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Review

Understanding the Genomic Landscape of Glioblastoma: Opportunities for Targeted Therapies

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Abstract. *Glioblastoma (GBM) is categorized by the World Health Organization (WHO) as a grade 4 glioma and is a uniformly fatal tumor of the central nervous system. With the discovery of specific gene anomalies, GBM classification has been modified several times to provide better diagnostic and prognostic accuracy. Survival outcomes remain dismal despite the current therapeutic modalities, which include a combination of surgical resection, adjuvant chemotherapy, and radiotherapies, providing brief control of tumor progression. GBM remains aggressive and reoccurs primarily due to the presence of a unique population of untreatable glioblastoma stem cells (GSC). The presence of high mutation rates and a dysregulated transcriptional landscape increase GSC resistance to conventional chemotherapy and radiation therapy, contributing to poor outcomes seen in GBM patients. Accordingly, GSCs have emerged as targets of interest in new GBM treatment paradigms. Consequently, it is important to understand their distinct properties, such as GSC interactions with the hypoxic microenvironment, enhancing their growth. The epigenomic regulators and fundamental molecular components of the signaling pathways represent potential targets for GBM therapies. In this review, we aimed to describe the evolution of GBM classification and highlight the current therapeutic modalities, including gene and immunotherapies, and mammalian target of rapamycin (mTOR) inhibitors to target GBM. Furthermore, we explored the molecular pathway of GSCs and the ongoing investigation of circulating tumor cells (CTC), along with precision therapeutics, which aim to provide novel discoveries and effective treatments for GBM with improved survival.*

Glioblastoma (GBM) is the most common and aggressive cancer of the central nervous system (CNS), with an annual incidence of about 3.23 per 100,000 population in the United States, and accounts for 57.7% of gliomas and 82% of malignant gliomas (1-4). Despite aggressive multimodality treatment, consisting of maximal surgical resection with adjuvant temozolomide (TMZ) chemotherapy and radiation therapy (RT), survival remains dismal, with a median survival of 14-16 months and a five-year survival rate of less than 5% (3, 5, 6). GBM remains incurable due to its high recurrence rate of about 90% as well as its infiltrative nature, which renders gross total resection highly difficult. Therefore, with these properties paired with its rapid growth and resistance to currently available therapies, GBM carries an invariably fatal prognosis regardless of intervention.

The World Health Organization (WHO) classifies GBM as a grade 4 glioma. This diagnosis was initially primarily based on histopathological features, including high vascularity, pseudopalisading necrosis, and invasion of adjacent normal brain tissue. The WHO provided updated guidelines in 2021. This 5th edition of the WHO Central Nervous System (WHO CNS5) tumors classification incorporates genetic mutations or alterations into the grading system, thus classifying GBM based on its recently defined genetic markers, such as isocitrate dehydrogenase (IDH) wild-type, telomerase reverse transcriptase (TERT) promoter mutation, epidermal growth factor receptor (EGFR) gene amplification, or chromosome copy-number variations, in addition to its histological appearance. These classifications reflect the significance of genetic drivers on GBM

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development and progression. These genetic markers are increasingly being utilized to stratify GBM and to not only predict prognosis but also personalize therapies.

GBM exhibits a broad range of oncogenic driver mutations, and thus, displays high inter- and intra-tumoral heterogeneity at the molecular as well as cellular levels. Conventional classification of GBM tumors included two major categories: primary IDH wild-type GBM that arise de novo represent approximately 90%, and secondary, which progress from lower-grade gliomas and carry IDH mutations, represent 5-10% (7). Both subtypes display histologically similar characteristics. Primary GBM typically affects older individuals and is genetically characterized by EGFR amplification, phosphatase, and tensin homologue (PTEN) mutation, and p16INK4a deletion, while secondary GBM has an average age of onset of 45 years and typically display mutations in TP53 and RB2. In addition, both subtypes commonly demonstrate loss of chromosome 10q, although the percentage of occurrence varies (7). In a landmark discovery, Parsons *et al*. demonstrated that mutation in IDH1, also found in more than 70% of WHO grade 2 and 3 astrocytomas and oligodendrogliomas, is commonly seen in secondary GBM progressed from lower-grade tumors (8). Furthermore, tumors that lack IDH1 mutations often have mutations at the analogous amino acid site of the IDH2 gene. Presence of IDH1/2 mutations is associated with more favorable survival (9).

Studies of gene expression profiling during the past three decades have distinguished GBM into separate subclasses with unique genetic signatures and survival outcomes. Initial classification of GBM describes three discrete subclasses, including proliferative, mesenchymal, and proneural based on gene overexpression profiling (10). The Cancer Genome Atlas (TCGA) further classified GBM into four subtypes following extensive genomic analysis. These included classical (CL), mesenchymal (ME), proneural (PN), and neural (NE) types based on distinct gene expression signatures of different cell origins: astrocytic, astroglial, oligodendrocytic, and neural, respectively (11, 12). Discrete genetic abnormalities, such as specific oncogene or tumor suppressor gene mutations, and partial or whole chromosomal loss or gain was observed in these defined subtypes. The CL subtype, which accounts for about 21% of GBMs, is associated with EGFR amplification, seen in 97% of these tumors. Other common characteristics of this subtype include increased expression of Notch and Sonic hedgehog signaling pathways, chromosome 10q23 loss of heterozygosity (LOH), chromosome 7 gain with chromosome 10 loss, and chromosome 9p21.3 homozygous deletion, which encodes p16INK4a and p14ARF. Interestingly, this subtype lacks TP53 mutations. The ME subtype displays expression of CHI3L1/YKL-40, vascular endothelial growth factor (VEGF), and CD44, among other mesenchymal histologic markers (10-12). Also found predominantly in the ME subtype is partial loss of chromosome 17q11.2, where tumor suppressor gene neurofibromin 1 (NF1) resides. Notably, NF1 mutations are present in 32% of all GBMs. The PN subtype, atypical due to its increased expression of oligodendrocytic genes, is seen in approximately 31% of GBMs. PN samples also display mutations or LOH of the TP53 gene. Although present in all subtypes, a higher percentage (35%) of PN samples demonstrate amplification of a chromosome 4q12 locus containing the platelet-derived growth factor receptor A (PDGFRA) gene. Eleven of the twelve IDH1 mutations observed are commonly found in this class and serve as a diagnostic and prognostic marker (12). Approximately 30% of the PN subtype express glioma CpG island methylator phenotype (G-CIMP) and are associated with occurrence in younger patients with more favorable outcomes (13). G-CIMP phenotype is closely associated with methylated O6-methylguanine-DNA methyl-transferase (MGMT) promoter methylation, irrespective of glioma grade, similar to IDH mutation (14). The classic GBM signature was observed in only 54% of these tumors. Lastly, the NE subtype, expressing neural markers, accounts for 16% of GBMs and appears to originate from the neural tissue at the tumor margin. Gain of chromosome 7 with loss of chromosome 10 is frequently seen in the NE subtype (11, 12). A recent study identified the novel potentially targetable oncogenic fibroblast growth factor receptor 3 (FGFR3)-TACC3 (F3T3) fusions in a subset of GBM patients with a prevalence of 4.1%. Lower tumor mutational and copy-number alteration burdens are evident in F3T3-positive GBM relative to F3T3-wild-type GBM. F3T3-positive GBMs are often seen in the ME or receptor tyrosine kinase (RTK) II subclass and are less commonly seen with p53 alterations. Patients with F3T3-positive GBMs have better outcomes with 8 months longer survival compared to F3T3-wild-type GBMs (15).

Gene expression profiling studies have attempted to further characterize GBM in order to reflect survival outcomes. Motomura *et al*. analyzed 79 GBM samples for the expression of 16 proteins relevant to the TCGA classification and observed four subcategories: oligodendrocyte precursor (OPC) type, differentiated oligodendrocyte (DOC) type, astrocytic mesenchymal (AsMes) type, and mixed type (11, 12, 16). Notably, the histological classification contributes to prognosis, as those with the OPC type containing IDH mutation exhibited a longer survival of 19.9 months (16). This study and other genomic and proteomic analysis studies support the updated guidelines for the WHO classification of CNS tumors. Other markers to be considered are mutations of IDH1, MGMT, and 1p/19q co-deletion or ATRX loss, displaying significant diagnostic, prognostic, and predictive abilities (17).

The major hindrance in treatment of GBM is the recurrence of disease, which is partially attributed to the presence of cancer stem cells (CSC; termed glioblastoma stem cells, GSC), a unique population of tumor cells that are capable of proliferation and self-renewal. GSCs play a major role in tumor invasion, recurrence, and resistance to therapies. Like

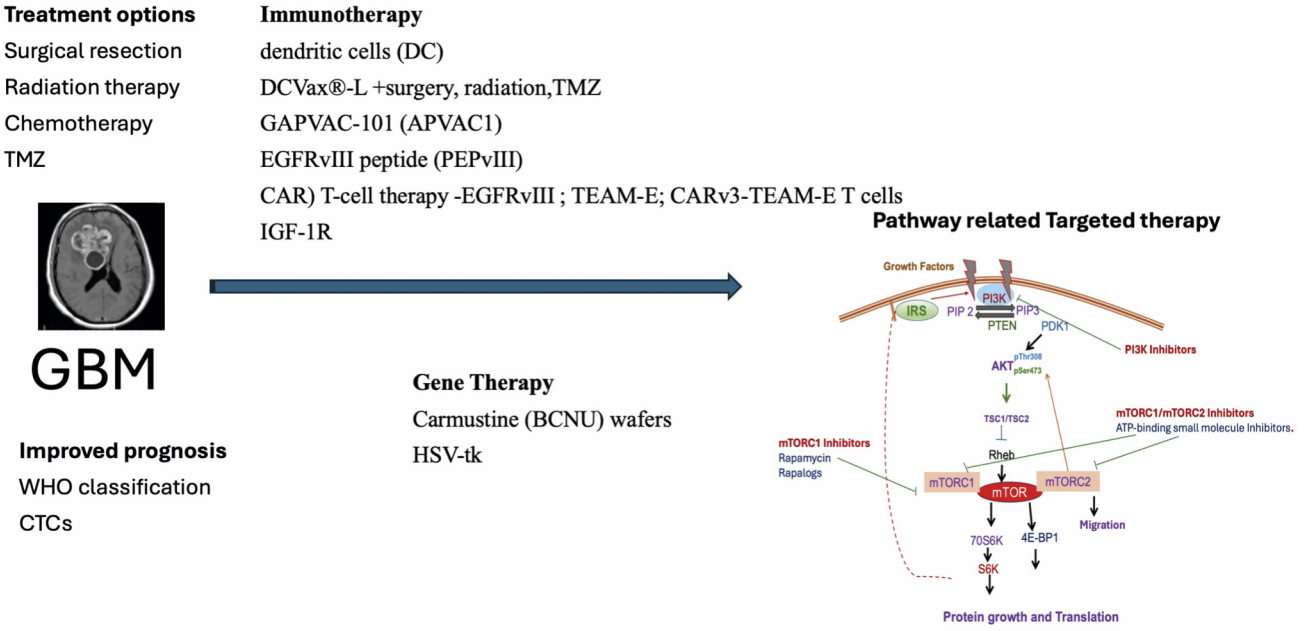

Figure 1. *The standard treatment options for GBM remain maximal surgical resection and radiation therapy, followed by chemotherapy with TMZ. The new WHO classification offers improved criteria for diagnosis, prognosis, and management of GBM. Analysis of CTCs during therapy additionally provides an enhanced prognosis. Novel therapies include immunotherapies and gene therapy. In recent years, pathway-related therapy has also emerged. Specifically, several inhibitors of the PI3K/mTOR pathway have been used in clinical trials. GBM, Glioblastoma; TMZ, temozolomide; CTC, circulating tumor cell; WHO, World Health Organization; DC, dendritic cells; GAPVAC, Glioma Actively Personalized Vaccine Consortium; EGFRvIII, epidermal growth factor receptor variant III; CAR, chimeric antigen receptor; TEAM, T-cell-engaging antibody molecule; IGF-1R, insulin-like growth factor 1 receptor; BCNU, carmustine; HSV-tk, herpes simplex virus thymidine kinase; IRS, insulin receptor substrate; PIP2, phosphoinositol bisphosphate; PIP3, PIP trisphosphate; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; PDK1, phosphoinositide-dependent kinase 1; TSC, tuberous sclerosis complex; mTOR, mechanistic target of rapamycin; mTORC, mTOR complex; S6K, ribosomal protein S6 kinase; 4EBP1, eukaryotic initiation factor 4E-binding protein 1; ATP, adenosine triphosphate.*

GBM, GSCs are genetically heterogeneous. In addition, GSCs possess a high mutation rate and demonstrate epigenetic changes that alter their genomic expression. Not only are GSCs present in the tumor, but they are also known to be present in the areas surrounding the tumor. Conventional treatments that target GBM often fail to eradicate GSCs due to their quiescent nature, allowing the recurrence of disease. The oncogenic properties of GSCs are driven by deregulation of several critical signaling pathways, which serve as potential therapeutic targets. Multi-pronged therapeutic approaches with simultaneous targeting of several pathways and molecules may overcome tumor resistance mechanisms in the treatment of GBM. In this review, we present the recent WHO GBM classification updates, immunotherapies, vaccines, and targeted molecular pathway therapies, as depicted in Figure 1.

Updated WHO Classification of GBM

Gliomas are CNS tumors arising from glial cells and are classified along with glioneuronal and neuronal tumors in the 2021 WHO Classification, which is revised from the 2016 WHO Classification (17). Gliomas encompass a large family of tumors, which are further sub-classified based on tumor grade, growth pattern, and age predilection. GBM, an adult-type diffuse glioma, represents the most common glioma subtype. New advances in molecular genomics have redefined how clinicians identify CNS tumors, leading to a greater understanding of the diagnosis, prognosis, and management (19-22).

The classification of CNS tumors was previously based on histologic and immunohistochemical (IHC) characteristics determined by the microscopic properties and presumed cellular origin (23, 24). It was not until the 2016 update of the WHO classification of CNS tumors that genetic alterations were incorporated into the diagnostic criteria. Notably, grading of diffuse gliomas included an additional emphasis on the driver mutations in IDH1/2 genes. Glioblastoma WHO grade IV was divided into IDH-wild-type, accounting for 90% of cases generally discovered as primary neoplasms in older patients, and IDH-mutant, accounting for 10% of cases and more common in younger patients often secondary to progression of a lower-grade

glioma (23). A third group existed for those with an incomplete or unattainable evaluation of the IDH gene and was thus designated as not otherwise specified (NOS) (23). The combination of phenotypic and genotypic parameters was also first introduced in the 2016 CNS WHO classification, providing improved diagnosis and a better understanding of applicable treatment options (23).

The 2021 fifth edition of the WHO classification of CNS tumors (WHO CNS5) further incorporates molecular variations in the categorization and grading of tumors to create an integrated diagnosis (17, 19). In an attempt to conform CNS tumor grading to that used for non-CNS tumors, the revised classification shifted to within tumor type grading and naming was changed from Roman numerals to Arabic numerals. For example, grade IV glioma is now referred to as grade 4. Thus, astrocytoma IDH-mutant CNS WHO grade 4 has replaced the previous GBM IDH-mutant; GBM now refers specifically to the IDH-wild type entity. Another important update was the combination of histological and molecular grading of gliomas due to increased awareness of molecular variations and their resultant clinical behavior (25, 26). For example, in the new classification system, adult diffuse astrocytic gliomas are considered WHO grade 4 not only if there is histological appearance of microvascular proliferation and necrosis, but also if there is presence of either EGFR gene amplification, TERT promoter mutation, or entire gain of chromosome 7 with loss of chromosome 10 [+7/–10] (17, 19). Furthermore, in pediatric-type diffuse high-grade gliomas, the term "glioblastoma" is no longer used as a classification, and instead, tumors in this group are identified by their distinct molecular profiles (17). The changes made in the WHO CNS5 further delineate the significance of molecular characteristics of GBM to improve diagnosis, prognosis, and decision-making in clinical management. This updated classification, therefore, underscores the importance of molecular aberrations in the pathogenesis and treatment of GBM.

Circulating Tumor Cells of GBM: Diagnostic, Prognostic, and/or Predictive Therapeutic Responses

Circulating tumor cells (CTCs) are detached or passively released tumor cells in the bloodstream or circulation of GBM patients that can be used as important molecular markers of disease progression as well as therapeutic response (27, 28). Gliomas, like other tumors, discharge molecular information into the circulation, including tumor-associated biomarkers, proteins, nucleic acids, and tumor-derived extracellular vesicles that are collected in plasma, serum, blood platelets, urine and/or cerebrospinal fluid (CSF) (29). CTCs have been detected in the blood of glioma patients and serve as an important tool for diagnostic, prognostic, and/or predictive biomarkers to regulate patient management. Several biological analytes, such as CTCs, circulating cell-free DNA (cfDNA) that contain circulating tumor DNA (ctDNA), circulating cell-free tumor RNA (ctRNA) containing mRNAs and mainly microRNAs, extracellular vehicles (EVs), proteins, metabolites, and tumor-educated platelets (TEPs) are seen in body fluids that can be sampled via liquid biopsy (30). Therefore, this approach may help to circumvent problems related to tumor heterogeneity and sampling error at the time of diagnosis. Also, liquid biopsies may allow for serial monitoring of treatment response and changes in the molecular characteristics of gliomas over time. This review summarizes the literature on circulating tumor cell genetic markers and their potential value to improve the management of diffuse glioma patients. Incorporating the study of circulating molecular biomarkers into clinical trials is essential for further assessment of the utility of liquid biopsies in this context. Ultimately, CTCs acquired through liquid biopsy may provide information on GBM stratification and real-time therapy response. Several clinical trials investigating the biomarkers in CTCs of GBM have provided evidence of significant genetic markers relating to diagnosis, prognosis, and therapeutic response (29). CTCs of GBM can provide important genetic information regarding the presence of driver mutations or the presence of multiple crucial genetic markers during therapy and remission. CTCs of GBM can serve as biomarkers to provide a basis for precision medicine or patient-tailored therapies for GBM patients.

In a recent study, where imipridone (ONC201) was used in treatment of children with H3K27M-mutant diffuse midline glioma (DMG), the decreased presence of the H3K27M mutation in CSF and plasma tDNA was associated with a better chance of survival compared to the patients with increased H3K27M mutation in circulating DNA (31). Another recent study assessed tissue paired with cDNA collected from CSF via lumbar puncture [41], intraoperative extraction [3], and Ommaya reservoir [1]. The presence of several gene mutations was noted in the CSF of 45 brain tumor patients, including H3K27-altered DMG [14], GBM [1], H3-wild-type astrocytoma [10], ependymoma [11], and other lesions [9] (32).

Immunotherapy in the Treatment of GBM

Multiple ongoing clinical trials in various phases (Phase I, II, and III) are currently evaluating the application of cancer immune vaccines for GBM patients. The status of some of these trials was reviewed by Galluzzi *et al*., 2012 and Aranda *et al*., 2013, and "Clinicaltrials.gov" provides updated information on past and current clinical trials (33, 34). The principle behind the cancer vaccine hypothesis in GBM is to stimulate the patient's immune system to target residual tumor cells by creating a vaccine with patients' dendritic cells (DC) programmed against their specific cancer stem

cells. These immune-stimulating DC are harvested and isolated from patients' tumors or blood via leukapheresis. The isolated DC is then combined with the tumor antigen or cancer stem cells and matures to generate an immune response against them. The resulting vaccine is then implanted in the patient. DC vaccines have shown promising results in malignant glioma patients, demonstrating improvement in median survival to 525 days and 5-year survival to 18.8% in newly diagnosed or recurrent grade 4 gliomas (35). The successful outcome of these vaccines is reliant on the body's natural immune system to mount a response targeting the neoplastic cells.

A Phase III trial recently investigated DCVax®-L for patients with newly diagnosed GBM in conjunction with the current standard-of-care, including surgery, TMZ, and radiation. In this trial, a patient's DCs are mixed with the patient's extracted tumor lysate so that they recognize GBM cells. The immune-modified DCs are then administered to the patient. The major endpoints of this trial were to investigate progression-free survival (PFS) and OS following treatment with DCVax®-L. Phase I/II clinical trials were previously completed in patients (n=39) that included 20 newly diagnosed and 19 cases of recurrent GBM or other gliomas. In these early studies, patients who were newly diagnosed and received DCVax in conjunction with standard therapies typically had delayed tumor recurrence until a median of approximately 2 years (more than triple the usual time compared with standard-of-care treatments), and the median OS was also extended to approximately 3 years (about 2.5 times the median survival with the standard-of-care treatment). In the intention-to-treat Phase III trial, 331 GBM patients were included, with 232 patients receiving DCVax®-L compared to 99 patients receiving placebo; all patients received standard-of-care therapy. Results demonstrated a median OS of 19.3 months from randomization (or 22.4 months from surgery) for those who received the vaccine compared to an external control of 16.5 months. Also, the median OS was further extended to 30.2 months from randomization in patients with MGMT. Approximately 15.7% of vaccinated patients survived up to 48 months compared to 9.9% of control patients. Patients with recurrence were allowed to cross over to the experimental arm and were found to have a median OS of 13.2 months from relapse compared to 7.8 months in controls. PFS was no longer used as an endpoint due to ambiguity in distinguishing true progression versus pseudo-progression (36, 37). Another trial evaluated the use of DC vaccine therapy in 56 patients with relapsed GBM, which typically has worse survival with a median PFS of 2 months and nearly 100% mortality by 18 months, with total resection with minimal residual disease burden before vaccination was the only significant predictor of improved PFS where survival at the 2- or 3-year mark was observed in 14.8% and 11.1%, respectively (38).

In a phase I trial conducted by the Glioma Actively Personalized Vaccine Consortium (GAPVAC), GAPVAC-101, personalized vaccines were integrated into the standard treatment regimen for patients with newly diagnosed GBM. The study involved fifteen patients with GBM expressing human leukocyte antigen (HLA)-A02:01 or HLA-A24:02. These patients were treated initially with a vaccine (APVAC1) created from a pre-manufactured library of unmutated antigens, followed by a second vaccine (APVAC2) that specifically targeted neoepitopes. The vaccines were tailored based on mutations and detailed analyses of the transcriptomes and immunopeptidomes of each patient's tumor. The vaccine approach was determined to be feasible, which included poly-ICLC and granulocyte colony-stimulating factor (GCSF) as adjuvants, showing favorable safety profiles and strong immunogenicity. APVAC1 triggered sustained responses from central memory CD8+ T cells, while APVAC2 mainly induced CD4+ T cell responses against the predicted neoepitopes (39).

Technical issues, as well as the comparatively high cost of tumor-based DC vaccine therapy, make immunotherapeutic approaches targeting the unique deletion mutant EGFR class III variant (EGFRvIII) a promising and more accessible option. Using a peptide-based vaccine, EGFRvIII peptide (PEPvIII) is an EGFRvIII-specific, 14-mer peptide (H-Leu-Glu-Glu-Lys-Lys-Gln-Asn-Tyr-Val-Val-Thr-Asp-His-Cys-OH) coupled to keyhole limpet hemocyanin (KLH) that prompts an immune response to EGFRvIII, a tumor-specific antigen implicated in many malignancies including GBM (40). Phase I/II studies (VICTORI, ACTIVATE, and ACT II) have been completed without serious adverse events. ACTIVATE, a Phase II trial consisting of 19 patients with newly diagnosed EGFRvIII-expressing GBM who underwent standard-of-care plus the KLH-conjugated peptide vaccine, demonstrated a delayed median time to progression after surgery of 12 months compared to historical controls of 7.1 months. Phase III clinical trials are currently underway (41).

Chimeric antigen receptor (CAR) T-cell therapy, successful in various hematologic cancers, faces challenges in solid tumors due to complex microenvironments, cellular and genetic heterogeneity, and immunosuppressive factors. In GBM, targeting of the tumor-specific protein EGFRvIII has been a focus, although the tumors often develop resistance. In a recent novel approach by Choi *et al*., T cells were engineered to secrete a bispecific antibody, called T-cell-engaging antibody molecule (TEAM-E), which recognizes both EGFR and CD3 and enhances interaction between T cells and tumor cells, aiming to overcome heterogeneity and efficacy by ensuring localized delivery of the treatment. Three GBM patients were treated with CARv3-TEAM-E T cells, demonstrating significant tumor regression in all patients. While one patient experienced a durable response, only transient benefit was observed in the other two patients. Still, the treatment showed a manageable

safety profile with no dose-limiting toxic effects. These promising early results suggest the potential for CAR T-cell therapy in GBM, highlighting the need for future research to enhance and prolong treatment efficacy (42).

Finally, insulin-like growth factor 1 receptor (IGF-1R) represents another commonly overexpressed surface receptor recognized in malignancies, including GBM. While IGF-1R has been an unsuccessful target of monotherapy, current ongoing clinical trials are evaluating IGV-001, a mixture of patient GBM tumor cells treated with an anti-IGF-1R antisense oligonucleotide that is placed into biodiffusion chambers and implanted into the abdomen for 24 to 48 h. A Phase I trial consisting of 33 subjects receiving IGV-001 in addition to standard-of-care initially showed an improved PFS of 9.8 months compared to 6.5 months in previously published studies. IGV-001 is now undergoing Phase IIb trial (43). Some of the current ongoing trials are presented in Table I.

Gene Therapy in the Treatment of GBM

The treatment goal for malignancies, including GBM, is to provide tumor-specific cytotoxicity without damage to normal cells; thus, systemic chemotherapy is not ideal for brain tumors. Further, even normal cells in the tumor bed are not spared from localized radiotherapy. Carmustine (BCNU) wafers placed in the resection cavity at the time of surgery represent the only approved local intra-cavity chemotherapy and have been effective in GBM treatment with a marginally increased rate of wound healing complications. While this represents a form of localized chemotherapy, its activity remains non-specific for targeting tumor cells (44). Alternatively, several therapeutic approaches, including gene therapies, have been explored to more specifically target tumor cells for patients with GBM (45).

Suicide gene therapy using viral vectors to transfect tumor cells has been utilized with little success. A recent phase III trial investigated the local administration of an adenovirus vector into the resection cavity of GBM patients (44). This cDNA coding for herpes simplex virus thymidine kinase (HSV-tk) is incorporated into DNA of the proliferating tumor cells and phosphorylates ganciclovir to ganciclovir triphosphate, a cytotoxic analog that selectively induces apoptosis in cells with transfection and their neighboring dividing tumor cells; this procedure has been shown to spare normal neurons since they remained unproliferative and ineffective to the toxic metabolites (46-48). GBM patients across multiple countries were randomized into the treatment group, with 119 patients, and the non-treatment group, with 117 patients. The treatment group received local injections in the resection cavity walls up to a depth of 2 cm. A five-day period was allowed for transduction followed by twice daily intravenous ganciclovir administration from day 5 to 19 postoperatively. There was variability in both groups regarding the standard-of-care received, as some patients did not receive either TMZ or RT. The study was successful in achieving its primary endpoint of increased median time to re-intervention or death after administration of the vector, regardless of TMZ administration, from 268 days in the control group to 308 days in the experimental group ($p=0.006$); however, improvement in overall survival (OS) was not achieved. In addition, a significant number of patients experienced adverse events, 88 patients (71%) in the experimental group versus 51 patients (43%) in controls. Although most adverse events were self-limited, hemiparesis (8 in experimental, 3 in control) and aphasia (6 in experimental, 2 in control) were most commonly observed. Thus, the results of this study concluded that the marginal benefit of gene therapy is associated with moderate risk. Therefore, gene therapy requires further evaluation and optimization prior to incorporation into standard-of-care GBM therapy.

mTOR Inhibitors in the Treatment of GBM

A recent strategy utilized in the development of antineoplastic therapies is the targeting of signaling pathways that are integral to the growth of tumor cells; one such target is the phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway. The PI3K/mTOR pathway is subject to activation by EGFR, a previously identified target for antineoplastic drugs, and inhibition by PTEN, a tumor suppressor (49-51). Loss of PTEN occurs in 36-60% of GBM tumors, causing sustained activation of the PI3K/Akt/mTOR pathway and, therefore, implies that inhibition of the pathway may have therapeutic potential. Numerous clinical trials have been initiated to investigate the use of inhibitors of this pathway in the treatment of GBM. These trials were mostly conducted in recurrent GBM with some newly diagnosed cases. The efficacy of these inhibitors was investigated in various regimens, including as a stand-alone therapy and in conjunction with standard-of-care or other pathway inhibitors. This article highlights some of these trials.

Sirolimus, also known as rapamycin, is an mTOR inhibitor evaluated in the treatment of recurrent GBM. A phase I clinical trial investigated the safety of daily neoadjuvant sirolimus (2, 5, or 10 mg oral) in 15 recurrent GBM patients with PTEN loss for approximately 1 week before re-resection and continuing until progression with grade 3 toxicities in 5 patients (52). While 7 patients were noted to have a decreased Ki-67 proliferative index, time to progression (TTP) was reduced. Notably, IHC showed increased expression of activated Akt, as demonstrated by $pAkt^{Ser473}$ expression in tumor specimens. Furthermore, expression of pPRAS40, another marker of mTORC1 activation, was significantly higher in patients who demonstrated a shorter time to disease recurrence. It appeared that prolonged use of sirolimus disrupted a negative feedback loop, leading to the activation of Akt in patients following treatment.

Table I. *Clinical trials of immunotherapy in glioblastoma*.

Immunotherapy/ Target	Drug/ Intervention	Cancer type	Patients (n)	Clinical Trial Number(s) (Recruitment status, Phase)
Cytomegalovirus antigens	PEP-CMV vaccine	Newly diagnosed MGMT-unmethylated GBM	26*	NCT06132438 (not yet recruiting, Phase I)
Actively personalized vaccination	APVAC1 vaccine plus Poly-ICLC and GM-CSF	HLA-A*02:01 or HLA-A*24:02 positive patients with newly diagnosed GBM	16	NCT02149225 (completed, Phase 1)
Dendritic cell based tumor vaccine	(I) ADCTA-G (II) ADCTA-SSI-G1 (III) ICT-107 (IV) Autologous DC loaded with an autologous tumor lysate (V) Dendritic cell immunization	(I) Primary GBM (II) Primary GBM (III) HLA-A1 or HLA-A2 positive patients with newly diagnosed GBM (IV) Newly diagnosed GBM (V) GBM IDH wild-type, with unmethylated MGMT-gene promotor	(I) 50* (II) 118* (III) 124 (IV) 76* (V) 60*	(I) NCT02772094 (unknown, Phase II) (II) NCT04277221 (unknown, Phase II) (III) NCT01280552 (completed, Phase II) (IV) NCT04801147 (unknown, Phase I/II) (V) NCT03548571 (recruiting, Phase II/III)
Proteome-based personalized immunotherapy	Proteome-based Dendritic vaccine, Autologous hematopoietic stem cells, Cytotoxic lymphocytes	Recurrent GBM	60*	NCT01759810 (unknown, Phase II/III)
Immunostimulating Agent	CpG-ODN	Primary GBM	80	NCT00190424 (completed, Phase II)
Anti-PD-1 immunotherapy	Pembrolizumab	Recurrent GBM	30*	NCT04977375 (recruiting, Phase 1/II)
Anti-CD38 immunotherapy	Daratumumab	Primary GBM	16*	NCT04922723 (recruiting, Phase I/II)
CD200AR-L and allogenic tumor lysate vaccine	hP1A8, GBM6-AD	Recurrent GBM	24*	NCT04642937 (active, not recruiting, Phase I)

*Estimated number of patients to be enrolled. GBM, Glioblastoma; PEP-CMV, peptide vaccine derived from cytomegalovirus; MGMT, O6-methylguanine-DNA methyltransferase; APVAC, actively personalized vaccine; Poly-ICLC, poly-L-lysine; GM-CSF, granulocyte-macrophage colony-stimulating factor; HLA, human leukocyte antigen; ADCTA, autologous dendritic cells/tumor cell antigen; IDH, isocitrate dehydrogenase; CpG-ODN, CpG oligodeoxynucleotides.

In a separate phase I trial, sirolimus was evaluated in combination with an EGFR inhibitor, erlotinib, in the treatment of recurrent WHO grade 3 and 4 gliomas, as defined by the Macdonald criteria, to determine the maximum tolerated dose (MTD) of the combination (53). Nineteen patients, including 14 GBM patients, participated in the study. The first 7 days consisted of erlotinib alone, followed by a 15-mg loading dose of sirolimus on day 8 with subsequent daily 5 mg maintenance. This trial was unsuccessful, as it was terminated after five dose-limiting toxicities (DLT) were seen. A phase II clinical trial performed by another group evaluated the combination without a corresponding phase I trial, using the MTD determined from single-agent sirolimus and erlotinib studies (54). The study included 32 participants with recurrent GBM after receiving previous standard-of-care treatment. Sirolimus was administered daily at 10 or 5 mg for patients taking or not taking CYP3A-inducing anti-epileptic drugs (EIAEDs), respectively. While the doses were well tolerated, the primary endpoint of PFS at 6 months (PFS6) was only 3.1%, with a median OS of 33.8 weeks. PFS was slightly improved in patients not on EIAEDs, which may be related to interactions with drug metabolism.

Sirolimus has also been investigated in combination with the EGFR/VEGF inhibitor vandetanib in a phase I trial with

a phase II component for recurrent GBM patients (55). The MTD was determined in phase I/II patients who did not receive CYP3A-inducing medications. The MTD, vandetanib 200 mg and sirolimus 2 mg, was administered to 19 patients, with a radiographic partial response (PR) in two patients. The median PFS and OS were 2.1 and 7.7 months, respectively, with a PFS6 of 15.8%. This trial concluded that co-administration of sirolimus with the multi-kinase inhibitor vandetanib is safe.

Temsirolimus, an ester of sirolimus, has undergone many clinical trials to determine its dosing limitations as monotherapy or in combination with other therapeutics and its efficacy both in recurrent GBM or in comparison to standard-of-care. Compared to sirolimus, temsirolimus displayed fewer negative immunosuppressive effects, specifically on T-lymphocyte function (56). Further, phosphorylated mTOR status has been identified as a favorable biomarker for response to temsirolimus therapy.

Temsirolimus was trialed in 65 recurrent GBM patients in a phase II study using 250 mg intravenous (IV) weekly (57). Overall, there was neither a reported radiographic response nor an improvement in OS. Interestingly, radiographic improvement was observed in 36% of study patients. Increased presence of phosphorylated S6 kinase (pS6K), a downstream substrate of the mTOR pathway, in baseline tumor samples was significantly associated with radiographic improvement and responsiveness to temsirolimus therapy, while such association was not evident with PTEN and pAkt expression (57). Responders had a significantly longer TTP of 5.4 months compared to 1.9 months in non-responders.

Another phase II study investigated the efficacy and toxicity of temsirolimus in 43 patients with radiographically and pathologically confirmed GBM recurrence (58). Although temsirolimus was well tolerated, for patients who were taking EIAEDs or who had experienced toxicities, their dose was reduced to 170 mg IV weekly. Radiographically, 20 patients displayed stable disease (SD), while 2 had PR. Of note, the molecular status with regards to the PI3K/Akt/mTOR pathway was not incorporated into the analysis. Overall, the TTP was 9 weeks with a PFS6 of 2.3%.

Several clinical trials have combined the use of rapalogues with other inhibitors. In one such phase I/II trial, temsirolimus and erlotinib were evaluated in recurrent malignant gliomas (59). Combination with erlotinib lowered the MTD of temsirolimus (15 mg weekly) compared to previous trials. Twelve patients from phase I received the MTD of temsirolimus and were added to the phase II trial of patients (n=59), which included 43 patients with recurrent GBM. SD was seen in only 12 patients. Biomarker evaluation revealed EGFR amplification in 48% of GBMs, with both EGFRvIII and EGFR amplification in approximately 30%. Following treatment, the expression of pS6K remained unchanged between samples, and patients without progression displayed phosphorylated ERK; however, there were no significant therapy response markers for activation of the PI3K/mTOR or mitogen-activated protein kinase (MAPK) pathways. Eight weeks median PFS was achieved, including a PFS6 of 13%. In summary, the combination of these two inhibitors decreased the MTD of temsirolimus, thus potentially preventing the achievement of therapeutic levels.

Temsirolimus was also used in combination with the MAPK inhibitor, sorafenib, in a phase I/II trial of recurrent GBM patients (50). Patients were divided into two separate cohorts based on prior anti-VEGF treatment with bevacizumab. Those who had received prior bevacizumab had a worse PFS6 of 9.5% compared to 17.1% in those without prior treatment. The combined treatment decreased MTD, potentially limiting the efficacy. This drug combination was studied in another phase I/II study of recurrent GBM patients without prior anti-VEGF therapy, which determined the MTD of temsirolimus to be 25 mg weekly (49). Of the eighteen patients in the phase II stage, PR was achieved in only 2 patients, and the median PFS was 8 weeks. Similar to the previously described trial, the reduced MTD of temsirolimus with poor CNS penetration of sorafenib was credited for the limited efficacy of the combination. When studied in combination with bevacizumab, the 25 mg MTD of temsirolimus given every 8 days was unsuccessful as none of the patients showed partial remission (60).

The combination of temsirolimus with perifosine, an Akt inhibitor, was investigated in a phase I study in patients with recurrent gliomas, including 17 recurrent GBM patients, which was able to establish a higher MTD; temsirolimus 115 mg weekly was tolerated concurrently with a 600 mg load of perifosine followed by 100 mg daily (61). This combination aimed to target and sequentially blockade the PI3K/mTOR pathway. Median PFS and OS were 2.7 and 10.4 months, respectively. Notably, one patient with PTEN loss, MGMT promoter unmethylated, IDH-wild type recurrent GBM experienced a long-term PR for 4.5 years. DLT of the combination of temsirolimus with perifosine included severe lung infection, which prompted the incorporation of pneumocystis jiroveci pneumonia (PJP) prophylaxis.

While previous temsirolimus trials discussed in this review have targeted recurrent GBM, the use of temsirolimus in newly diagnosed GBM is also being investigated. In a phase I trial led by Sarkaria *et al*., 12 newly diagnosed GBM patients were given a combination of temsirolimus and standard-of-care therapy, establishing the MTD of temsirolimus to be 50 mg weekly (62). Patients demonstrated significant immunosuppression with an elevated infection risk, which prompted the use of prophylactic antibiotics. A separate phase II trial comparing temsirolimus with RT versus TMZ with RT in MGMT promoter unmethylated newly diagnosed GBM patients had similar OS between groups, 14.8 months for the

temsirolimus group versus 16.0 months for the TMZ group (63). Analysis of pathway component biomarkers within the subgroup demonstrated a significant increase in OS following temsirolimus treatment for patients' tumors who expressed phospho-mTORSer2448. Of the tumors, 37.6% showed expression of phospho-mTORSer2448.

Everolimus is another derivative of sirolimus that acts as an mTOR inhibitor and has been investigated in the treatment of GBM. Everolimus and EGFR inhibitor gefitinib were studied in patients with recurrent GBM in a phase I/II clinical trial (64). Results demonstrated PR in 14% and SD in 36%, but only one patient reached PFS6. The investigators noted that the EGFR and PTEN biomarker status failed to predict treatment outcomes.

Everolimus has also been evaluated in newly diagnosed GBM. Everolimus was combined with standard-of-care therapy in a phase I study of 18 patients, and a dose of 70 mg weekly was reported to be well tolerated (65). Stable disease was noted in 14 patients. Tissue metabolism was measured by fluorodeoxyglucose (FDG)-positron emission tomography (PET), displaying a partial response in 4 patients. Of these patients with metabolic responses, an increased serum everolimus level was observed.

A phase II study of 68 newly diagnosed GBM patients sought to evaluate the addition of bevacizumab to standard-of-care followed by everolimus after completion of RT. This combination demonstrated an acceptable safety profile and efficacy. Median PFS was favorable at 11.3 months, and 61% of patients displayed a fair radiographic response; however, OS was not significantly changed (66).

The recommended dose of everolimus 70 mg weekly was tested starting 1 week prior to standard-of-care and continuing in conjunction with adjuvant TMZ until progression in a phase II trial of 104 newly diagnosed GBM patients (67). Of those who were imaged with 3'-deoxy-3'-(18)F-fluorothymidine [(18)FLT]-PET, less than 40% had a partial response following everolimus (two doses); these patients had no obvious alteration PI3K/Akt pathway. This study concluded that everolimus had moderate toxicity. The (18)FLT-PET findings suggested that everolimus had an initial anti-proliferative effect with no survival benefit (67). In a separate phase II trial where 171 patients were given standard-of-care therapy with or without everolimus, the study concluded that the addition of everolimus caused increased toxicity and no significant difference in PFS or OS compared to standard-of-care alone (68).

Other drug combinations have been explored with everolimus in advanced solid tumors, including a phase I trial evaluating everolimus in conjunction with dactolisib, a PI3K inhibitor (69). These inhibitors demonstrated a potential interaction with poor tolerance and efficacy. Currently, there are ongoing clinical trials that continue to evaluate everolimus in the treatment of GBM (NCT03834740).

Stem Cell Therapy in the Treatment of GBM

GSCs are believed to play a pivotal role in the recurrence and regeneration of GBM (70-72). The existence of CSCs has been well-established in both GBM and medulloblastoma (70, 71, 73-76). Like stem cells, GSCs are characterized as pluripotent cells, which have a capacity for self-renewal. Along with typical properties of neuronal stem cells, GSCs possess enhanced DNA repair and mitochondrial reserve and have been expressed not only in the tumor mass but also in peritumoral areas (18, 77). Their ability to display uncontrolled growth and to replicate after chemotherapeutic treatments or tumor resection is a critical factor contributing to tumor regenesis (71, 74, 78). Particularly, studies have demonstrated GSCs can enter a quiescent state, rendering a refractory status with lower susceptibility to therapies (79).

In recent years, the search to identify chemical targets against GSCs has found specific pathways and markers of interest. Specifically, the stem cell marker Nestin and the MAPK pathway have emerged as valuable prognostic tools for GBM, as has CXCR1, which is increasingly recognized as a potential CSC marker (18, 80). Additionally, prior studies have demonstrated resistance of tumorigenic CD133+ enriched CSCs against irradiation and chemotherapeutic agents for GBM (12, 77). Tumor suppressors, such as PTEN and p53, have also been implicated in their potential regulatory role of self-renewal in CSCs (81).

Of particular interest, the PI3K/Akt/mTOR and EGFR pathways have been found to significantly influence the survival and preservation of GSCs (82). Of these, the mTOR signaling pathway, which is essential in the regulation of growth and migration in normal neural stem cells, is also a key factor in the unregulated growth and invasion of GSCs (83). Thus, inhibition of the mTOR pathway serves as a promising target against proliferation for various cancers (84). In our previous study, we investigated this regulatory function of the mTOR pathway in GSCs. In applying inhibitors of the PI3k/Akt/mTOR pathways, such as rapamycin, a selective mTORC1 inhibitor, PP242, an ATP-binding mTORC1/2 inhibitor, LY294002, a PI3K inhibitor, and U0126, a MAPK inhibitor, we observed the effective targeting of GSCs using Torin2, a novel small molecule inhibitor of mTORC1/2, leading to disruption (85).

In one study, GSC lines obtained from surgery most commonly revealed mutations in TP53 (44%), followed by PTEN (35%) and RB1 (17%). One GSC sample with the BRAFV600E mutation responded to BRAF inhibitors in an in vitro study. In addition, RNA sequencing revealed that the GSC lines formed three "clusters", distinguished by different upregulated genes. Increased mutations in "mismatch repair, cell cycle, p53, and methylation related pathways" were seen in GSCs obtained in surgery I, while samples from surgery displayed mutations in receptor tyrosine kinase and MAPK signaling pathways (86).

Conclusion

The WHO CNS5 integrated molecular data with histology in the classification of CNS tumors, including GBM. Tumors are clustered by biologically and molecularly distinct characteristics coupled with patients' natural histories, with refined new tumor types and subtypes. The major aim of these updated classifications is to provide a better understanding of the diagnosis of GBM patients, which may allow for improved prognostication and development of ideal targeted therapies. With the new WHO CNS5, IDH-wild type adult diffuse astrocytic tumors, void of the typical histologic features of GBM, are now classified as GBM if they display one or more of three genetic abnormalities, such as TERT promoter mutation, EGFR gene amplification, or combined gain of entire chromosome 7 and loss of entire chromosome 10 [+7/–10]. Furthermore, WHO CNS5 classifies all IDH-mutant diffuse astrocytic tumors as a single type (astrocytoma, IDH-mutant) with different grades, 2-4, depending on molecular findings, such as homozygous deletion of CDKN2A/B, which confers a worse prognosis. Even in the absence of such histological features of microvascular proliferation or necrosis, it is important to note that IDH-mutant astrocytomas can still be defined as WHO CNS5 grade 4 tumors. Extensive interventions have been explored to achieve better survival outcomes for GBM patients. Several clinical trials using immunotherapies and novel pathway-related targeting aim to provide precision medicine tailored toward individual patients based on the genetic makeup of their GBM tumors. The development of novel therapies has recently focused on targeting GSCs, which play a significant role in not only tumor recurrence but also GBM resistance to therapy. As shown in Figure 1, recent investigations of the presence of genetic markers in the CTCs from patient CSF or serum have provided crucial genetic information on the diagnosis, prognosis, and disease recurrence, which would provide better management and improved survival of GBM patients.

Conflicts of Interest

All Authors (SZ, ES, AMC, SH, CG, MJU) declare no conflicts of interest in relation to this study.

Authors' Contributions

SZ and ES contributed to literature review, writing, reviewing, and editing. AMC contributed writing, editing, and preparation of figures for the manuscript. SH and CG contributed to funding acquisition, supervision, and reviewing. MJ-U contributed to conceptualization, data curation, formal analysis, funding acquisition, project administration, supervision, writing, reviewing & editing.

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