Targeting drug resistance in glioblastoma (Review)

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Abstract. Glioblastoma (GBM) is the most common malignancy of the central nervous system in adults. The current standard of care includes surgery, radiation therapy, temozolomide; and tumor-treating fields leads to dismal overall survival. There are far limited treatments upon recurrence. Therapies to date are ineffective as a result of several factors, including the presence of the blood-brain barrier, blood tumor barrier, glioma stem-like cells and genetic heterogeneity in GBM. In the present review, the potential mechanisms that lead to treatment resistance in GBM and the measures which have been taken so far to attempt to overcome the resistance were discussed. The complex biology of GBM and lack of comprehensive understanding of the development of therapeutic resistance in GBM demands discovery of novel antigens that are targetable and provide effective therapeutic strategies.

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1. Introduction

Glioblastoma (GBM) is a fatal primary cancer of the brain. Unlike other cancers, such as breast and lung, where therapies have improved overall survival (OS) and progression-free survival (PFS), the same trend is observed in GBM (1). GBM is indeed a difficult disease due to its complex biology, anatomical barriers and poor functional status of the affected patient population (2).

The median age of diagnosis for GBM is ~64 years, with the average incidence rate being 3.19/100,000 of the population. These patients have a median OS time of ~15 months (3). GBM occurs more frequently in men than in women (1,3). The only confirmed risk factor for the development of GBM is exposure to high doses of ionizing radiation (4). Clinical presentation of GBM varies greatly depending on the brain location and can be manifested as weakness, numbness, loss of vision, slurring of words, mood disorders, fatigue, memory disorders and seizures (5). Upon presentation, magnetic resonance imaging (MRI) of the brain is preferred for radiographic assessment (1), followed by either biopsy or surgical resection. Upon confirmation of the diagnosis, treatment includes radiotherapy (RT) and temozolomide (TMZ) (6). This regimen provides suboptimal outcomes with the median survival of ~15 months (6).

Pathologically, GBM is derived from the unregulated growth of cells of astrocytic origin and is the most common primary brain neoplasm accounting for >60% of all brain tumors in adults (7,4). WHO 2021 reclassified GBMs as grade 4 primary brain tumors that are IDH-wild type (wt) with or without histological features of GBM (micro-vascularization and/or necrosis) encompassing one or more of the following molecular alterations, such as hTERT promoter mutation, EGFR amplification or alteration, the combined gain of chromosome 7 and the complete loss of chromosome 10 ($7^{+}/10^{-}$) (8). GBM can also be classified further into two distinct subtypes: Primary and secondary. Between the two subtypes of GBM, primary GBM occurs mainly in older patients at an average age of 62 years, accounting for 80% of GBM cases, while secondary GBM mainly occurs in younger patients at an average age of 45 years (3). Primary tumors, at the time of diagnosis, show little to no evidence of the tumor's origin

coming from a lower grade glioma, while secondary GBM develops from a lower grade glioma (WHO grade 2 or 3) into a grade 4 glioma (9). Primary GBM can be further classified as either neural, classical, mesenchymal or proneural transcriptional profiles whereas secondary GBM tends to have a more proneural transcriptional profile and a hypermethylation phenotype (10).

2. Standard of care

Newly diagnosed GBM. Signs and symptoms arising from GBM vary depending on its location within the brain. Focal neurological/cognitive deficits, headaches, seizures and increased intracranial pressure are common and often progress within days to weeks (11). Less commonly, ~25% of patients present with seizures and benefit from anticonvulsant medication administration (12). Corticosteroids may also be prescribed for symptom alleviation related to peritumoral edema (PTE) (13). Brain MRI typically reveals enhanced tumor regions with central mass necrosis and increased T2/FLAIR signal intensity in PTE (14-16). Pathological confirmation after biopsy or maximal safe surgical resection leads to diagnosis of GBM per WHO 2021 classification.

Following surgical resection, the current standard of care for high-grade gliomas includes adjuvant RT with concomitant and maintenance systemic TMZ. TMZ is an FDA-approved DNA alkylating agent, and when combined with RT, it has shown to increase OS in patients with GBM; with a median OS time of 14.6 months compared with 12.1 months with radiation alone (17-19). Treating patients with tumor-treating fields (TTF), or alternating electric fields, in combination with maintenance TMZ, has been shown to increase both PFS and OS time. The former increases to 6.7 months compared with 4 months with TMZ alone, while the latter increases to 20.9 months compared with 16 months with TMZ alone (20).

Treatment of recurrent GBM. Upon recurrence of GBM, there are two main pathways that begin to differentiate the treatment: i) local tumor recurrence and ii) diffuse/multiple tumors outside of the initial resection cavity. With local tumor recurrence, the patient must be assessed with brain MRI for the feasibility of additional surgical resection. After resection, non-invasive treatment should be considered. If the tumor is unable to be resected, not recommended, or not elected by the patient, then the patient is directly diverted to non-invasive treatments. Such treatments include clinical trials, systemic chemotherapy, reirradiation, alternating electric field therapy and palliative care. For diffuse/multiple tumors outside of the initial resection cavity, the treatments are the same as the non-invasive therapy with the addition of surgical resection of symptomatic or large lesions. Resection of the recurrent tumor also allows for genetic and histologic analysis to understand the progression of the tumor from the genetics identified at initial diagnosis.

Preferred regimens of systemic chemotherapy in recurrent high-grade gliomas include re-challenging with TMZ, lomustine or carmustine, PCV (procarbazine, carmustine and vincristine), regorafenib, or bevacizumab. If there has been a long interval between initial treatment with TMZ and recurrence of the glioma, then it is reasonable to consider rechallenging with TMZ therapy at variable proposed dosages, which was shown to have a response rate of 64% in the study reported by Perry *et al* (21). Another therapeutic option is the use of a nitrosourea such as lomustine or carmustine (22-25). This therapy should be particularly considered in those with methylguanine-DNA methyltransferase (MGMT) unmethylated promoter status, whose response to TMZ is suboptimal (26,27). In PCV therapy, carmustine may be substituted for lomustine. In a randomized, placebo-controlled clinical trial, traditional carmustine administration was compared with a carmustine-biodegradable polymer placed surgically at the tumor site in order to understand its impact on OS and effects of systemic toxicities in recurrent gliomas. The median survival time of the treatment group was 31 weeks compared with 23 weeks in the placebo group and 6-month survivability was 50% higher in the carmustine-polymer treatment group (28,29). Regorafenib was compared with lomustine in a randomized, open-label phase II clinical trial studying its effectiveness in treating recurrent gliomas and increased average survival time by 1.8 months compared with lomustine (30).

Bevacizumab, an FDA-approved, anti-VEGF monoclonal antibody, has failed to show a survival advantage against recurrent GBMs while it has led to significantly improved PFS (31-33). The increase in PFS may be due to its mechanism of action, which alters the blood-brain barrier (BBB), and it may also play a role in the prevention or improvement of rapid neurologic deterioration (34,35).

RT should be considered if there is a sufficient time period since the last RT in order to prevent additional RT-related complications, or if there was a favorable response to RT in the past. RT may be used in combination with bevacizumab even when bevacizumab monotherapy fails, if retaining the steroid-sparing effects of bevacizumab are desired. An FDA-approved biosimilar agent may be used in place of bevacizumab. A meta-analysis reviewed 50 eligible non-comparative studies including 2,095 patients to determine the efficacy and toxicity of reirradiation of recurrent GBM (36). The meta-analysis demonstrated an OS at 6 and 12 months of 73 and 36%, respectively. A PFS 6-month rate of 43% and PFS 12-month rate of 17% was also observed.

Alternating electric field therapy has been FDA-approved for safety in recurrent glioma therapy based on the results of the EF-11 clinical trial (37). This treatment is a non-invasive, low-intensity and intermediate frequency electric field therapy which interferes with cell division by interrupting microtubule formation during mitosis (38). This trial compared chemotherapy-free treatment using alternating electric field therapy vs. traditional chemotherapy in recurrent GBM. There was no increase of survival between the two groups. Median survival time was 6.6 vs. 6.0 months, respectively [hazard ratio, 0.86; 95% confidence interval (CI), 0.66-1.12; P=0.27]. The analysis of the Patient Registry Dataset on all patients with recurrent GBM receiving TTF from 2011-2013 showed 1/3 of patients received treatment at first recurrence as opposed to 9% in the EF-11 clinical trial. The overall median survival in the study was 9.6 months, displaying a significant improvement as compared with the results of the EF-11 trial.

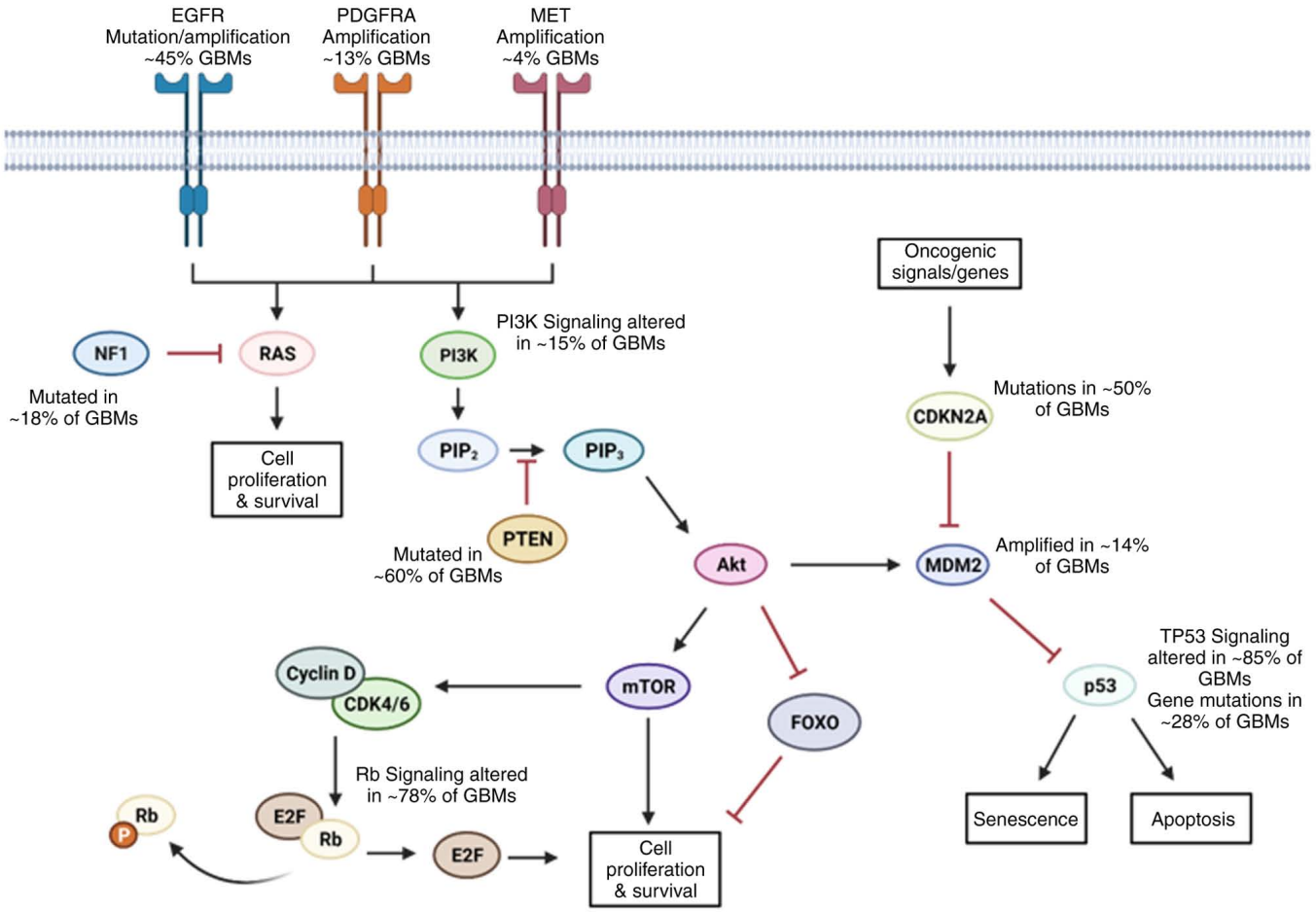

Figure 1. Clinically relevant dysregulated pathways in GBM. EGFR, PDGFRA and MET are some of the most common RTK receptors mutated and/or amplified in GBM. These mutations and/or amplifications alter downstream signaling pathways responsible for tumor growth and survival. GBMs with these receptor modifications can activate the RAS signaling pathway, promoting cell proliferation and survival while retaining mutations in the inhibitory protein of RAS, NF1. PI3K signaling is also upregulated by these receptor alterations, while also harboring alterations in the signaling pathway itself. In its wild-type form, PTEN inhibits conversion of PIP_2 to PIP_3. When mutated, it activates a downstream cascade of signaling pathways. Increase in Akt activity inhibits FOXO, assisting in uncontrolled cell proliferation. While Mouse Double Minute 2 (MDM2) has an increase in activity due to the increase in Akt activity, MDM2 is also amplified in ~14% of GBMs. MDM2 is typically inhibited by CDKN2A in response to activated oncogenic genes/signals, but CDKN2A is mutated in ~50% of GBMs. MDM2 inhibits p53 while the p53 gene itself is identified to have mutations in ~28% of GBMs. The inhibition of p53 due to mutations and/or increased activity of MDM2 prevents p53 from sending cells into apoptosis, leading to tumor growth and survival. The protein mTOR is also activated by Akt which promotes the cell cycle, leading to phosphorylation of Rb, which allows activation of E2F, thus promoting cell proliferation. The Rb signaling pathway is also known to be altered in almost 80% of GBMs. The image was created using BioRender.com. GBM, glioblastoma; RTK, receptor tyrosine kinase; NF1, neurofibromin 1; PTEN, phosphatase and tensin homolog; Rb, retinoblastoma.

3. Dysfunctions in multiple pathways lead to treatment resistance

One of the most profound characteristics of GBM is its high intra- and inter-tumor heterogeneity. Classical, neural, proneural and mesenchymal subtypes further differ in their subclonal evolution which is time, location and treatment dependent (39). Snuderl *et al* (40) showed evidence of these distinct clonal populations within the same tumor in relationship to receptor tyrosine kinases (RTKs) and that while these relationships were mutually exclusive, they shared common mutations suggesting that these clones arise from the same precursor cells. Sottoriva *et al* (41) also reported their genetic analyses which showed that a single precursor cell gives rise to different subclonal populations. Their work highlights the significance of understanding intratumoral heterogeneity and its implications in a clinical setting. It also sheds light on the idea that intratumoral heterogeneity may allow these subclones to survive initial standard of care treatment, with increase in genetic/epigenetic aberrations in response to therapy aiding in therapeutic resistance and in turn leading to recurrence (41). In GBM, the most clinically relevant alterations are: Epidermal growth factor receptor (EGFR), retinoblastoma (Rb), TP53 and RTK/Ras/PI3K signaling pathways (42) as shown in Fig. 1.

Epidermal growth factor receptor. EGFR is the most common amplification/mutation in GBMs, observed in ~58% of cases (43). Tumors with EGFR amplification have also been identified to harbor the EGFRvIII mutation. This mutation is caused by the deletion of exons 2-7, producing a constitutively active form of EGFR (44). This form of EGFR has direct consequences on cell proliferation, tumor initiation and resistance to apoptosis due to continuous autophosphorylation of downstream signaling proteins (45-47).

Inda *et al* (48) reported heterogeneity of EGFR expression in GBM cells, showing co-expression of both wt-EGFR

and EGFRvIII. The expression of both forms of EGFR plays critical roles in GBM proliferation and creates a feedback loop between them. It was reported that cells overexpressing wt-EGFR had a higher proliferative ability, and that the cells expressing EGFRvIII produced cytokines, such as IL-6, which activate signaling pathways responsible for expressing wt-EGFR, indicating a harmonious relationship between the two populations of cells. This correlation was shown in cell lines, immortalized murine astrocytes and clinical samples (48).

Rb signaling pathway. Rb is a tumor suppressor protein encoded by the gene *RB1*. In healthy cells, Rb protein functions to control the cell cycle, only allowing cycle progression when appropriate. When the cell cycle progresses, Rb is inactivated via phosphorylation by the cyclin D and CDK4/6 complex, releasing E2F and allowing the cell cycle to enter the S phase. This protein is mutated in numerous different cancers, allowing cells to proliferate uncontrollably (49). In 2001, a study was performed on 56 samples of GBMs to investigate the loss of *RB1* expression. Results demonstrated that 85% of the samples without *RB1* expression were hypermethylated at the promoter region of *RB1* (50). Another study in 2010 evaluated genetic networks in known dysregulated pathways in GBM. Cyclins CDK4, CDK6, and CCDN2 were commonly mutated and negatively affected the Rb pathway (51). GBM samples analyzed by EGFR expression levels revealed a significant correlation between Rb dysregulation and tumor size. When the pathway was unaltered, tumor size averaged 2.5 cm^3. GBM samples harboring mutations in the Rb pathway had an average tumor size of 4.1 cm^3 (52). This tumor size discrepancy indicates that dysregulation of Rb leads to increased tumor growth, ultimately leading to more aggressive disease.

TP53 signaling pathway. The p53 protein isoforms are referred to as the 'guardians of the genome' and are encoded by the *TP53* gene. P53 proteins protect DNA by promoting cell cycle arrest and DNA repair mechanisms when damaged. They also initiate apoptosis if DNA damage is beyond repair. It is one of the most frequently mutated genes in all cancers (53). In GBM, the pathway is altered in 85% of patients. The p53 protein alone is modified in 28% of GBM samples, but its frequency varies depending on the molecular subtype: Proneural, 54%; mesenchymal, 32%; neural, 21%; and classical, 0% (54). In GBMs with an amplification of PDGFRA, which is a marker of the proneural classification, a loss of wt-p53 has been revealed to constitute a more invasive tumor (55). Most p53 mutations are gain-of-function (GOF) missense mutations in the DNA binding domain (55). This gene signature has also been associated with the increased inflammation and decreased OS in patients (56). Some studies have reported that reactivating p53 in GBM cell lines can increase drug sensitivity and inhibit growth *in vitro* (57,58). These studies have shown that the loss of wt-p53 and/or GOF of p53 aids in the progression of GBM and may be an effective clinical target.

PI3K/Akt signaling pathway. PI3K is a family of kinases upstream of the PI3K/Akt/mTOR pathway. This pathway coordinates pro-survival signaling throughout cells. PI3K is regulated by both PTEN and PIK3R1 (59). The function of PTEN is to prevent uncontrolled proliferation in healthy cells (60). In GBM, PI3K frequently has GOF mutations in its catalytic domain, promoting Akt overactivation and cell growth (61). The loss of heterozygosity mutation in PTEN is found in ~60% of GBMs. Mutations in PTEN tend to occur more frequently at later stages of cancer development and have a similar effect as the PI3K GOF mutations (62). The repressive subunit of PI3K, PIK3R1, has also been demonstrated to be mutated/altered in GBM samples at a higher frequency than PI3K alterations (59,63,64). Numerous studies have demonstrated that inhibition of the PI3K/Akt pathway in GBM cell lines inhibits growth *in vitro* and *in vivo* (65,66). However, clinical trials have shown that inhibitors of this pathway are ineffective in improving the long-term survival of patients (67).

There are several other genetic and epigenetic alterations that lead to propagation of growth, viability and invasion of GBM and carry prognostic and therapeutic implications. For the sake of discussion in this paper, we will be focusing on these aforementioned pathways as they are most significantly upregulated or downregulated in GBMs. Altogether, the complex heterogeneity of GBM leads to the survival and proliferation of malignant cells. These alterations/mutations not only affect the tumorigenicity of cells, but allow them to evade chemotherapeutics and RT. The intricate web of interactions these pathways have on one another and have independent of one another indicates the dire need for novel therapeutic targets. It also strengthens the need for multimodal treatment strategies to combat the heterogenous nature of GBM. The literature shows that not all cells harbor the same mutations within the same tumor. Consequently, by targeting a specific protein, this treatment strategy may only be targeting a subpopulation of tumor cells present and lead to inevitable relapse and treatment-resistant tumors.

4. Emergent resistance mechanisms to current treatment modalities

TMZ. TMZ, which was granted FDA approval in 2005, is the standard chemotherapeutic drug used concurrently with RT for patients with GBM. At physiological pH, TMZ is converted into methyl-diazonium ions. Methyl-diazonium ions cause formation of DNA adducts by transferring a methyl group to DNA, which ultimately causes cytotoxicity. Methylation of the O^3 position of adenine and the N^7 and O^6 position of guanine causes DNA breaks, leading to G2/M cell cycle arrest and apoptosis (68).

Although TMZ is an important part of GBM therapy, resistance to this drug is common. After exposure, almost 50% patients exposed to TMZ stop responding positively. MGMT is the primary contributor to resistance to TMZ (Fig. 2). MGMT is involved in direct repair of DNA in the presence of methylated residues. If MGMT is present, it directly removes O^6-methylguanine residues, essentially rendering the drug ineffective. Hypermethylation of MGMT's promoter region leads to decreased expression of MGMT, preventing the removal of O^6-methylguanine residues and resulting in cells being sensitive to TMZ treatment. Stupp *et al* (19) showed the clinical implications of MGMT promoter methylation status while diagnosing and treating patients. They demonstrated that when comparing methylation status alone,

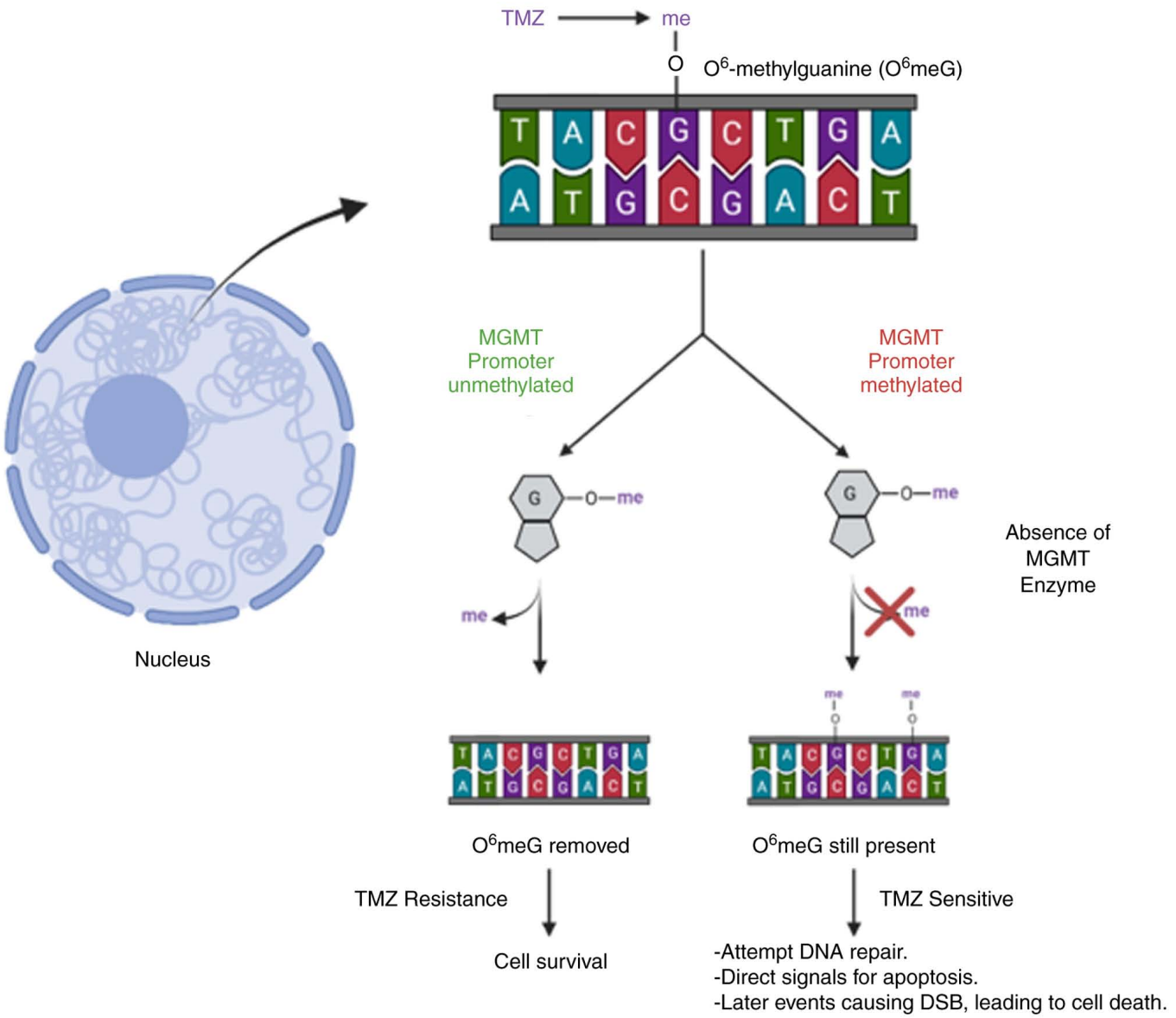

Figure 2. MGMT promoter methylation status contributes to the sensitivity of GBM cells to TMZ. TMZ adds a methyl group to the O^6 position on guanine. In tumors where the MGMT promoter is unmethylated, there is active MGMT, and the methyl groups will be removed, preventing DNA damage. The presence of MGMT leads to GBM cells that are resistant to TMZ. In tumors where the MGMT promoter is hypermethylated, there is an absence of MGMT, causing the guanine residues to stay methylated. This leads to the recruitment of DNA repair enzymes or direct signals for apoptosis. If repair enzymes fail to remove O^6meG or the cells are not directly signaling for apoptosis, O^6meG can lead to mismatch pairing with thymine residues leading to recruitment of MMR. If MMR is unsuccessful, DSBs can occur, leading to homologous recombination, non-homologous end-joining or cell death. The image was created using BioRender.com. MGMT, methylguanine-DNA methyltransferase; TMZ, temozolomide; GBM, glioblastoma; MMR, mismatch repair enzymes; DSBs, double-strand breaks.

patients who had the MGMT promoter region methylated had an increase of OS by 6 months compared with patients with an unmethylated MGMT status. The effects of methylation status in response to treatment were also reported and an improved response was observed to both TMZ and radiation therapy in patients who harbored a methylated MGMT promoter status (69). Several published clinical trials have stratified treatment plans according to MGMT methylation status, signifying its importance in drug efficacy and patient response (69-71).

Besides DNA repair proteins, cancer stem cells also contribute to TMZ resistance. Ligands of the Wnt/β-catenin pathway such as Wnt3a, Wnt7a and Wnt1 are known to induce stemness and have been reported to be highly expressed in GBM cells. As compared with healthy cells, Wnt signaling is upregulated in glioma stem cells (GSCs). This upregulation results in higher self-renewal capabilities, motility and altered epithelial-to-mesenchymal transition (EMT) activator expression (72). A previous study showed that EMT in GBM promotes resistance to chemotherapy, including TMZ, which can be reversed by knocking out β-catenin. Moreover, β-catenin has been linked to genes involved in EMT such as *ZEB1*, *Snail*, *Slug* and *Twist* (73).

Bevacizumab. Bevacizumab, a humanized monoclonal antibody against VEGF-A, has received FDA approval for the

treatment of a variety of cancers (74). The FDA approved bevacizumab in 2009 to treat recurrent GBMs (75,76). While the use of anti-angiogenetic therapies provides some benefit to PFS, patients ultimately become resistant to these anti-angiogenic therapies (77).

The use of bevacizumab leads to the creation of intratumoral hypoxia due to a decrease in blood vessel formation (78). This creation of a hypoxic microenvironment results in an increased expression of hypoxia-inducible factor 1-alpha (HIF1α), which increases the expression of VEGF (79). Alongside an increase in HIF1α and VEGF, hypoxia also causes upregulation of c-Met and phospho-c-Met (p-Met). Increases in expression of these proteins have been reported to have direct consequences on downstream signaling pathways that are involved in resistance to anti-angiogenic treatments, leading to more invasive tumors. The hepatocyte growth factor (HGF) and c-Met pathway has been extensively examined in the context of anti-angiogenic-resistant tumors (78). Multiple studies have shown the effects of the HGF/c-Met pathway on invasiveness and tumor growth. HGF interacts with c-Met, causing activation of several pathways such as the pathways mentioned previously as well as the MAPK/ERK and NF-κB pathways (78,80,81).

Interestingly, VEGF has been reported by Lu *et al* (82) to inhibit c-Met in GBM murine models and human GBM samples. It was reported that the inhibitory effect of VEGF on c-Met actually prevented tumor invasion. The group reported that when cells were stimulated with 10 ng/ml of HGF and increasing concentrations of VEGF, there was a decrease in p-Met. However, p-Met levels were elevated when cells were stimulated with increasing concentrations of HGF and 100 ng/ml of VEGF; indicating that there is an antagonistic relationship between HGF and VEGF. Analysis of samples of patients with GBM that had relapsed with bevacizumab treatment revealed a 70% increase in p-Met staining. Tumor samples also revealed an increase in proteins associated with mesenchymal GBM (82). This finding indicated that anti-VEGF bevacizumab may actually increase the functionality of the HGF/c-Met pathway in GBM of recurrent patients, leading to bevacizumab failure.

Etoposide. Etoposide, also known as VP-16, was approved by the FDA in 1983 (83) and has been used to treat a wide variety of cancers. The mechanism of action is through the inhibition of topoisomerase type II (TopoII). By inhibiting the TopoII cleavage complexes (TopoIIcc), replication fork stalling occurs, leading to double-strand breaks (DSBs) and the failure of TopoII to re-ligate the DNA, ultimately resulting in cell death (84,85). It has been reported that cancer cells have used autophagy as a way around etoposide treatment conferring to decreased sensitivity to etoposide. Biasoli *et al* (85) reported that Rb may play a role in GBM's resistance to etoposide by regulating autophagy and apoptosis. They reported that a knockdown of Rb produced an increase in apoptosis with etoposide. It was also reported that p62, which is degraded in auto-phagolysosomes, was decreased in their negative control groups treated with etoposide. In their Rb knockdown cells treated with etoposide, p62 levels were increased, suggesting that Rb may play a role in blocking etoposide-induced autophagy (85).

Mouse Double Minute 2 (MDM2) has been shown to be amplified in ~14% of GBMs (86). Senturk *et al* (87) showed that osteosarcoma cell lines that had amplification of MDM2 were responsive to DNA-damaging agents, but not as sensitive to TopoII inhibitors such as etoposide. Conradt *et al* (88) reported that MDM2 may be involved in repairing DNA damage caused by etoposide in murine models of pancreatic ductal adenocarcinoma. It was shown that MDM2 interacts with Nijmegen breakage syndrome 1, which is part of a DNS DBS repair complex. They identified that using an MDM2 inhibitor (PXN822) resulted in a decrease of DNA repair capabilities and an increase in sensitivity to etoposide (88). MDM2 has also been reported to be responsible for resistance to etoposide in GBM by Kondo *et al* (89); it was demonstrated that MDM2 causes an increase in expression of p-glycoprotein (P-gp) by transfecting human MDM2 into U87-MG GBM cells that do not express MDM2. In addition to the expression of P-gp, cells that expressed MDM2 were less sensitive to etoposide compared with U87-MG parental cells (89). These findings indicated that MDM2 may be responsible not only for an increase in tumor proliferation, but also in the resistance to certain drugs such as etoposide.

Carboplatin. Approved by the FDA in the 1980s, carboplatin has been used for different cancers over the years. The drug creates DNA lesions, thus disrupting replication and transcription which ultimately leads to cell death. Although carboplatin is an established drug, resistance after persistent use is common. In total, three primary mechanisms are involved: Decreased drug availability, DNA repair mechanism alteration and changes in microenvironmental responses. CTR1 downregulation, ATP7A/7B and MRP2 upregulation help cancer cells in keeping the intracellular concentration of the drug low by reduced uptake and increased efflux. Any drug that reaches the tumor site and gets inside of the cell is neutralized by high levels of glutathione (GSH). Carboplatin-resistant cells have high levels of GSH and GSH-supporting proteins such as γ-glutamyl-cysteine synthetase and glutathione s-transferases (90,91).

Similar to other DNA-damaging chemotherapeutics, a key resistance mechanism involves DNA repair. Most carboplatin-induced lesions are excised by nucleotide excision repair (NER). Proteins involved in NER such as ERCC1, ERCC4 and XPF are observed to be upregulated in resistant tumors. High levels of ERCC1 are a marker of poor prognosis in numerous cancers, rendering drugs such as carboplatin and cisplatin ineffective. Other enzymes including MMR proteins MSH2 and MLH 1, and specific DNA polymerases such as REV1 and REV3 also play a part, albeit an indirect one, in chemoresistance (92). High expression levels of these proteins cause an increase in DNA replication, which can prevent tumor recurrence. However, in the event of relapse, they make DNA-damaging chemotherapies obsolete.

RT. The initial therapy for GBM consists of maximal surgical resection followed by adjuvant RT to a dose of 60 Gy with concurrent TMZ, per the so-called Stupp protocol, followed by maintenance TMZ and the use of TTF. This regimen became the standard of care following a series of landmark trials which established the role for the respective

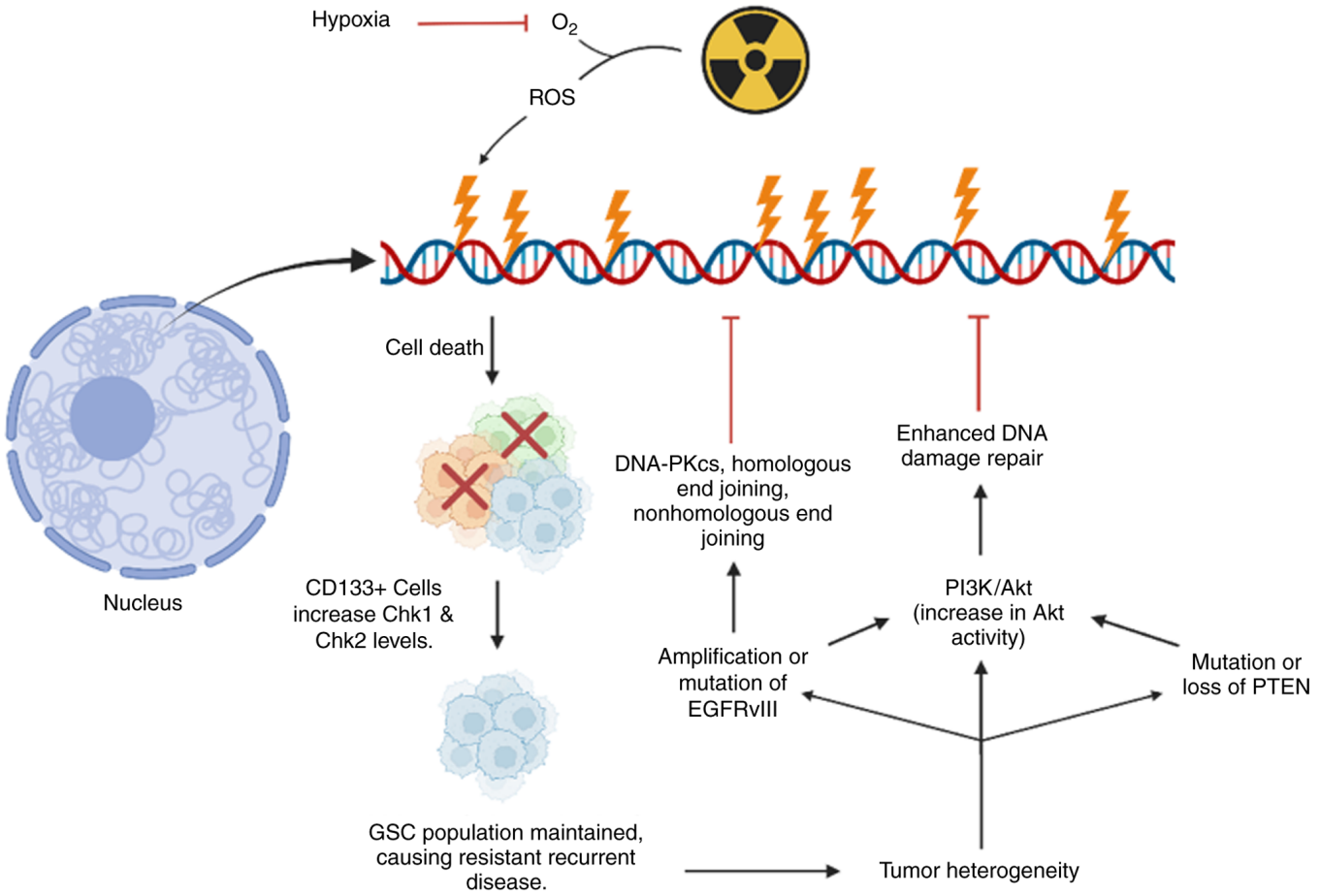

Figure 3. Pathways involved in radiation therapy resistance in GBM. Radiation treatment produces ROS that cause DNA damage and ultimately result in cell death. GBM has hypoxic regions that can be observed on MRI. Hypoxic regions cause a decrease in oxygen thus preventing the formation of ROS, which leads to futile cell death. Within GBM tumors, there are GSCs that have stem-like properties. Radiation treatment eliminates differentiated cells, while GSCs are radioresistant. Tumor heterogeneity is a leading contributor in treatment resistance in GBM. Through several permutations, tumors have multiple ways of becoming radioresistant. The image was created using BioRender.com. GBM, glioblastoma; ROS, reactive oxygen species; GSCs, glioma stem cells; PTEN, phosphatase and tensin homolog.

adjuvant therapies. The benefit of adjuvant RT was initially demonstrated in trials conducted by the Brain Tumor Study Group, which found that the addition of RT roughly doubled survival time compared with post-operative observation (93). This benefit was also found to be dose-dependent, with patients receiving higher RT doses living longer (94). After preclinical data demonstrated reduced tumor cell survival with the combination of TMZ and RT, this combination was investigated clinically (95).

This work ultimately culminated in the landmark 2005 EORTC-NCIC trial by Stupp *et al* (19), which demonstrated significant improvements in 2-year OS with combination of adjuvant TMZ and RT without significant toxicity. Unfortunately, disease progression is virtually inevitable despite the current standard of care, with a median time to recurrence/progression of <12 months for patients receiving maximal safe resection and definitive adjuvant therapies (96). When recurrence or progression occur, effective salvage options are limited, with median OS after recurrence of 6 months (97). This poor survival is due in part to the development of resistance to systemic therapies as previously outlined and also radioresistance. While radioresistance has been empirically observed, the mechanisms underlying this phenomenon are not entirely understood. Thus far, several relevant pathways have been identified.

Solid tumors have abnormal vasculature, and consequently varying degrees of oxygenation. GBM is a rapidly growing, hypoxic tumor, and the degree of hypoxia is further associated with increased neoangiogenesis and accelerated endothelial proliferation. In turn, this neoangiogenesis causes remodeling of the extracellular matrix, and increased overall invasiveness of tumor cells (98). Furthermore, hypoxia also reduces the lethal effect of irradiation by reducing the generation of the reactive oxygen species that mediate RT-induced DNA damage (99,100). Thus, tumor hypoxia is a crucial driver of aggressiveness of GBM and reduces RT effectiveness (Fig. 3). Hypoxia also results in downstream signaling via HIFs, with multiple targets including vascular endothelial growth factor A (VEGF) (101).

Given these findings, agents targeting this pathway have been developed, most prominently bevacizumab. This humanized monoclonal antibody binds the circulating VEGF-A ligand, reducing its ability to bind to receptors, altering the kinetics of ligand binding to endothelial cells and downregulating angiogenesis. Despite compelling preclinical data of the benefits of RT with bevacizumab, real-world outcomes have been

less than optimal. A total of two phase III clinical trials both failed to demonstrate significant improvements in OS, though improvements in PFS were noted (102,75). Interestingly, the use of bevacizumab actually reduced survival time in the most favorable subgroup (MGMT-methylated with favorable gene signatures from the 9-gene molecular profile) from 25 months to 16.7 months. The 9-gene molecular profile used for stratification in the trial came from the work of Colman *et al* (103), who reported the development of a 9-gene array that can be used as a predictor of survival in patients with GBM. The 9 genes that were selected as prognostic indicators were: Aquaporin 1 (*AQP1*), YKL-40 (*CHI3L1*), epithelial membrane protein 3 (*EMP3*), glycoprotein (*GPNMB*), insulin-like growth factor binding protein 2 (*IGFBP2*), galectin 3 (*LGALS3*), oligodendrocyte lineage transcription factor 2 (*OLIG2*), podoplanin (*PDPN*) and reticulon 1 (*RTN1*). These genes were shown to give survival time predictions independently of MGMT status in patients and were observed to provide similar predictions of survival as MGMT methylation status (103). Further investigation is necessary to successfully exploit hypoxia therapeutically.

Moreover, hypoxia has been linked to increased stemness characteristics of GBM (104). Cancer stem cells (CSCs) are considered to exist as a subpopulation of tumor cells with the ability to potently repopulate and have been demonstrated as a common feature of several tumor histologies (105). These stem cells contribute significantly to therapy resistance, and thus represent a potential target for intervention (106). Attempts to distinctly identify and preferentially eradicate CSCs have been largely unsuccessful, due to genotypic and phenotypic heterogeneity and plasticity in response to therapy and environmental cues (107). CSCs are particularly refractory to cancer therapies because cancer therapies are largely mediated by DNA damage and subsequent mitotic catastrophe (108). In response to genotoxic insults, CSCs adopt a quiescent, dormant phenotype, which may subsequently repopulate tumors long after initial treatment (109).

CD133 (prominin-1) is identified as a putative hallmark of stem cells, both in tumors and neural progenitor cells (106,110,111). CD133-positive ($CD133^+$) GSCs have more robust DNA repair mechanisms and greater growth checkpoint activation following DNA damage vs. CD133-negative ($CD133^-$) GBM tumor cells (112). Consequently, irradiation exerts a potent evolutionary pressure that inadvertently selects for the survival of $CD133^+$ cells. This ultimately contributes to eventual repopulation of the surviving niche of tumor cells, which likely underlies the observation of the distinct genetic profile of recurrent GBM (113). More specifically, exome sequencing has demonstrated that some recurrent tumors appear to originate from clonal expansion of specific subpopulations of the original tumor (113).

Specific targeting of the DNA repair mechanisms upregulated in $CD133^+$ GSCs is a compelling proposition that may prove useful. This is perhaps especially advantageous in the recurrent setting, in which tumors are $CD133^+$ enriched (114,112). In response to irradiation, $CD133^+$ glioma cells have more robust activation of ATM, Rad17, Chk1 and Chk2 compared with $CD133^-$ glioma cells, preferentially inducing cell cycle arrest and repair (112). Irradiation of $CD133^+$ glioma cells pretreated with the Chk 1/2 inhibitor debromohymenialdisine has been shown to significantly increase efficacy of irradiation *in vitro* (112).

Other molecular signaling pathways have also been shown to contribute to radioresistance, both in the *de novo* and recurrent setting. Alterations of the EGFR are among the most common mutations in GBM, present in >50% of tumors (115). Specifically, mutations of EGFR-wt to a specific, constitutively active variant, EGFRvIII are highly oncogenic (116). EGFRvIII has been demonstrated to confer radioresistance compared with EGFR-wt by multifold activation of pro-proliferative signaling via mitogen-activated protein kinase (MAPK). It has also been shown to cause robust stimulation of anti-apoptotic pathways via the Akt/phosphatidylinositol-3-kinase pathways (PI3K-Akt) in response to irradiation (117). This hyperactivation of the PI3K/Akt signaling pathway by EGFRvIII reduces radiosensitivity via enhanced repair of DNA DSBs (118). Unfortunately, response to EGFR inhibition has generally been modest (119). This has been attributed to poor tumor penetrance as well as due to redundant mutations of these downstream signaling cascades. However, inhibition of downstream PI3K-Akt has been demonstrated to radio-sensitize glioma cells *in vitro* (120). Ongoing investigations of the Akt pathways and other molecular cascades downstream of EGFR may prove productive.

While targeting the mechanisms underlying the radioresistance of GBM remains a largely preclinical endeavor, the inevitability of recurrence has prompted clinical efforts to improve the efficacy of RT. In the upfront setting, this was primarily investigated from the perspective of RT target delineation. It has been demonstrated that GBM exists as distinct subpopulations of cells with unique roles in the growth, signaling and invasiveness of the tumor (121). Thus, one area of inquiry is that of incorporating novel imaging modalities to localize and characterize GBM more granularly. For example, multiparametric MRI sequences to assess hyper-cellularity and hyper-perfusion has been shown to be predictive of subsequent sites of failure (122). Similarly, hypoxia imaging is an ongoing area of investigation, given its association with increased invasiveness and radioresistance (123). Imaging that more robustly correlates the known intratumoral heterogeneity with location and may allow for more optimal, biologically-driven, RT dose distribution, often referred to as 'dose-painting'. Novel PET agents such as [^{11}C] methionine-PET (MET-PET); [^{18}F] fluoro-ethyl-L-tyrosine (FET-PET), and [^{18}F]-FDOPA-PET are being investigated in dose-escalation trials (124).

In the recurrent setting, several reirradiation approaches have been attempted as salvage options. These are complicated by considerations of the location of the recurrent lesion relative to the initial course (in-field, marginal, out-of-field), prior RT dose to adjacent organs-at-risk, volume of recurrent/progressive disease and changes in tumor biology. GBMs fail overwhelmingly in-field, and thus additional radiation doses often overlap significantly with the initial course (125). Consequently, this may increase the risk of radio-necrosis or other toxicity if full-dose, conventionally fractionated reirradiation, namely 60 Gy/30 fractions, was attempted (125). The use of more conformal, stereotactic approaches, whether single-fraction radiosurgery or fractionated stereotactic RT (SRS/SRT), may limit the toxicity of reirradiation (126). For larger volume recurrences, more conservative hypo-fractionated approaches

should be utilized to reduce toxicity (127). Additionally, the changes in biology at the time of recurrence may dictate response to RT. Specifically, recurrent GBM has been observed to shift to a more aggressive, mesenchymal phenotype (126). Intriguingly, it has been observed that RT itself (as well as other therapies) may play a crucial role in the mesenchymal transition of recurrent GBM, which in turn is more treatment-resistant (128).

5. Preclinical investigations to overcome treatment resistance

Regardless of a patient's response to prior treatment, ~90% of patients will show disease recurrence within the first 2 years of treatment (18). This, alongside the poor survival rate of GBM itself, is what fuels the search for novel targets and therapeutic strategies. Histone deacetylases (HDAC) have become a target of extreme interest in drug development for cancer. Wang *et al* (129) showed that overexpression of HDAC6 promotes proliferation and treatment resistance in GBM. Further studies by Yang *et al* (130) revealed an increase in activity of the HDAC1/2/6 and Sp1 axis that leads to tumor growth and drug resistance in GBM. It was revealed that inhibiting HDAC1/2/6 significantly reduced the proliferative abilities of both GBM and TMZ-resistant GBM cells. The greatest efficacy in their TMZ-resistant orthotopic GBM model was observed when comparing OS between TMZ and TMZ plus their HDAC inhibitor (MPT0B291) (130). These results indicated that HDAC pathways may be a valuable target in the fight against GBM and recurrent disease.

Another area that has gained interest in the fight against cancer is immunotherapy. Programmed cell death protein 1, programmed death-ligand 1 (PD-L1) and T-cell immunoglobulin mucin receptor 3 have been found to be overexpressed on GBM tissues (131-133). While this suggests that immunotherapy may be a great asset in the fight against GBM, it has suboptimal results due to the extreme immunosuppressive nature of GBM and the immune-privileged environment of the CNS (134). Tong *et al* (135) reported that the use of ACT001, which is currently in a phase I/II clinical trial (NCT05053880), significantly reduces the expression of PD-L1 in GBM. It inhibits the phosphorylation of STAT3, preventing transcription of PD-L1. This was shown to cause a decrease in a protumor immune responses and an increase in antitumor immune responses (135).

There have been multiple studies on how GBM and other malignancies evade the response of anti-angiogenic therapies such as bevacizumab. These studies highlight the need for the development of therapeutic strategies to target these evasive mechanisms either alone or in combination with other therapies. Scholz *et al* (136) investigated the potential use of targeting angiopoietin-2 (Ang-2) in both treatment-naïve and bevacizumab-resistant GBM. The aforementioned study showed an increase in survival when targeting both VEGF and Ang-2 (136). Other studies have also looked at targeting pathways in response to the hypoxic environment caused by bevacizumab treatment. Piao *et al* (137) showed that using altiratinib, an inhibitor of MET, VEGFR2, TIE2 and tropomyosin receptor kinases, was significantly effective in decreasing cell viability *in vitro*. It was also identified that altiratinib in combination with bevacizumab provided the best overall results in reducing tumor volume, invasiveness and mesenchymal markers compared with bevacizumab treatment alone. It was also demonstrated in their xenograft models that the combination treatment provided the greatest benefit to OS (137). Carbonell *et al* (138) exploited β1 integrins in bevacizumab-resistant GBM cells. The group reported that β1 integrin expression was increased after becoming resistant to bevacizumab, in their bevacizumab-resistant clinical and xenograft samples. It was reported that targeting β1 integrins had a significant effect on the proliferation and mesenchymal-like properties of bevacizumab-resistant cells (138). While these findings are optimistic, markedly further investigation is required regarding the use of immunotherapies in treating CNS malignancies such as GBM. Emphasis must focus on understanding the tumor microenvironment (TME) in these tumors in order to develop more effective single-agent or combination therapies.

With EGFR and EGFRvIII being the most common alterations in GBM, they would appear to be valuable targets in treating GBM. However, EGFR inhibitors have been shown to be less effective than anticipated. Zanca *et al* (139) reported that EGFRvIII-positive cells secreted interleukin-6 (IL-6) that activated NFκB, which in turn activated survivin and decreased the sensitivity to EGFR inhibitors (139). Liu *et al* (140) tested a third-generation EGFR inhibitor, AZD9291 (Osimertinib), and compared the response to erlotinib and gefitinib. Osimertinib easily crosses the BBB, making it an attractive compound for treating GBM. Compared with earlier versions of EGFR inhibitors, AZD9291 continued to inhibit the EGFR/ERK pathway, leading to an improved response in their murine models and an increase in OS (140). While EGFR again would be a sound target for GBM, treatment is suboptimal until multiple aspects are inhibited by the therapy. These results indicate the need for innovative next-generation compounds that can inhibit multiple aspects of the pathways.

Another fast-growing field in the treatment of cancers is the examination of different classes of RNA molecules such as long non-coding RNAs (lncRNAs) and microRNAs (miRs). LncRNAs do not code for protein and are >200 base pairs (bp) in length (141). Lu *et al* (142) revealed that small nucleolar RNA host gene 12 (*SNHG12*) was upregulated in TMZ-resistant cells compared with non-treated cells. It was found that the promoter region of this lncRNA had a decrease in methylation, allowing easier access by transcription factors such as Sp1. It was later showed that this lncRNA does indeed play a role in TMZ resistance when expression was knocked down using short hairpin RNA (142). By knocking down expression of Sp1, an increased sensitivity to TMZ compared with the control was revealed. It was also found that lncRNA SNHG12 interacts with miR-129-5p, and this interaction stops miR-129-5p from inhibiting MAPK1 or E2F7 (142). By preventing this inhibition, the MAPK signaling pathway has an increased level of activity, allowing for the inhibition of apoptotic proteins. The combined activity of E2F7 and MAPK allows for cell proliferation and survival through G1/S phase transitions. Mazor *et al* (143) showed that the presence of lncRNA TP73-AS1 correlates to TMZ resistance in GSCs. It was demonstrated that when lncRNA TP73-AS1 was knocked

down, there was a significant decrease in cell viability when treating with TMZ compared with the control cells. It was also reported that following knockdown of this lncRNA, metabolic processes were affected via RNA sequencing data. It was shown that one of the major proteins regulated by lncRNA, TP73-AS1, was aldehyde dehydrogenase 1 family member A1, which has been previously reported as a stem cell marker in cancers and corresponds to treatment resistance (143).

MicroRNAs have also gained traction in understanding the mechanisms behind therapeutic resistance. MiRs are small, single stranded RNA sequences which bind to 3'-untranslated regions (UTR) of mRNA that effect gene expression post-transcriptionally. Li *et al* (144) demonstrated that miR-1268a regulates the expression of ABCC1 in GBM cells. ABCC1, also known as MRP1, is a drug efflux pump that removes drugs from the cells. It was identified that upon treatment with TMZ, miR-1268a was downregulated while protein expression of ABCC1 was upregulated. This was also confirmed in patient samples which compared primary tumors to recurrent tumors. When miR-1268a mimics were overexpressed, a decrease in ABCC1 protein levels was observed. The mimics also allowed the cells to become sensitive to TMZ treatment both *in vitro* and *in vivo* (144). Luo *et al* (72) reported another miR, miR-126-3p, that is involved in TMZ resistance in GBM. It was shown that in patient samples, miR-126-3p was decreased in TMZ-resistant samples compared with TMZ-sensitive samples. TMZ-resistant cell lines were also created and it was revealed that compared with their TMZ-sensitive parental cell lines, miR-126-3p was decreased. When miR-126-3p mimics were transfected into the TMZ-resistant cell lines, it was observed that the expression of miR-126-3p made the cells sensitive to TMZ compared with controls by affecting cell viability and proliferative abilities. It was later demonstrated that miR-126-3p binds to the 3'-UTR of *SOX2* and downregulates its expression while a decrease in miR-126-3p showed an increase in SOX2 protein levels. Following this discovery, it was found that when miR-126-3p decreases SOX2 levels, the Wnt/β-catenin signaling was inhibited. These results suggested that miR-126-3p promotes TMZ sensitivity by inhibiting SOX2 expression, which prevents Wnt/β-catenin signaling (72).

As shown in the literature, GBM is notorious for having multiple mechanisms at its disposal to evade current treatment strategies (145-147). In brief, multiple studies have reported that to overcome this treatment-resistant characteristic of GBM, it is needed to find ways to target pathways and/or proteins that are involved in these resistance mechanisms. Using multimodal treatment strategies has shown to re-sensitize cells to therapies and increase OS preclinically.

6. Clinical trials investigating targeted therapies

While preclinical investigations appear promising, clinical trial results have been dismal when it comes to GBM. Currently there are 320 actively enrolling clinical trials for GBM according to clinicaltrials.gov. Of these 320, 117 of them include recurrent GBM. In the fight against GBM and recurrent GBM, novel treatment strategies are a must. It has been shown preclinically that some of the best responses come from a multimodal treatment approach. Investigators must start incorporating these therapeutic resistance mechanisms into consideration for their trials or the outcomes will continue to be suboptimal.

Another area that has hindered the progress of successful clinical trials is the presence of the blood-brain barrier (BBB). There is a current phase I/II clinical trial (NCT04440358) where the aim is to establish the safety and efficacy of using microbubbles in order to disrupt the BBB in patients with recurrent GBM undergoing intravenous carboplatin therapy. The primary goal of this trial is to open up the BBB prior to chemotherapy administration, allowing for improved drug delivery.

With advancements made in the ability to deliver therapies more effectively to GBM tumors and by targeting these resistance mechanisms, it is hopeful that current and future clinical trials will lead to improved outcomes with regard to PFS and OS. Current clinical trials that are actively recruiting patients with a focus on targeting aspects of therapeutic resistance are presented in Table I (clinicaltrials.gov).

7. Discussion and conclusions

GBM is a highly aggressive tumor characterized by poor patient survival. One of the leading causes of the dismal outcome is the heterogeneous biology of the TME and mutations in regulatory signaling pathways. Collectively, these promote resistance to radiation and standard drug treatments. Dysregulation is observed in tumor signaling pathways, including PI3K/Akt, Tp53, Rb, STAT/Notch, CDKN2A and reelin (146-148). Altered signaling promotes tumorigenesis by enhancing migration, proliferation and invasion and prevents apoptosis in tumor cells (145,146). Profiling the transcriptional, genetic and epigenetic changes within the TME has led to new insights in the diagnosis of GBM. Cellular variation rising from intratumoral and intertumoral mutations have led to investigations into novel subtype specific therapies. Ongoing clinical trials for drugs which target specific molecular markers and genes involve patients having neurofibromin 1, EGFRvIII and BRAFv600 mutations, as well as EGFR gene amplification (39). In most cases, patients within these trials are classified based on the mutation of *hTERT* promoter gene, *MGMT* promoter methylation status, IDH1/2 status, and aberration of EGFR/PDGFR signaling. However, the clinical translation of targeted treatment remains unknown. Furthermore, the effect of such therapies on the host immune system and secondary neuroinflammatory responses needs to be elucidated.

GBM stem cells are hypothesized to influence intratumoral cellular variation due to their high tumorigenic potential. Preliminary studies demonstrated that GBM stem cells impact cell growth dynamics and evade cell death mediated by radiation and chemotherapy. This indicates that it may be essential to target GBM stem cells with genetic and molecular tumor subtypes. Investigation into multimodal therapy has also given rise to novel therapeutic regimens to treat GBM. One such treatment involves using electric TTFs that interfere with cell division through misalignment of

Table I. Current clinical trials actively enrolling patients with recurrent GBM targeting resistance aspects.

NCT number	Phase	Target	Drug	Primary objective(s)
NCT03961971	Phase I	Anti-TIM3 & Anti-PD1	MBG453 & Spartalizumab	Estimate overall and progression-free survival. To estimate Radiographic Response (RANO & iRANO). To evaluate pain for patients undergoing the treatment of anti-TIM3 and anti-PD1 in combination with SRS.
NCT04492163	Phase II	TTFields	OPTUNE with high-intensity transducer arrays	Progression-free survival.
NCT03834740	Phase 0/I	Cyclin D1/CDK4/CDK6 & mTOR	Ribociclib (LEE011) and Everolimus	Pharmacokinetic analyses. Median concentration of ribociclib and everolimus for all patients for unbound plasma, CSF, unbound NE, unbound enhancing. Percentage of pRB and pS6 positive cells will be quantified in resected post-treatment recurrent tumor tissue compared to baseline. MTD: highest dose of the drug that did not cause a DLT in >33% of patients.
NCT05053880	Phase I/II	PD-1 and PD-L1	ACT001 and Pembrolizumab	TEAEs, DLTs, mean changes in vital sign measurements, mean changes in electrocardiogram parameters, mean changes in Karnofsky Performance Scale score and progression free survival.
NCT04051606	Phase II	EGFR & VEGFR	Regorafenib	Median overall survival.
NCT04074785	Early Phase I	CDK2/4/6 and VEGF	Abemaciclib and Bevacizumab	Number of patients with adverse events.
NCT03643549	Phase I/II	Unmethylated MGMT	Bortezomib and Temozolomide	Maximum tolerated dose, overall survival, progression-free survival and time to progression.
NCT03618667	Phase II	EGFR	GC1118 (anti-EGFR monoclonal antibody)	Progression-free survival.
NCT03914742	Phase I/II	PARP	BGB-290 and Temozolomide	Maximum tolerated dose, percentage of patients with adverse events and tumor radiographic response.

NCT, national clinical trial; MTD, maximum tolerated dose; DLT, dose limiting toxicities; TEAE, treatment emergence adverse event.

mitotic spindles. Another modality uses focused ultrasound to disrupt the BBB (low-intensity) or ablate the tumor mass (high-intensity). These techniques seek to aid drug delivery, overcome resistance and increase drug efficacy for tumors. New treatment modalities in conjunction with targeted immunotherapy/chemotherapy may be essential for improving the outcomes of patients with GBM.

No matter the response to initial treatment, patients will ultimately succumb to recurrent disease. Recurrent disease tends to be more aggressive and resistant to treatment compared with the initial tumor. This leaves first-line therapies ineffective and give patients only a limited number of second-line treatment options. The continued poor OS indicates the dire need for novel targeted therapeutic strategies to overcome these resistance mechanisms. This review has highlighted key mechanisms behind treatment resistance in GBM, indicating the dire need for novel treatment strategies against these key resistance mechanisms.

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SA conceived the study. JS, TA, AB and SA wrote the original draft of the manuscript. JS and SA wrote, reviewed and edited the manuscript. PL and SA supervised the study. PL and SA conducted project administration. SA acquired funding. All authors read and approved the final manuscript. Data authentication is not applicable.

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Competing interests

The authors declare that they have no competing interests.

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