the infiltrative nature of GBM cells, which limits the feasibility of complete surgical resection, despite the advances in neurosurgery techniques (8).

The Cancer Genome Atlas (TCGA) published a study analyzing main mutational events in GBM, according to which three main genetic events occur in human glioblastomas: i) Amplification and mutational activation of RTK genes; ii) activation of the PI3K pathway; and iii) inactivation of the p53 and retinoblastoma tumor suppressor pathways (59). In terms of epigenetic events, the MGMT promoter is methylated in ~50% of newly diagnosed GBM cases. MGMT encodes a DNA repair protein that disrupts the therapeutic process by removing alkyl groups from guanine-rich areas in DNA, a target for alkylating agents such as TMZ. The DNA methylation status of this gene may be a useful biomarker of the chemotherapy response and explain in part why patients with a methylated MGMT gene promoter may have longer OS (60).

Glioblastoma may harbor the codeletion 1p/19q. This codeletion has an association with chemosensitivity and favorable prognosis in oligodendroglioma (61). Therefore, it was proposed that in GBMs with oligodendroglioma component 1p/19q codeletion may also have a prognostic value. However, this still remains to be fully demonstrated (62). In one study, the frequency of 1p/19q codeletion in GBM was 3%, but neither codeletion, nor isolated mutations were associated with increased survival and had no prognostic value (63). The tumor suppressor gene TP53, encoding the transcription factor p53, is the most frequently mutated gene in various types of

malignancies, and in GBM, it is the second-most commonly mutated gene (28,3%) after phosphatase and tensin homolog (PTEN) (30.7%) (64). In accordance with its primary role in suppressing oncogenesis, mutations that disrupt the function of wt-p53 are common in human malignant tumors (65). TCGA project data indicated that the p53 signaling pathway (including CDKN2A, MDM2 and TP53) is disrupted in ~85% of glioblastoma cases (66).

Within the glioblastoma tumors reside ontogenically distinct immunoregulatory macrophages [spalt-like 1-positive (Sall1⁺) tumor microglia, Sall1⁻ monocyte-derived macrophages), immunosuppressive T-regulatory cells (e.g., C-C chemokine receptor 8-positive)] and dysfunctional T-cell populations [high levels of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1] (67). Computational analysis grouped glioblastoma tumors into three immune response-related subgroups: i) Negative (defined by a relative paucity of immune cells; enriched in TCGA-proneural cells and cyclin-dependent kinase 4-membrane associated ring-CH-type finger 9 amplification); ii) humoral (defined by a high B-cell and CD4⁺ T-cell compartment; enriched in TCGA-mesenchymal cells); and iii) cellular-like (defined by a higher 'negative regulation of T-cell activation' and 'gamma delta T-cell' cluster; enriched by classical TCGA-CL cells and samples with a high macrophage content) (7). For further information on additional signaling pathways please refer to Garofano *et al* (68).

CDKN2A is a gene located on chromosome 9, band p21.3. It is ubiquitously expressed in numerous tissues and cell types (50). Germinal CDKN2A mutations have been described to be associated with familial glioblastoma (69). The presence of multiple altered signaling pathways in GBM emphasizes the notion of tumor dependency on dysregulation of multiple molecular targets that may alter tumor biology, as illustrated in Fig. 2.

6. EGFR in GBM

EGFR has an important role in the division, migration, adhesion, differentiation and apoptosis of cells. EGFR consists of the extracellular domain, which binds ligands, a transmembrane

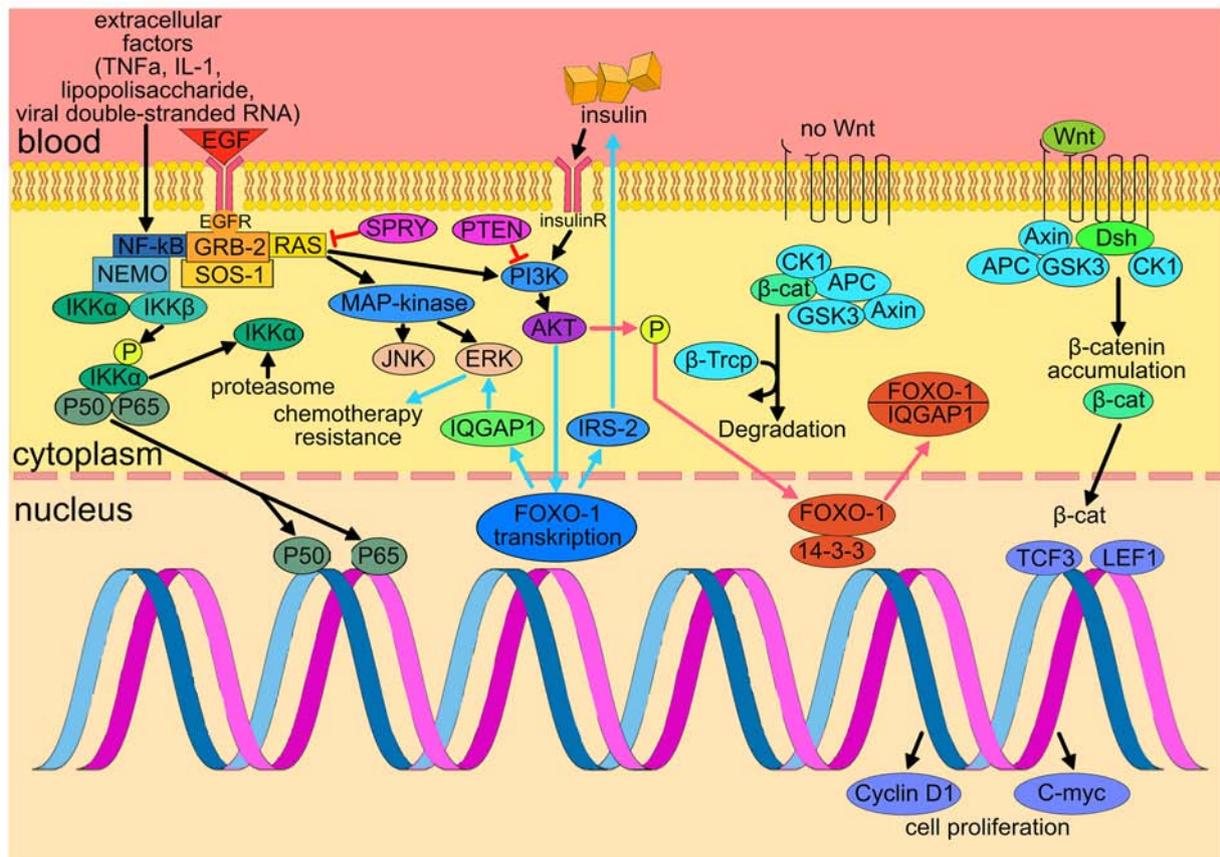


Figure 2. Schematic representation of major molecular mechanisms involved in glioblastoma development. Binding of EGF to the EGFR results in activation of numerous downstream signaling pathways, including SOS1, GRB2 and PI3K-Akt-mTOR. Various extracellular factors lead to NF- κ B activation. NF- κ B dimers (p65-p50) are inactive in normal cells due to binding to I κ B inhibitory factors in the cytoplasm, which blocks the nuclear localization sequence and prevents the transfer of NF- κ B into the nucleus. In the nucleus, NF- κ B dimers bind to κ B-sites in the regulatory regions of genes participating in a wide range of cellular processes. Ras is a key player in the RTK-mediated PI3K/AKT and MAPK signaling pathways. The activation of all isoforms of RAS protein by the exchange of GDP with GTP results in the activation of MAPKs that also activate downstream ERK via phosphorylation. PI3K-activated AKT phosphorylates FOXO proteins at 3 serine/threonine residues, resulting in the promotion of nuclear exclusion and inactivation of the transactivation-dependent (genomic) tumor suppressor activities of FOXO proteins in the nucleus. Wnt signaling is inactivated in the absence of Wnt ligands. When inactive, cytoplasmic β -catenin is degraded by a β -catenin destruction complex, which includes Axin, adenomatosis polyposis coli, protein phosphatase 2A, GSK3 and casein kinase 1 α . Phosphorylation of β -catenin within this complex by casein kinase and GSK3 targets it for ubiquitination and subsequent proteolytic destruction by the proteasomal apparatus. The active Wnt signaling pathway activates β -catenin, which is shuttled into the nucleus, leading to transcriptional activation of WNT signaling-target genes. SOS1, Son of Sevenless 1; GRB2, growth factor receptor bound protein 2; GSK3, glycogen synthase kinase 3; GDP, guanosine diphosphate; FOX, forkhead box.

domain and the intracellular tyrosine kinase domain. Activation of EGFR leads to the activation of numerous downstream signaling pathways, such as PI3K-AKT-mTOR, leading to cancer proliferation and therapy resistance (47).

Amplification of EGFR and/or its overexpression at the protein level are common alterations that occur in 35-45% of cases of GBM (70). Amplification of its active mutant EGFRvIII in GBM (characterized by an in-frame deletion of exons 2-7) is also distinctive for GBM and is found in 50% of cases (71). While EGFR and EGFRvIII have a crucial role in the pathogenesis of GBM, inhibitors of EGFR tyrosine kinase and antibodies demonstrated low efficacy in clinical trials (72). Overexpression of EGFR results in an abnormal activity of downstream signaling pathways, including son of sevenless 1 (SOS1), growth factor receptor bound protein 2 (GRB2), RAS protein and AKT (73). GRB2 is a molecular adapter protein that coordinates other signaling molecules, including SOS1, NF- κ B, Ras and Akt. Thus, GRB2 enhances other types of cell activity, such as

cell proliferation, epithelial to mesenchymal transition and tumor development (74).

Overexpression of EGFR/mutant EGFRvIII is associated with increases in proliferation and migration of GBM cells. These properties affect the malignant phenotype of these tumor cells (75). Expression of EGFRvIII also stimulated and accelerated angiogenesis in preclinical models of GBM *in vivo* (76). Potential therapies that target EGFR or EGFRvIII, including tyrosine kinase inhibitors (TKIs), such as erlotinib, gefitinib and lapatinib, as well as monoclonal antibodies, vaccines and RNA-based agents, are currently in development or in clinical trials for the treatment of GBM (72).

Erlotinib exhibited minimal activity only against tumors that overexpressed EGFR and PTEN (77). Given that PTEN mutations are found in 41% of patients with GBM (78), erlotinib may not be a good therapeutic option for the majority of GBM cases with overexpression of EGFR. Erlotinib was indicated to be ineffective as a monotherapy in patients with recurrent GBM and only slightly beneficial as a follow-up to

RT in patients with non-progressive GBM (79). Unlike erlotinib, gefitinib has anti-tumor activity regardless of the level of EGFR expression (80); however, only minor clinical effects were observed in phase II trials. Several phase I/II trials have indicated that while adding gefitinib to RT improves tolerability, it only has a minor effect on the survival rate (81,82).

One of the difficulties of the analysis of the impact of EGFR amplification on targeted therapy is that amplification may be lost when cells from EGFR-amplified GBM are placed in the cell culture (70). As a result of this constraint, preclinical models studying the biology of EGFR in GBM substantially relied on the ectopic overexpression of EGFR and/or EGFRvIII in non-amplified cell lines of GBM and subsequent blockage of overexpressed proteins (83). Another reason for the failure of targeting EGFR is the heterogeneous distribution of EGFR in the tumor. This may lead to differential EGFR sensitivity, which may eventually result in treatment failure (84).

Depatuzumab mafodotin (Depatux-M) is a tumor-specific combination made up of an antibody directed against EGFR antibody (ABT-806), conjugated to the toxin monomethylauristatin-F. In a phase II trial, this drug was tested in patients with centrally confirmed EGFR-amplified glioblastoma at first recurrence after concurrent chemoradiation with TMZ. The efficacy of Depatux-M monotherapy was comparable to that of the control group [hazard ratio (HR)=1.04, 95% CI=0.73-1.48; P=0.83], thus failing to meet the primary endpoint (85).

7. Targeting signaling pathways in GBM

NF-κB in GBM. Aberrant constitutive activation of the NF-κB signaling pathway is common in GBM. The constitutive NF-κB hyperactivation is oncogenic due to the stimulation of tumor growth and invasion, apoptosis suppression and development of resistance to therapy (53). The most common form of NF-κB protein dimer is the heterodimer of p65-p50. This dimer is able to bind to a specific sequence (i.e., NF-κB sites) of the target gene to regulate gene transcription. NF-κB regulates cell activity through slight differences in the binding of these NF-κB dimers to target sequences (52). In non-stimulated cells, NF-κB dimers are inactive due to binding to three inhibitory factors (IκBα, IκBβ and IκBε) of NF-κB in the cytoplasm, which blocks the nuclear localization sequence and prevents the transfer of NF-κB into the nucleus. In the nucleus, NF-κB dimers bind to κB-sites in the regulatory regions of genes participating in a wide range of cellular processes (86). NF-κB in neurons maintains neuronal health, synapse growth and plasticity-related functions (87).

Activation of the NF-κB signaling pathway is common in various cancer types. Numerous mechanisms have been proposed that may lead to the disruption of NF-κB signal regulation in gliomas. For instance, RTK, primarily EGFR and PDGFR that are frequently activated in glioblastomas, may be triggered secondary to the activation of NF-κB through several mechanisms such as AKT-related and -unrelated signaling pathways. In murine models, it has been demonstrated that inhibition of NF-κB by depletion of IκB kinase 2, expression of an IκBaM super repressor or using an NF-κB essential modifier-binding domain attenuated tumor proliferation and prolonged survival (88). In one of the studies, NF-κB p65 subunit was indicated to be overexpressed in 81% of cases of GBM (89).

Oncogenic mechanisms of EGFR and PDGFR signal transduction make a major contribution to the growth and invasion of GBM. The NF-κB pathway interplays with these receptors, which may lead to the development of GBM (90). Loss of the tumor suppressors, such as neurofibromin 1 is also associated with the disruption of activation of NF-κB in GBM due to the upregulated activity of PI3K (91). Disruption of the tumor suppressor Krüppel-like factor 6, which serves as a negative regulator of NF-κB, also contributes to the activation of NF-κB (92).

Exposure of GBM cell lines to NF-κB-p65 small interfering (si)RNA and NF-κB inhibitors resulted in a significant decrease in GBM-cell viability. Treatment of GBM with NF-κB inhibitors overcame cisplatin resistance and led to an increase in the effects of cisplatin and doxorubicin. Importantly, normal astrocytes were less sensitive to NF-κB inhibition, implying tumor cell selectivity (93).

It has been proposed that numerous other mechanisms have an important role in the disruption of signal transduction of NF-κB, including facilitation of NF-κB by peptidyl-prolyl cis-trans isomerase NIMA-interacting 1, mixed lineage kinase 4 and heterozygous deletion of the NFKBIA gene, which encodes IκBα (94). These observations emphasize the potential role of abnormal NF-κB signaling pathways in various mechanisms of GBM pathogenesis.

Amentofavone is a flavonoid that is able to cross the BBB and inhibit the NF-κB pathway by inhibiting IκB kinase degradation. Treatment with this compound reduces the viability and proliferation of GBM cells, resulting in the emergence of sub-G1 population, which indicates apoptosis. To date, amentofavone has not been evaluated in any randomized clinical trial (95).

Wnt pathway alterations in GBM. The Wnt signaling pathway is a primordial instructive genetic program (96). Depending on the type of interaction between Wnt and Frizzled protein and consecutive involvement of β-catenin, there are one β-catenin-dependent canonical pathway and two β-catenin-independent noncanonical pathways (planar cell polarity and Wnt/Ca²⁺).

Wnt/β-catenin pathway activity has been linked to neural progenitor cell proliferation in the early stages of brain development, while it reduces the self-renewal capacity and promotes neuronal differentiation in later stages (97). Recent reports from a small cohort have reported mutations of adenomatous polyposis coli (which is responsible for Wnt activation) in ~13% of GBM cases with a mutation frequency of close to 14.5% (98). It has been reported that the increased activity of the canonical Wnt pathway is responsible for GBM resistance to chemotherapy and RT (99), contributing to the growth, aggressiveness and invasive potential of GBM (100). It also generates CSCs from differentiated cells (97).

It should be noted that the hypoxic environment influences Wnt-induced differentiation. Furthermore, hypoxia-inducible factor 1α is required to sustain the expression of transcriptional associates of β-catenin the transcription factor T cell factor and lymphoid enhancer binding factor) (101). In addition, Wnt-induced differentiation inhibits Notch signal transduction and thus, enhancement of Wnt and Notch suppression lead to the activation of pre-neuronal differentiation (102).

Although the Wnt pathway has been thoroughly studied and numerous molecular targeted drugs have entered the clinical trial stage, insight into the efficacy of these medications is lacking (40). In the canonical Wnt signaling pathway, the Wnt protein interacts with the Frizzled and low-density lipoprotein receptor-related protein 5/6 receptor. This binding was inhibited by monoclonal antibodies, including vanticumab (OMP18R5) and ipafricept (OMP54F28), thus blocking the Wnt signaling pathway (103). Vanticumab has been indicated to be well tolerated in a phase I trial with nearly no gastrointestinal adverse effects at the effective dose (104). While clinical trials are ongoing, it remains elusive at this time whether these drugs have brain efficacy or whether any GBM trials are planned with these agents.

Telomerase reverse transcriptase (TERT) promoter mutations in GBM. TERT encodes the catalytic subunit of the telomerase complex. Since telomerase activity is a function of this catalytic subunit, mutations of the TERT gene promoter are frequently associated with cancer. These mutations are most often represented by nucleotide substitutions in the two most common 'hot spots': At position -124 up from the transcription initiation site (nucleotide polymorphism chr5: 1,295,228 G>A, also called C228T) and at region -146 (nucleotide polymorphism chr5: 1,295,250 G>A, known as C250T) (105).

The TERT promoter (TERTp) mutation (pTERTmut) was originally detected in melanoma. Follow-up studies also revealed a high frequency of pTERTmut in IDH-wt GBM, as well as in IDHmut oligodendroglioma and oligodendroglioma with 1p/19q co-deletion, and demonstrated its potential use for glioma classification (106).

Most glioblastomas may be divided into molecular subgroups based on mutations in TERTp and IDH 1/2. These molecular subgroups use different genetic mechanisms to maintain telomeres: This is either a TERTp mutation leading to telomerase activation or an α -thalassemia/mental retardation, X-linked mutation leading to an alternative extension of the telomere phenotype. TERTp-mutant GBM demonstrates telomerase activation due to the *de novo* generation of transcription factor binding sites, leading to increased TERT expression. These tumors, designated glioblastomas TERT pWT-IDH WT, do not have any well-established genetic biomarkers or specific mechanisms for maintaining telomeres (107).

In one of the studies, overexpression of the C-terminal fragment of human TERT (hTERTC27) was demonstrated to inhibit the growth and oncogenicity of HeLa cells (108). The therapeutic effect and molecular mechanisms of gene therapy for hTERTC27-mediated malignant tumors were further studied *in vivo* on human glioblastoma xenografts in thymus-free mice. Intra-tumor injection of the hTERTC27-carrying adeno-associated virus (rAAV-hTERTC27) has been demonstrated to be effective in slowing the growth of subcutaneously transplanted glioblastoma tumors. Histological analysis suggested that treatment with rAAV-hTERTC27 led to deep necrosis, apoptosis, infiltration of polymorphonuclear neutrophils and a decrease in the density of microvessels in tumor samples (109). Another pre-clinical study reported increased expression of the hTERT gene in patients with high-grade glioma that may be associated with the aggressiveness of the

tumor (patients with low hTERT mRNA levels in the tumors had a median PFS of 24 months and patients with low hTERT levels had a PFS of 11 months). It was indicated that when hTERT mRNA expression was reduced by siRNA, this led to a decrease in the cell viability. Therefore, targeting TERT using small molecules or other approaches may lead to the development of novel therapeutic agents in the future (110).

Liu *et al* (103) demonstrated the feasibility of gene editing as a pre-clinical therapeutic approach, utilizing CRISPR-associated protein 9 from *Streptococcus pyogenes* or *Campylobacter jejuni*, together with chimeric guide RNA (sgRNA), which is a programmable endonuclease that may be used to modify, regulate or label genomic loci in a variety of cells. Local injection of adeno-associated viruses expressing sgRNA-controlled *Campylobacter jejuni* CRISPR-associated protein 9-fused adenine base editor suppressed the growth of gliomas carrying mutations of the TERT promoter (111).

PI3K/AKT/mTOR signaling pathway in GBM. The PI3K/AKT/mTOR intracellular signaling pathway is responsible for growth, cell proliferation and metabolism (54). There are three classes (I, II and III) of PI3K, which differ in substrate specificity and products. Class I kinases are the most well-studied; they are heterodimers of regulatory and catalytic subunits. These enzymes may be activated by G-protein-associated receptors and RTK. After ligand binding, RTK autophosphorylate tyrosine residues in their cytoplasmic domains. The regulatory subunits of PI3K contain an SH2 domain that allows them to recognize and bind phosphotyrosine residues of RTK. As a result, kinases are in close proximity to the membrane, and hence their substrates, so the synthesis of PI3K begins (112). PI3K inhibitors may be classified into pan-PI3K, isoform-selective and dual PI3K/mTOR types (113). The frequency of mutations of PIK3CA in GBM (encodes p110 α , which is part of a catalytic subunit of class IA PI3K) ranges from 4 to 27% (113). Through decreased AKT and FAK activation, PIK3CA knockdown significantly decreases cell survival, migration and invasion of GBM cells (114). The p110 α isoform-selective inhibitors A66 or PIK-75 effectively suppressed GBM cell growth, survival and migration *in vitro* (115). In the absence of PTEN, p110 β has a critical role in GBM cell proliferation, survival and migration. *In vitro* and *in vivo*, knockdown of PIK3CB (encodes p110 β) suppresses cell proliferation and triggers caspase-dependent apoptosis in GBM, and it works in tandem with PTEN restoration (116). The selective inhibitor of p110 β TGX-221 significantly reduces cell migration in GBM cells while having a minimal effect on survival and invasion (115). Thus, AKT-phosphorylated forkhead box O proteins may have tumor suppressor functions unless they are degraded by E3 ubiquitin ligases (117).

mTOR also belongs to the PI3K-related kinases (118). mTOR is a core component of two functionally different multi-subunit protein complexes named mTORC1 and mTORC2 (119). Various extracellular stimuli, such as growth factors, nutrients or amino acids, cause a strong association of mTOR with various protein molecules. PI3K/AKT/mTOR regulates various growth signals by directly phosphorylating the immediate substrates (120). In a normal cell, various RTKs, such as EGFR, insulin receptor and G-protein coupled receptor avails extracellular stimuli from various growth

factors. RTKs stimulate the recruitment of a family of lipid kinases known as class 1 PI3Ks to the plasma membrane, where they phosphorylate the glycerophospholipid phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] at the D-3 position of the inositol ring, converting it to PtdIns(3,4,5)P₃. PTEN, the tumor suppressor which reverses phosphatidylinositol 3,4,5-trisphosphate to phosphatidylinositol 4,5-bisphosphate, counteracts this activity (121). Hyperactivation of the mTOR signaling pathway occurs in ~90% of glioblastomas. The mTOR inhibitor rapamycin has failed in clinical studies to demonstrate efficacy in patients with GBM, partially as a consequence of persistent mTORC2 signaling (122). Expression of activated mTORC2 was indicated to be nearly undetectable in normal brain tissue but was high in tumor cell lines. These same investigators discovered that 86% of tumor samples had rapamycin-insensitive companion of mammalian target of rapamycin overexpression and 70% of them had strong mTORC2 activity, which matched the *in vitro* observations (123).

The deregulation of the mTOR pathway was also correlated with radioresistance and in pre-clinical studies, it has also been proposed that PI3K/mTOR inhibition rendered GBM tumors radiosensitive (124).

mTORC1 inhibitors include rapamycin (sirolimus) and its analogues, such as RAD001 (everolimus), CCL-779 (temsirolimus) and AP23573 (ridaforolimus) (55). These medications are first-generation mTOR inhibitors. A total of 171 patients with newly diagnosed GBM took part in the everolimus phase II study. RT with concurrent and adjuvant TMZ, with or without daily everolimus (10 mg), was provided to patients. When comparing patients in the everolimus group to those in the control group, there was no significant difference in PFS. Patients who received everolimus, on the other hand, had a considerable increase in toxicities (125). The only known peri-surgical phase I study of ridaforolimus in grade IV malignant glioma was suspended due to slower than expected patient accrual and postsurgical drug administration challenges (126). In the phase II study of RT with temsirolimus vs. radiochemotherapy with TMZ, a total of 257 patients were enrolled. In the temsirolimus arm, the median OS was 14.8 months, while in the control arm, it was 16.0 months. The temsirolimus arm had a median PFS of 5.4 months, while the control arm had a median PFS of 6.0 months (127). The combination of RT and temsirolimus is currently being further studied in a phase I/IIa trial that seeks to selectively match patients with targeted therapies based on known alterations (N2M2 trial) (128). Over the past several years, great effort has been put into developing second-generation ATP-competitive mTOR kinase inhibitors (TORKi), including INK128, Torin 1 and AZD8055, and third-generation bivalent mTOR inhibitors that specifically target mTOR resistance mutations (129). However, TORKi have not yet demonstrated clinical effectiveness in GBM, likely due to the limited capacity of mTOR inhibitors to cross the BBB and compensatory AKT activation (130).

Targeting c-mesenchymal-epithelial transition factor (c-Met) in glioblastoma. c-Met is an RTK, expressed on the surfaces of various cells. Hepatocyte growth factor (HGF) is the ligand for this receptor. HGF binding leads to a sequence of intracellular signals that mediate embryogenesis and wound

healing in normal cells. In cancer cells, aberrant HGF/c-Met axis activation, which is closely related to c-Met gene mutations, overexpression and amplification, promotes tumor development and progression by stimulating several signaling pathways (131). Approximately 37% of patients with GBM have c-Met overexpression (132). c-Met also has a role in the resistance mechanism that drives GBM invasion in xenografts. Resistance to anti-angiogenic drugs may be mediated by upregulation of c-Met gene expression (acquired resistance) or due to selective survival (intrinsic resistance) of tumor cell subpopulations overexpressing c-Met (133). It is worth noting that the shortest time to progression and OS was observed in GBM accompanied by overexpression of c-Met and VEGFR2, which indicates the primary/innate activation of the two pathways (134).

Several drugs targeting c-Met have been studied in clinical trials. Onartuzumab, which is an anti-c-met monoclonal antibody, is highly specific for binding c-Met. This antibody is able to block the binding of c-Met-HGF by blocking the HGF α -chain and forming a complex with the Sema-PSI domain of c-Met (135). This process does not lead to any agonistic activity or trigger c-Met dimerization. Recent clinical trials did not indicate any clinical benefit with onartuzumab in GBM (136). Other types of drugs that may affect c-Met are small molecule inhibitors. Crizotinib is an effective small molecule inhibitor of c-Met, derived from the first-generation series c-Met inhibitor, PHA-66752. Crizotinib targets the TK domain of c-Met and is approved by the FDA for the treatment of advanced non-small-cell lung carcinoma (131). In the context of GBM, a recent phase Ib dose-escalation study followed by an extension phase with crizotinib was added to standard RT and TMZ. This study indicated highly promising efficacy for newly diagnosed GBM, warranting further investigation (137). Other small molecule inhibitors of c-Met include cabozantinib, foretinib, LY280163, MK2461, capmatinib, tivantinib, which may be tested in future GBM trials (131).

Targeting fibroblast growth factor receptor (FGFR) and BRAF in glioblastoma. FGFRs control numerous biological functions, including cell proliferation, survival and cytoskeletal regulation. The FGFR signal is important in the embryonic development of the CNS and serves as the survival mechanism of adult neurons and astrocytes (138). In addition, it was indicated that FGFR signaling may promote the self-renewal and fate specification of neural stem cells (139). FGFR expression changes in astrocytes may prompt malignant transformation and GBM progression due to the activation of mitogenic, migratory and antiapoptotic reactions (140). Whole-genome analyses of patient samples have uncovered that the rate of FGFR mutations and amplifications are exceptionally low in GBM (<2%) (141). Several FGFR inhibitors have been developed over the past years. Fisolatinib is an inhibitor of the FGFR4 gene. Clinical trials suggested that fisolatinib has high activity and selectivity, resulting in considerable anti-tumor efficacy (142). Fisolatinib is able to covalently bind to a specific cysteine residue identified in FGFR4 (Cys 552), giving it a high degree of selectivity over other FGFR family members (133). Currently, this drug has only been studied in phase I clinical trials for the treatment of hepatocellular carcinoma (143). However, the study in mice indicated that brain accumulation

of fisogatinib is significantly limited by ATP binding cassette subfamily B member 1 P-glycoprotein in the BBB, while oral availability of fisogatinib is markedly constrained by CYP3A activity (144). Futibatinib is also an irreversible inhibitor of FGFR. Several tumor cell lines with distinct genetic abnormalities of FGFR exhibited effective and specific growth suppression with futibatinib (145). However, to date, it has not been studied in the settings of GBM or CNS tumors. Another drug is AZD4547, which is a selective FGFR1-3 inhibitor. In an FGFR3-transforming acidic coiled-coil containing protein 3 (TACC3) glioma xenograft model, oral administration of AZD4547 resulted in longer survival compared with that of mice that were given the vehicle control (146). AZD4547 has been studied in patients with recurrent IDH-wt gliomas with FGFR1-TACC1 or FGFR3-TACC3 fusions (147).

The BRAF proto-oncogene serine/threonine kinase (BRAF) is a member of the Raf kinase family (148), which consists of three kinases: ARAF, CRAF (RAF-1) and BRAF. BRAF has an important role in regulating of the MAPK/ERK pathway. Hyperactivation of this pathway may cause cell cycle arrest, while aberrant regulation of the pathway may lead to carcinogenesis (149). BRAF activation in human neural stem and progenitor cells not only triggers tumor growth, but also subsequently leads to oncogene-induced senescence in certain low-grade brain tumors (150). This may explain the relatively high frequency of BRAF mutant brain tumors associated with good prognosis. BRAF gene alterations, on the other hand, are also seen in diffusely developing malignancies in adults, which are associated with poor prognosis (151).

In total, >40 mutations have been discovered in the BRAF gene, with a single thymine-to-adenine nucleotide base change at position 1,799 accounting for 90% of them. This missense mutation causes a substitution of glutamine for valine at position 600 (V600E). BRAF^{V600E} leads to a ~500-fold increase in gene activity. It allows signaling cascade activation in the absence of external stimuli such as growth signals, allowing cells to become self-sufficient in this pathway (152). The BRAF^{V600E} mutation is rare in primary and metastatic CNS neoplasms, found in 4% of cases (151).

BRAF^{V600E} mutation has a higher frequency in certain brain tumors, such as pleomorphic xanthoastrocytoma, ganglioglioma and pilocytic astrocytoma, and in epithelioid and giant cell glioblastoma (148). Genetic analyses have indicated a particularly high percentage of BRAF^{V600E} (50-93%) in epithelioid glioblastoma (153). This tumor also possesses TERT promoter mutations (70%) and homozygous deletions of CDKN2A/2B (79%) (153).

BRAF inhibitors targeting the BRAF^{V600E} mutation, such as dabrafenib and vemurafenib, provided a big step forward in the treatment of patients with malignant melanoma. Currently, BRAF inhibition is a treatment option for a small subset of patients with recurrent GBM if the V600E mutation is present (154).

Another study reported a marked radiological response and a stable clinical outcome in patients with malignant BRAF V600E-mutated glioma with leptomeningeal tumor appearance who received dabrafenib alone for up to 27 months (155). In one of the three instances in this case series, histology revealed glioblastoma, whereas the other diagnoses were compatible with anaplastic pleomorphic xanthoastrocytomas.

Primary treatment with BRAF and MEK inhibitors has been indicated to lead to tumor regression in patients with BRAF V600E mutant glioblastoma. As a result, it was proposed that all young patients with GBM, particularly those with an unusually aggressive tumor behaviour, should be tested for BRAF^{V600E} mutation (156).

Targeting proto-oncogene tyrosine-protein kinase (Src) in glioblastoma. Preclinical and human tumor studies support a potentially important role for Src in human glioblastoma. In transgenic mice expressing v-Src, glioblastomas may potentially develop due to this alteration (157). Dasatinib is a TKI that reduces Src autophosphorylation and downstream signaling to AKT and phosphor-S6 in GBM cell lines, also reducing the growth and invasion of glioblastoma cells. Inhibition of src-family kinases by dasatinib also causes death of autophagic glioblastoma cells *in vitro* (158). Src signaling is also markedly enhanced in patients with invasive glioblastoma after administration of bevacizumab. The Src family kinase inhibitor dasatinib effectively blocked bevacizumab-induced invasion of glioma in preclinical models, leading to the hypothesis that combining bevacizumab with dasatinib may increase the efficacy of bevacizumab in patients with recurrent GBM (159).

Despite encouraging pre-clinical data, in the clinical study of Galanis *et al* (159), the combination of bevacizumab with dasatinib did not significantly improve outcomes in patients with recurrent GBM compared to treatment with bevacizumab alone. Although the combination had an acceptable tolerance profile in this sample of 121 patients and an improvement in PFS at 6 months was observed in the bevacizumab-dasatinib group, the efficacy threshold was not reached with a PFS-6 of 28.9% for bevacizumab with dasatinib vs. 18.4% for bevacizumab alone (P=0.22).

8. Glioblastoma vaccines, immunotherapy and checkpoint inhibitors

Several experimental therapeutic vaccines have been developed over the past decades to treat GBM. Dendritic cell vaccines have been able to modify the immune response in patients with malignant neoplasms and induce anti-tumor immunity (160). Recent advances in vaccination with dendritic cells have achieved encouraging results in clinical trials and may improve the survival of patients with GBM (160). A recent double-blind, randomized phase II trial (161) evaluated the efficacy of the ICT-107 vaccine based on autologous dendritic cells loaded with six epitopes targeting GBM-associated antigens: melanoma antigen gene 1, human epidermal growth factor receptor 2 (HER-2), absent in melanoma 2, tyrosinase-related protein-2, gp100 (also known as premelanosome protein) and interleukin-13 receptor subunit α -2 in patients with newly diagnosed GBM, whose major histocompatibility complex serotype was human leukocyte antigen (HLA)-A1+ and/or HLA-A2+. The vaccine increased PFS by 2.2 months (P=0.011) but did not increase OS (17 months for the treatment group and 15 for the control group).

The frequency of expression of the primary tumor antigen HLA-A2 in patients (>90%) was higher than that of HLA-A1 (37.8%). Patients with HLA-A2 exhibited a more pronounced

immune response to the vaccine (assessed using Elispot) and a significant therapeutic effect was observed in patients with a methylated MGMT gene promoter (PFS, 24.1 vs. 8.5 months in the control group) and with an unmethylated one (PFS, 10.5 vs. 6 months in the control group). This study demonstrated the possible clinical efficacy of ICT-107 in patients with the HLA-A2 serotype (152).

KHS101 exerts its cytotoxic effects by disrupting the mitochondrial chaperone heat shock protein family D member 1 (HSPD1). Research identified that KHS101 exerts cytotoxic activity in several GBM cell lines obtained from patients, disrupting cellular metabolism and promoting GBM cell autophagy. The mechanism of action of KHS101 is to affect HSPD1, which also influences the mitochondrial protein, leading to the disruption of mitochondrial metabolism and the activation of autophagy mechanisms. *In vivo* injection of KHS101 reduced tumor growth and increased survival in patient-derived xenograft tumor GBM models in mice (162).

Another novel approach in GBM research is VB-111, an anti-cancer gene therapy. The mechanism of action of VB-111 is determined by two main mechanisms: An anti-angiogenic action leading to oxygen starvation of the tumor and the induction of a tumor-directed immune response. VB-111 is based on a non-integrating adenovirus type 5 vector carrying a chimeric Fas receptor transgene that binds to the human TNF-1 receptor (163). Based on preclinical results in combination with dose escalation, VB-111 demonstrated efficacy as an anti-angiogenic agent in the treatment of GBM. According to the results of phase I/II clinical trials, VB-111 monotherapy, continued after tumor progression with the addition of bevacizumab, was associated with promising improvements in OS and PFS, a favorable safety profile and typical radiological responses. The observed radiological response and the survival advantage of the combined regimen with primer VB-111 prompted further study in the randomized controlled GLOBE trial (164). However, the GLOBE study did not reproduce the promising results seen in a phase II study (164).

Rindopepimut (also known as CDX-110) is a vaccine targeting EGFRvIII deletion mutation and consists of an EGFRvIII-specific peptide conjugated to snail lymph hemocyanin (165). Preclinical studies demonstrated that intradermal administration of an EGFRvIII-specific vaccine-induced humoral immunity and prolonged survival of mice with intracerebral tumors (166). A randomized phase II trial confirmed the possibility of its usage in patients with GBM. A total of 73 patients were randomized (36 rindopepimut, 37 controls). PFS was 28% (10/36) for rindopepimut vs. 16% (6/37) for the control group ($P=0.12$). In the experimental group, tumor response to treatment based on objective assessment was 30% (9/30) vs. 18% in the control group (6/34; $P=0.38$) and the median response duration was 7.8 months (95% CI, 3.5-22.2) compared to 5.6 for the control group (95% CI, 3.7-7.4); at 6 months, steroid intake was discontinued in 33% (6/18) vs. 0% (0/19) in the control group (167).

Another large study (165) enrolled 745 patients [405 with minimal residual disease (<2 cm² of residual enhancing tumor on post-chemoradiation imaging), 338 with significant residual disease (SRD; ≥ 2 cm² of residual enhancing tumor on post-chemoradiation imaging), and two not evaluated who were randomly assigned to a group], the subjects were stratified into a rindopepimut + TMZ group ($n=371$) and control group

treated with TMZ ($n=374$). There was no significant difference for patients with minimal residual disease; 20.1 months (95% CI 18.5-22.1 months) in the group with rindopepimut vs. 20.0 months (95% CI 18.1-21.9 months) in the control group (HR=1.01, 95% CI 0.79-1.30; $P=0.93$). Rindopepimut did not increase survival in patients with newly diagnosed glioblastoma. Despite the initial success in the early studies, further research indicated low therapeutic efficacy of rindopepimut in the treatment of GBM.

Immunotherapy with checkpoint inhibitors of programmed death-ligand 1 and CTLA-4 has improved outcomes in numerous tumor types. However, in GBM, the exploratory phase I Checkmate 143 study with nivolumab \pm ipilimumab enrolled 40 patients with GBM and demonstrated limited efficacy (3/40 responders) (168,169).

A total of 369 patients with recurrent glioblastoma were randomized to receive nivolumab or bevacizumab in the open-label phase 3 CheckMate 143 clinical study. Nivolumab is an immune checkpoint inhibitor, a fully human IgG4 monoclonal antibody against programmed cell death-1. The primary endpoint of median OS did not differ substantially between the two medications at the study's conclusion: 9.8 months for nivolumab and 10.0 months for bevacizumab with a considerably lower PFS in the nivolumab group (1.5 vs. 3.5 months) (170).

In the CheckMate-498 trial, 560 patients were randomized into either the RT + TMZ + nivolumab or RT + TMZ + placebo group. The median OS in the treatment group was 13.4 months, while in the control group, the median OS totalled 14.9 months. PFS was also longer in the control group (6.2 vs. 6 months) (171).

In another study, 80 bevacizumab-naïve patients at the 1st/2nd recurrence were randomized to receive pembrolizumab with or without bevacizumab. The group that received bevacizumab had a median OS of 8.8 months, while the other group had a median OS of 10.3 months. Pembrolizumab was well tolerated, although its efficacy as a monotherapy for recurrent GBM was limited (172). All these trials failed to support the utility of checkpoint inhibitors in glioblastoma. Checkpoint inhibitors were also tested in the neo-adjuvant approach. In one of those trials, a pre-surgical dose of nivolumab was followed by postsurgical nivolumab until disease progression or unacceptable toxicity in 30 patients. Neoadjuvant nivolumab increased chemokine transcript expression, immune cell infiltration and TCR clonal diversity among tumor-infiltrating T cells, indicating that the treatment had a local immunomodulatory effect. However, there was no evidence of a therapeutic benefit. In evaluated patients, median PFS was 4.1 months and median OS was 7.3 months (173). Another study was aimed at evaluating immune responses and survival following neoadjuvant and/or adjuvant therapy with pembrolizumab in 35 patients. In this study, neoadjuvant use was also associated with an immunomodulatory effect. Patients in the adjuvant-only group had a median OS of 7.5 months, whereas those in the neoadjuvant arm had a median OS of 13.7 months (174).

9. Potential additional novel targets for GBM therapy

Insulin-like growth factors (IGFs) are associated with aberrant signaling, cell growth and resistance to therapy, and are widely

expressed in glioblastoma (175), including both a ligand and a receptor (IGF1R). IGF1R expression also correlates with a worse response to therapy and the expression of the receptor is associated with a decrease in the effectiveness of therapy aimed at EGFR (176), mTOR and HER-2. Preclinical studies have provided encouraging results for anti-IGF1R therapy (177), but clinical trials have not confirmed the benefits of the therapy (178).

There are 14 types of Ephrin family receptors (Eph), divided into subcategories EphA and EphB (179). EphA receptors are expressed largely in stem cells and de-differentiated phenotypes and are absent in differentiated cell populations (180). EphA2 and EphA3 in glioblastomas are associated with self-renewal of tumor stem cells (181,182). Ifabotuzumab (KB004) the anti-EphA3 monoclonal antibody achieved stable disease for 23 weeks in GBM (NCT03374943) (183). Additional studies targeting EphA are warranted in GBM.

10. Conclusions

Despite a large amount of research regarding the biology and associated disrupted signaling pathways, glioblastoma remains one of the most difficult tumors to treat, leading to dismal prognosis. Existing treatment options may only modestly prolong survival and only a small proportion of patients are cured with current standard of care therapy. Therefore, there is an unmet requirement for further research to investigate the mechanisms of glioblastoma pathogenesis and to look for new treatment targets in novel signaling pathways that are implicated in glioblastoma progression. Recent data suggest the use of a personalized approach for the treatment of GBM with targeted drugs may be promising, but this will require further research into the safety and efficacy of novel compounds in selected GBM populations with distinct molecular targets. Finally, deeper biological studies of CNS development, as well as GBM cancer biology, are important to discover novel approaches for the treatment of glioblastomas.

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Competing interests

YB has been a speaker for Amgen and Regeneron and has been an Advisor/Board Member for Alphagenon, G1 Therapeutics and Jazz Pharmaceuticals. The remaining authors have no competing interests to declare.

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