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REVIEW ARTICLE

Feasibility of Targeting Glioblastoma Stem Cells: From Concept to Clinical Trials

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Abstract: ***Objective*:** Glioblastoma is a highly aggressive and invasive brain and central nervous system (CNS) tumor. Current treatment options do not prolong overall survival significantly because the disease is highly prone to relapse. Therefore, research to find new therapies is of paramount importance. It has been discovered that glioblastomas contain a population of cells with stem-like properties and that these cells are may be responsible for tumor recurrence.

***Method*:** A review of relevant papers and clinical trials in the field was conducted. A PubMed search with related keywords was used to gather the data. For example, "glioblastoma stem cells AND WNT signaling" is an example used to find information on clinical trials using the database ClinicalTrials.gov.

***Results*:** Cancer stem cell research has several fundamental issues and uncertainties that should be taken into consideration. Theoretically, a number of treatment options that target glioblastoma stem cells are available for patients. However, only a few of them have obtained promising results in clinical trials. Several strategies are still under investigation.

***Conclusion*:** The majority of treatments to target cancer stem cells have failed during clinical trials. Taking into account a number of biases in the field and the number of unsuccessful investigations, the application of the cancer stem cells concept is questionable in clinical settings, at least with respect to glioblastoma.

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1. INTRODUCTION

Glioblastoma multiforme (GBM) is one of the most aggressive forms of cancer, with a prevalence of 15.8% among all primary central nervous system (CNS) and brain tumors [1]. The current therapies to treat glioblastoma include surgical removal of the tumor and radiotherapy followed by temozolomide (TMZ) chemotherapy. Moreover, after first-line treatment, the vast majority of glioblastoma patients may experience the recurrence of the disease, and there is no salvage therapy with promising clinical outcomes for such patients. Additionally, it should be noted that currently available treatments have considerable limitations as well as the disadvantage of adverse effects [2].

The recurrence of GBM could be explained by various reasons. However, the main reason is considered to be tumor heterogeneity. Some tumor cells are intrinsically resistant to radiotherapy or chemotherapy. This phenomenon is due to versatile molecular and cellular alterations within tumor cells [3]. In 2012, Jian Chen *et al.,* using a genetically-engineered mouse model of glioma, showed that, after TMZ treatment, a population of glioblastoma cells having stem-cell properties promoted tumor regrowth. Importantly, the tumor bulk was mostly made up of the progenies of these cells [4]. This is an

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illustrative example of how one tumor cell clone could result in the recurrence of the disease. This is the example of cancer stem cells (CSCs) in action.

CSCs were discovered due to their ability to efficiently seed new tumors upon xenograft transplantation [5]. Moreover, CSCs commonly may be in a quiescent state with low proliferation rates. They have high levels of antiapoptotic as well as DNA-repair proteins. Therefore, CSCs are believed to resist diverse therapeutic approaches [5, 6]. However, the fundamental conjectures regarding CSCs are not unambiguous and are not always supported by investigations.

For instance, Kelly et al. showed that more than 10% of the bulk tumor cells of a murine model of lymphoma are able to initiate malignancies upon transplantation to syngeneic mice [7]. Having shown this effect, the authors presumed that xenograft transplantation studies might not be the ideal solution to identify CSCs because mice and human organisms are not fully compatible. The mice's tissue microenvironment (TME) could be hostile to human cancer cells. Thus, the CSC concept might be not accurate. Shortly after the publication of Kelly et al, Kennedy et al. published a comment on the work where they questioned the methods used in the aforementioned article, stating that the fundamental concept underlying the CSC hypothesis is not related to the absolute frequency of these cells [8]. Their findings indicated that a similar percentage of cells are able to initiate malignancies even in a case of xenograft transplantation, and thus disproved the idea proposed by Kelly et al. [7].

Nothing is clear with the CSC concept: for instance, some researchers even believe these cells to be non-cancerous, whereas others assume they are. The various key issues related to the concept itself are discussed in a number of authoritative reviews [5, 6, 9-11]. Pioneering studies CSCs established a lucrative target to treat various cancers. However, in the course of several years, it became clear that CSCs represent many difficult challenges: a very "high-hanging" fruit indeed. In this critical review, we will discuss attempts to target CSCs in glioblastoma as well as some aspects of stem cell research in general that must be taken into consideration.

2. GLIOBLASTOMA STEM CELL RESEARCH

It is known that cancer cells are heterogenic in nature. Basically, there are two models to describe the origin of clonal heterogeneity: the stochastic model and the hierarchical model. According to the hierarchical model, there is a strong pecking order within the cancer cell population where cancer stem cells give rise to more differentiated progenies and are responsible for tumor formation [11]. Analyzing the hierarchical model, one may think that targeting CSCs is a promising approach to eliminate cancer's proliferative potential. However, the first challenge is the definition of cancer stem cells.

There are several terms to describe these cells. There is no strong evidence of these terms, reflecting a single population or even distinct populations. Some researchers apply the term "cancer stem cells", whereas others use "cancer stem-like cells". Secondly, the so-called "tumor-initiating cells" and "tumor-propagating cells" are common. Importantly, these terms could have different meanings [10]. They do not reveal the molecular landscapes of the cells that they are said to represent. Sometimes these terms are mixed, creating more uncertainties. In addition, authors may subtly interchange terms, for example, the term *"stem cell"* in the title may be transformed to "stem cell-like brain tumor-initiating cells" in the abstract [12]. In order to distinguish these terms without bias, one must compare and contrast the molecular basics that underlie the differences (if they exist) between cell subtypes.

A bulk of uncertainties is at least partially due to limited techniques available for the identification of cancer stem cells. Studies focusing on the identification of GSCs or tumor-initiating cells rely on the methods initially designed to identify neural stem cells [13, 14]. Tilghman et al stated that GSCs represent tumor-propagating cells with stem-like characteristics, albeit they used similar techniques as in the previous examples (REF 13,14) [15]. Importantly, the cells used for experiments were shown to be capable of unipotent as well as multipotent differentiation, suggesting their stem cell status [16]. Thus, the ambiguous terminology remains.

The preliminary identification and characterization of GSCs may be based on the isolation of cells expressing various surface markers such as CD133, nestin, and other known stem cell signatures. However, these biomarkers might not represent the functional capabilities of the purified cells. For example, tumor-seeding and multipotent differentiation capabilities have been shown for CD133-positive cells [17-19]. Interestingly, CD133-negative cells induced the generation of CD133-positive cells upon passaging, thus suggesting that expression of this protein might be a dynamic characteristic that is not required for brain tumor initiation [19]. Of note, CD133 might have a prognostic value in clinical settings [20]. Reasonably, one should use biomarkers that strongly participate in maintaining stem cell properties, thus the loss of their expression would result in detectable changes in the GSC functionality.

The sphere-forming assay is one of the crucial methods used to characterize the clonogenic capacity of stem cells both in normal brain and in the disease. However, the technique has several significant flaws and limitations that could lead to unreliable or biased results. For instance, the assay is sensitive to cell density; and it is not applicable in determining quiescent stem cells. Besides stem cells, progenitor cells are able to form spheres as well [21]. It is worth noting that mitogens used in the assay could alter differentiation signatures of cells to be tested [21]. To illustrate, various GSCs populations regarding EGFR expression could differentially respond to EGF concentrations in the culture medium that should be taken into account when conducting research on EGFR inhibitors [22]. Nonetheless, it has been shown that GSCs are able to grow independently of exogenous mitogens [18, 23]. This may be related to autocrine signaling common for cancer cells [23]. However, the techniques employed in the study (REF 21), in particular, establishing GBM culture, were performed with the use of fetal bovine serum (FBS), which is not appreciated by a number of researchers. The main obstacle related to the application of FBS is deviations in concentrations of its components and their biological activities as well [24]. Upon culturing in an FBS-containing

medium, GBM cells become less likely to represent the initial disease; namely, they show the altered expression of NSC-related genes. They exhibit changed cellular characteristics [25].

Another hindrance in cancer stem research is that neither sphere-forming assay nor xenograft transplantations are able to show hierarchy within the tumor reasonably. In order to improve the accuracy and identify *bona fide* targets, one should apply lineage tracing or other techniques to track cellular fate during the assays performed [26]. Tirosh et al analyzed 4347 single cells from oligodendroglioma using RNA sequencing and showed developmental programs that could drive heterogeneity in the tumor and affect its growth [27]. Of note, the technique is not readily available for straightforward application.

When analyzing CSCs, it is important to choose relevant *in vitro* models that reflect the signatures of the original disease. For example, the commonly used U87 cell line does not represent the cellular and molecular characteristics of glioblastoma in comparison with patient-derived cells [16]. During *in vivo* experiments, U87-derived tumors displayed reduced infiltration potential, the cells did not leave the area of injection, and they did not show the same immunoreactivity as was observed in patient-derived stem cell-established grafts. CSCs exist in so-called niches that provide functional and molecular integrity within the tumor [10]. Sadly, it is well known that *in vitro* studies do not preserve the real conditions in which tumor cells exist.

In order to circumvent this issue, one could use slice models, which presumably retain some features of tumor microenvironment [28]. Admittedly, it requires specific culture conditions to be adjusted and even application of additional cytokines as well as fetal serum [29]. Even under ideal conditions, *in vivo* models are not able to reproduce the full range of factors in the tumor microenvironment (TME).

Finally, the strain of immunodeficient mice should be taken into consideration. Residual immune factors could affect the growth of a tumor, at least in some cases [30]. Fig. (**1**) provides a summary of the most relevant issues in CSC research.

3. TARGETING EMBRYONIC SIGNALING PATHWAYS

Various signaling pathways, including those involved in embryonic development, determine GSCs' phenotypical and functional capabilities. To develop cutting edge treatments and improve clinical outcomes, one needs to develop and implicate the targeting of multiple molecules that are the members of these cascades. In this section, we will discuss potential approaches to target signaling in GSCs' clinical trials listed in Table **1**.

3.1. Targeting Notch Signaling

The Notch pathway is one of the most crucial signaling cascades involved in stem cell maintenance. This is one of the major elements during embryonic development. The apex of the pathway is the Notch receptor, which is composed of two subunits whose precursors have undergone protease cleavage (S1 cleavage). There are four types of Notch receptors, which have a role in human cancers. Upon interaction with Notch ligands (Delta (or Delta-like) and Jagged/Serrate families of membrane-bound ligands), the receptor subsequently is cleaved twice (S2 and S3 cleavages) to release the Notch intracellular domain (NICD). It then translocates into the nucleus to induce the expression of a number of regulatory genes. This pathway could drive tumorigenesis even though it has been shown to exhibit tumor-suppressive functions as well [31, 32]. Moreover, Notch signaling interplays with other tumorigenic pathways, which should be taken into consideration during the development of targeted therapy protocols.

There are several strategies to target Notch signaling. These include monoclonal antibodies (mAbs) to Notch ligands or the receptors, inhibitors to disintegrin and metalloproteinase (ADAM) enzymes (α-secretases, mediate S2 cleavage), inhibitors to γ-secretase complex (mediate S3 cleavage), as well as to Mastermind-like 1 (MAML1, a protein involved in transcription of target genes)[33]. There are no data reports regarding investigations of targeting GSCs or glioblastoma with mAbs to Notch ligands.

Regarding Notch receptors, it should be taken into account that these are represented by 4 structurally different proteins that are important during the development of targeted therapies [33]. The mAbs to Notch receptors have been tested clinically in various cancers. For example, brontictuzumab, a mAbs targeting Notch1, have been assayed in solid tumors. However, the overall performance of the drug was quite discouraging: only 2 patients (5%) achieved an unconfirmed partial response, 10 patients (28%) had stable disease, whereas 24 patients (67%) had disease progression [34]. Such limited efficacy was noted only in adenoid cystic carcinoma and high NICD patients. The impact of brontictuzumab on GSCs and glioblastoma remains to be determined.

ADAM enzymes belong to α-secretases and provide the first cleavage of the Notch receptor, releasing the extracellular domain. To date, at least one inhibitor of α-secretases (INCB3619) has been evaluated on GSCs and glioblastoma. Being delivered in magnetic liposomes (due to its low solubility), the compound showed antitumor activity in mice [35]. There is no clinical data regarding this drug.

One of the most targeted members of this pathway is γ-secretase. Besides cancer, its inhibitors have been tested in a number of preclinical trials and have shown antitumor activity: reduced cancer cell viability, enhanced apoptosis, reduced tumorsphere formation, and prolonged survival of animal models [36-39]. However, in a non-randomized clinical trial on glioblastoma, gliosarcoma, and adult brain tumors, an inhibitor of γ-secretase RO4929097 failed to show effectiveness: 33 patients (85%) experienced progression of the disease, and only one and three achieved complete and partial responses, respectively [40]. Interestingly, the drug alone reduced numbers of CD133 positive cells, whereas combination either with TMZ or with TMZ plus radiation therapy did not show any additional improvements. The inability of the drug to suppress tumor growth could be explained by the activation of Notch-independent angiogenesis [41]. The authors hypothesized that additional anti-angiogenic treatment could improve clinical outcomes.

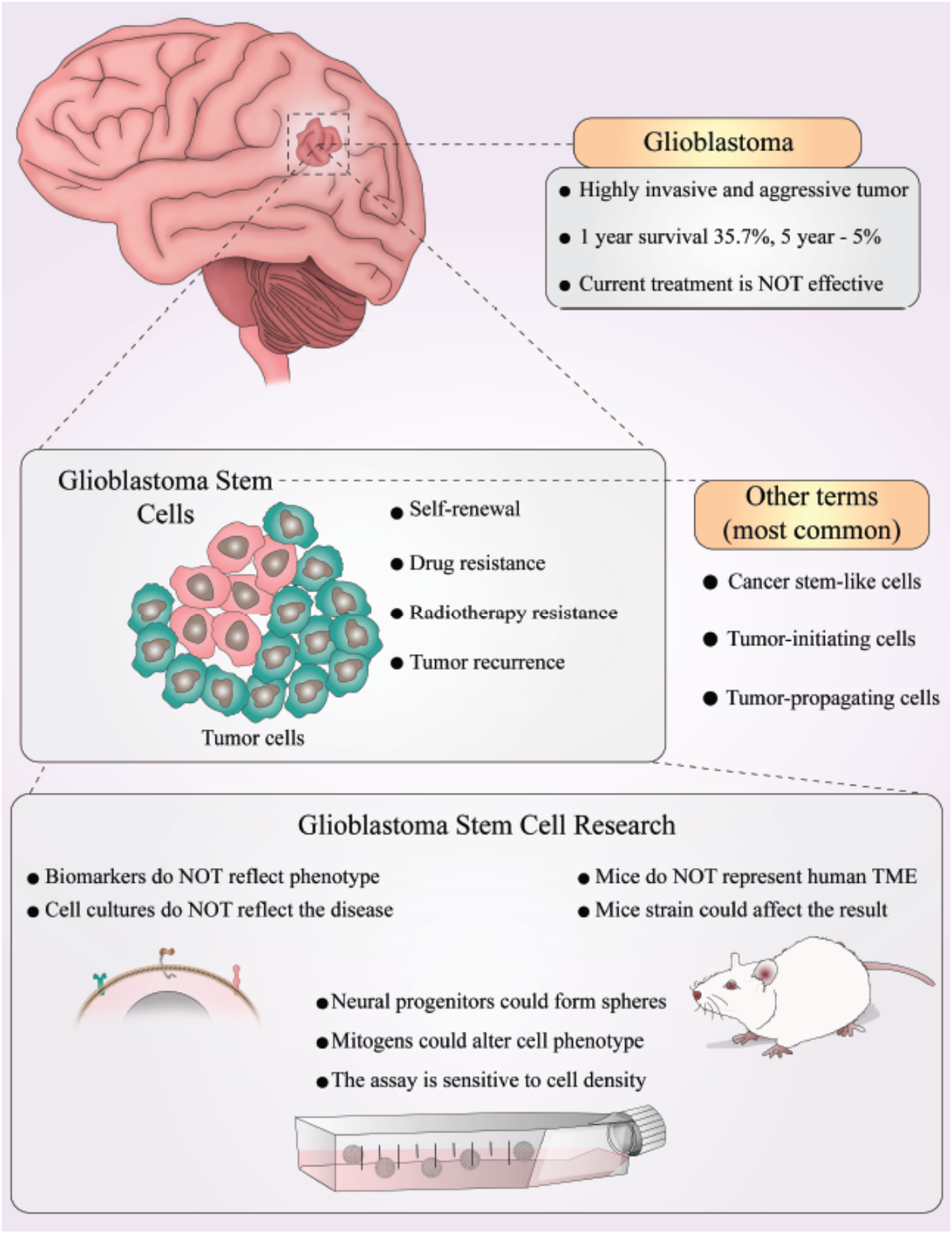

Fig. (1). Glioblastoma Stem Cell Research Overview.
Glioblastoma is a highly invasive and life-treating tumor. Cancer stem cells in glioblastoma are thought to be responsible for tumor relapse. Several terms could be used to name these cells (depicted the most prominent). Isolation of glioblastoma stem cells (GSCs) is based on cell markers, which could not reflect bona fide GSCs. Several hindrances are standing toward cancer stem cell research both *in vitro* and *in vivo*. TME; tumor microenvironment.

However, the results in a separate study, where a combination of RO4929097 with bevacizumab has been applied, were not satisfactory in patients with malignant gliomas (including glioblastoma) [42].

3.2. Targeting Wnt Signaling

Wnt is another signaling pathway to mediate basic cell developmental processes, such as cell-fate specification, the proliferation of progenitor cells, and the control of asymmet-

ric cell division. A number of articles support the role of Wnt in the maintenance of stem cell identity. For instance, Rheinbay and coworkers analyzed epigenetic and transcriptional landscapes of GSCs and identified several molecules to underpin the role of Wnt signaling in stemness [43]. The pathway contributes toward more invasive glioblastoma phenotype due to the differentiation of GSCs into endothelial cells that support neovascularization necessary for tumor growth [44]. This phenomenon could represent CSC plasticity.

In the canonical Wnt pathway, activation of the receptor results in inhibition of β-catenin degradation complex. β-catenin, in turn, translocates to the nucleus and activates expression of cell cycle-related genes upon interaction with T-cell factor (TCF) transcription factors [45]. AXIN is a component of a protein complex called "destruction box", which promotes β-catenin phosphorylation. SEN461, a small-molecule inhibitor, was shown to induce stabilization of AXIN, thereby increasing phosphorylation and degradation of β-catenin, which resulted in GBM suppression both *in vitro* and *in vivo* [46]. Concomitant inhibition of Wnt signaling by XAV939 could sensitize GBM cells to radiotherapy [47]. To date, there are no direct Wnt inhibitors clinically tested in glioblastoma.

Conversely, celecoxib, an indirect Wnt inhibitor that belongs to nonsteroidal anti-inflammatory drugs (NSAIDs), has been tested either alone or in combination in glioblastoma patients. NSAIDs are known to inhibit cyclooxygenase enzymes. However, the underlying mechanism of NSAID antitumor effect remains to be incompletely understood. There could be an interplay between cyclooxygenase-2 (COX-2) activity and Wnt signaling. For example, in colon cancer, prostaglandin E2 (PGE_2) stimulated the growth of tumor cells via β-catenin/TCF-dependent transcription [48]. It was reported, that inhibition of COX-2 by diclofenac or celecoxib resulted in a reduction of glioblastoma cell growth *in vitro* [49]. However, a phase II factorial clinical trial showed that patients did not benefit from combination therapies employing celecoxib [50].

In another phase II trial, a combination of celecoxib plus TMZ, capecitabine, lomustine, and 6-thioguanine did not show effectiveness since only 14 percent of patients achieved 6 months of progression-free survival (PFS)[51]. A combination of celecoxib with adjuvant TMZ and thalidomide did not significantly improve the survival of patients, although the regimen was well tolerated. However, the authors questioned if they used an appropriate dose of celecoxib [52].

3.3. Targeting Hedgehog Signaling

Hedgehog (HH) signaling is implicated in tissue patterning and stem cell development, and controls cell proliferation. This pathway was shown to sustain stemness in GSCs, and its inhibition prolonged survival of mice grafted with tumor cells [53]. HH pathway is initiated upon binding of its ligands to Patched (PTCH1), resulting in degradation of this receptor and subsequent release of Smoothened (SMO) to enter the cilia. Then, SMO induces dissociation of Suppressor of fused (SUFU)–glioma-associated oncogene homolog (GLI) complex, which, in turn, lets GLI2 and GLI3 enter the nucleus and regulate expression of target genes [54].

Vismodegib is a first-in-class inhibitor of SMO, which was approved by the US Food and Drug Administration (FDA) to treat patients with basal cell carcinoma [55]. However, the clinical results were discouraging in glioblastoma patients [56]. Interestingly, the percentage of CD133 positive neurospheres was almost four times lower in the pre-surgery vismodegib arm, suggesting the compound was active against GSCs. But these data are inconsistent with the results, questioning the clinical significance of CD133 positive CSCs altogether. Another phase I/II trial currently recruits patients to investigate various treatment options in glioblastoma. Vismodegib in combination with radiotherapy, is suggested to be used in individuals with increased HH signaling [57]. Glasdegib (PF-04449913) is an SMO inhibitor, which blocks SMO-mediated induction of HH downstream signaling. In phase I/II trial, this drug currently is being tested in combination with TMZ oral capsules in glioblastoma patients [58].

4. OTHER TARGETS IN GSCs

Several classic oncogenic pathways are involved in human cancers. Their components often acquire various mutations that lead to tumor progression. This cascade is essential for CSCs since they mediate their stemness and self-renewal properties. Besides cascades, several proteins were shown to regulate the CSC phenotype. Here we discuss some classical pathways that could be targeted to eliminate GSCs.

4.1. Targeting JAK/STAT Signaling

The JAK-STAT pathway is a chain of interactions that are involved in processes of immunity, cell division, cell death, and tumor formation. JAK-STAT often is activated in glioblastoma. This pathway could be triggered by various cytokines and hormones. Ligand-induced activation of the receptor leads to its dimerization followed by the recruitment of Janus kinase (JAK), which, in turn, phosphorylates tyrosine-rich residues of the receptor. Phosphorylated residues serve as docking sites for signal transducers and activators of transcription (STAT) molecules. Subsequently, STAT undergoes phosphorylation, homo- and hetero-dimerization, and then translocates into the nucleus to regulate the expression of target genes [59].

STAT3 is the most prominent member of this pathway that sustains GSC stemness. This protein has been found to be overexpressed in GSC, and its inhibition increased apoptosis and prevented neurosphere formation and halted the multipotency of these cells [60]. Similar results were obtained in other studies [61, 62]. Moreover, STAT3 was found to promote Notch signaling, supporting the evidence of its involvement in stemness [63]. We did not find any inhibitors of STAT3 to have been clinically investigated.

JAK2 is an important target in glioblastoma. WP1066, a JAK2 inhibitor, suppressed glioblastoma growth in mice [64]. At present, a phase I clinical trial on WP1066 monotherapy in treating patients with recurrent malignant glioma or progressive metastatic melanoma in the brain recruits participants [65].

4.2. Targeting PI3K/AKT/mTOR Signaling

Another of the key pathways to control cell proliferation, differentiation, and metabolic regulation is PI3K/AKT/mTOR. This pathway is initiated upon activation of various receptors that respond to different ligands, such as cytokines, growth factors, hormones, neurotransmitters, and so forth. The initial point of this cascade is the metabolic conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3,4,5-trisphosphate (PIP3) by phosphatidylinositol-4,5-bisphosphate 3-kinases (PI3K). There are several isoforms of PI3K that have different mutational frequencies in various types of cancers [66, 67].

The opposite conversion is provided by phosphatase and tensin homolog (PTEN). The loss of p53 tumor suppressor and PTEN leads to gliomagenesis in the mouse central nervous system. The resulting cells resembled the properties of GSCs [68]. However, it was reported that PTEN could regulate expression of PAX7, in which, upregulation results in tumorigenesis in human neural stem cells [69]. It was shown that the Notch pathway controls the expression of PTEN, and the loss of this protein could result in drug resistance to γ-secretase inhibitors [70]. The mammalian homolog of the retroviral transforming protein v-Akt (AKT) is a key signaling effector of PI3K. It is activated by binding with PIP3 and phosphorylation by phosphoinositide-dependent kinase 1 (PDK1) [66]. Inhibition of AKT by A-443654 reduced glioma growth both *in vitro* and *in vivo* [71]. However, there are no clinical data on this drug. AKT has multiple targets with various functions. For instance, survivin is an effector of AKT signaling, which probably is responsible for radioresistance in GSCs. Upon exposure to radiation, stem-like cells showed increased tumorigenicity in mice due to survivin activity [72]. However, YM155, an inhibitor of survivin, has not shown any effectiveness in different types of cancers during Phase I and Phase II clinical trials. In particular, in one trial, only 2 of 19 patients with solid tumors showed a partial response to the combined treatment regimen (YM155, carboplatin, paclitaxel)[73].

Several PI3K inhibitors either have been tested or are being tested in clinical settings. In a phase II trial, buparlisib (BKM120) was assayed on 65 patients. However, the treatment efficacy was minimal in PI3K-activated glioblastoma patients, despite good brain penetration [74]. The drug was tested in different combinations with chemotherapy or targeted therapy [75-77]. PX-866, another pan-inhibitor of PI3K, was tested in a clinic in a phase II trial. As for the previous drug, the response rate was low since only one patient achieved partial response (3%), eight patients had stable disease (24%), whereas twenty-four participants displayed a progressive disease (73%)[78]. Of note, there are no isoform-selective inhibitors of PI3K to be investigated in the clinic.

The mechanistic target of rapamycin (mTOR) is a kinase to regulate various metabolic processes within cells. There are two mTOR complexes (mTORC1 and mTORC2) that have distinct functions that are important during the development of targeted therapy regimens. The role of mTOR kinases is complex in GSC since these have a variety of downstream effectors to be involved in stemness [79]. One of the main functions of mTOR is a regulation of autophagy, a process implicated in cell homeostasis. For example, dopamine receptors were shown to control autophagy in GSCs via PDGFRβ2-ERK1/2 and mTOR signaling. DRD4 antagonism disrupted autophagy exclusively in GSCs and thereby induced G0/G1 cell cycle arrest as well as apoptosis. This intervention altered the expression of multiple genes in GSCs. But the current mechanism of action remains to be determined [80]. These results imply the dopamine antagonists as potential therapeutic agents in glioblastoma patients. Several clinical trials on mTOR inhibitors have been undertaken. AZD2014, a dual mTORC1/2 inhibitor, was shown to increase the sensitivity of GSCs to radiotherapy both *in vitro* and *in vivo* [81]. This drug is now being tested in GBM patients as a monotherapy in phase I trial [82]. The study, which employs a PI3K/mTOR inhibitor GDC-0084, currently recruits patients with glioblastoma. The compound was suggested to be used as a single agent [83]. Dual inhibitors have an advantage over selective drugs since they allow them to overcome possible drug resistance phenomena [84], albeit this conjecture has not been obtained with real clinical experience [85].

4.3. Targeting Aurora-A Kinase

The targeting of Aurora kinase could be another approach to eradicate CSCs in glioblastoma. Aurora-A regulates stem cell renewal due to interaction with the Wnt pathway. The ability of glioma cells to self-renew was abrogated after Aurora kinase silencing, indicating the role of the kinase in regulating stem cell features [86]. Concordantly, inhibition of Aurora kinase with small-molecule inhibitor (MLN8237) suppressed neurosphere formation and sensitized GSCs to radiotherapy [87]. This drug is in phase I clinical trial on patients with high-grade, recurrent gliomas [88].

4.4. Targeting the EphA2 Receptor

Eph receptors are known to participate in normal brain development and tissue homeostasis. They are found to affect cancer cells, as well. In particular, the EphA2 receptor was shown to be overexpressed in cancer stem cells. Using a soluble ephrinA1 (ligand) dimer fused to the Fc domain of immunoglobulins, authors showed that upon this high dose treatment, the receptor was downregulated, which resulted in a significant decrease of cell stemness and induced astroglial differentiation. The application of this dimer prolonged the survival of mice. The similar results were obtained when siRNA to target EphA2 was used. Authors noted that upon low dose treatment, they observed only scarce downregulation of the receptor, whereas the intracellular signaling was strong, in comparison with a high dose [89]. There are no direct inhibitors of the EphA2 receptor under clinical evaluation in glioblastoma patients. A multikinase inhibitor dasatinib was tested in a phase II trial. Its primary targets are Src family kinases. Nevertheless, this drug was shown to suppress the EphA2 receptor at higher concentrations. The treatment efficacy was not demonstrated in a phase II trial [90, 91].

5. IMMUNOTHERAPIES TO TARGET GSCs

Immunotherapy includes a number of distinct approaches to develop either active or passive immunity in a patient. In

this section, we will discuss two types of immunotherapy to be applied in targeting cancer stem cells in glioblastoma (see Table **3**).

5.1. Vaccines

One of the unique features of the immune system is the capability to develop an immune response against almost whatever antigen a human could encounter. Due to a wide variety of V, D, and J gene segments, random junctions of these segments, as well as somatic mutations in CDR3 (hypervariable region), combinatorial rearrangement of individual gene segments and combinatorial association between different heavy and light chains, our immune system can generate more than 10^{16} different immunoglobulins [92]. One of the solutions to take advantage of this feature is to design a vaccine. A vaccine offers several advantages over targeted therapies, the most significant of which is that it allows targeting multiple antigens within the highly heterogenic tumor. There are many variations of cancer vaccines depending on the type of antigen, formulation, adjuvants used, and delivery vehicles. For more detailed information, refer to a published comprehensive review [93].

Regarding GSCs, in clinical practice almost only dendritic cell-based formulations have been applied. Basically, tumor lysates, cells, proteins, peptides, or nucleic acids are delivered to autologous dendritic cells (DCs) that subsequently process these antigens and present tumor-derived peptides on the major histocompatibility complex class I (MHC-I) and class II (MHC-II) molecules. Next, loaded DCs are injected into a patient's lymph node, where these cells present the tumor-derived peptides on MHC-I molecules and MHC- II molecules to CD8+ and CD4+ T cells, respectively. This chain of events establishes an antitumor immune response [93]. Sadly, vaccines have a significant flaw in that only MHC-restricted peptides could be presented, and hence the number of potential antigens decreases. These medications could elicit an immune response against normal cells.

Previously, we described the role of survivin in GSCs. SurVaxM (SVN53-67/M57-KLH) is a peptide-based vaccine composed of a synthetic long peptide that mimics survivin, which is fused with keyhole limpet hemocyanin (KLH) that acts as a vaccine adjuvant. This peptide contains an amino acid substitution M57 leading to increased binding of surviving core epitope to HLA-A*0201 molecules, as well as multiple MHC-I epitopes, which could be presented by other MHC molecules [94]. During phase I trial, the vaccine was assayed on 9 patients, administered in emulsion with Montanide ISA 51 and sargramostim. The vaccine showed impressive results in comparison with previous therapies: median OS was 86.6 weeks and median PFS was 17.6 weeks; no serious adverse effects were observed [94]. Currently, this vaccine in combination with TMZ is under evaluation in a phase II trial [95].

The DEN-STEM vaccine, which employs monocyte-derived DCs loaded with survivin mRNA, was analyzed on glioblastoma patients in combination with TMZ and radiotherapy in phase I/II trial. The medication performed well: median PFS was 694 days (236 days in the control arm), median OS was 759 days (585 in the control group). This vaccine was tolerated without any serious adverse effects [96]. At present, DEN-STEM is under investigation in phase II/III trial [97].

Several tumor-derived vaccines have been tested on patients [98-101]. It has been shown that autologous GSCs are better in terms of vaccine development, despite the related difficulties. These cells allow the creation of patient-specific treatments, in contrast to allogenic material. For example, a trial accessing DC-based vaccine stimulated with apoptotic bodies from the allogenic GSC cell line (GBM6-AD) did not provide promising results since only one patient achieved partial response [98]. In a recent phase II trial, it was discovered that patient response to DC vaccination (DCs were stimulated with patient GSC lysates) could be dependent on the molecular profile of the particular participant. However, the study was limited due to its low size [102]. At present, the trial is ongoing and recruiting patients to assay the vaccine on the expanded randomized sample [99].

5.2. CAR T Cells

Chimeric antigen receptor (CAR) T cells open new avenues in cancer treatment. Since the initial success of CD19 CAR T therapy in B cell acute lymphoblastic leukemia, this strategy has become a promising treatment for other types of cancer with more than 400 ongoing clinical trials. Briefly, these are T cells engineered to express CARs recognizing a tumor-specific antigen. CAR is composed of the extracellular domain (recognition) and intracellular domains that activate and co-activate T cell-mediated immune response.

Depending on the CAR structure, CAR T cells may be classified into three generations; the second generation is the predominant form in testing. CAR T cell biology is complex. See reviews [103, 104] for details. In a pilot clinical trial, patients will receive CAR T cells that were specifically designed to recognize glioma-related antigens [105]. CAR T cell specification depends on the expression profile of the particular patient. Importantly, some of the antigens (CD133 and EphA2) are tightly interconnected with CSC biology, and hence CARs recognizing these molecules hold the capacity to target GSCs as well. Patients' benefits from such therapy remain to be determined.

CONCLUSION

Despite years of investigations, our understanding of GSCs has not progressed significantly. With the accumulation of knowledge, even more uncertainties become challenges for the scientific community. A number of potential pathways, biomarkers, targets, and treatment strategies have been identified and discovered during various studies. Unfortunately, only a small proportion have shown clinical significance. It is hard to determine whether the majority of therapies have failed due to imperfect techniques or due to limitations in the GSC concept itself. Nonetheless, some therapies are under active evaluation and hold the potential to improve patients' lives via targeting GSCs.

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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