Targeting glioblastoma cancer stem cells: the next great hope?

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Glioblastoma multiforme (GBM) is the most common primary brain tumor and is notorious for its poor prognosis. The highly invasive nature of GBM and its inherent resistance to therapy lead to very high rates of recurrence. Recently, a small cohort of tumor cells, called cancer stem cells (CSCs), has been recognized as a subset of tumor cells with self-renewal ability and multilineage capacity. These properties, along with the remarkable tumorigenicity of CSCs, are thought to account for the high rates of tumor recurrence after treatment. Research has been geared toward understanding the unique biological characteristics of CSCs to enable development of targeted therapy. Strategies include inhibition of CSC-specific pathways and receptors; agents that increase sensitivity of CSCs to chemotherapy and radiotherapy; CSC differentiation agents; and CSC-specific immunotherapy, virotherapy, and gene therapy. These approaches could inform the development of new therapeutics for GBM.

Key Words • glioblastoma multiforme • cancer stem cells • treatment • tumor-initiating cells • glioma stem cells

The CSC hypothesis is predicated on the presence of a small cohort of cancer cells that have properties of neural stem cells. Accordingly, these cells demonstrate self-renewal ability and multilineage capacity. 22 Self-renewal of CSCs maintains the small cohort of these cells, and the ability to differentiate into downstream progenitor cells gives rise to the diverse progeny that constitutes the bulk of the tumor. CSCs are thus thought to be the source of all GBM cells. CSCs are also known as tumor-initiating cells because of their potent ability to generate tumors in xenograft models (approximately 1000-fold more efficient than traditional [non-CSC] GBM cell lines). 70,140 Additionally, recent research has shown that CSCs are generally more resistant to conventional cytotoxic therapies and are invasive. Therefore, CSCs are thought to be the major driving force behind GBM resistance to therapy and high rates of GBM recurrence (Fig. 1).

In the arena of stem cell research, reliable recognition and characterization of putative CSCs is of paramount importance. 32 Functional assays such as serial implantation in nude mice are considered the gold standard. 22 Other techniques include Hoechst dye exclusion, 38,59,67,122,127 aldehydes dehydrogenase 1 assay, 114 spectroscopy, 76,77,130 and neurosphere cultures. 53,138 Cell sorting is another commonly used potent tool to enrich the population of CSCs (Table 1).

Among cell surface markers used to identify CSCs and neural stem cells, one of the most commonly used is CD133 (prominin-1). 93 CD133 was initially used to enrich CSCs in patients with leukemia 15,60 and has been observed...
because CD133 appears on neural stem cells, Singh et al. conducted in vitro and in vivo studies showing the presence of CSC on CD133-positive cells in gliomas and medulloblastomas. Nestin is another marker for CSCs in the CNS. Initially described as an antigen of rat-401 against embryonic spinal cord, nestin was later identified as a class VI intermediate filament protein.

**Treatment Considerations**

The current paradigm for treating GBM consists of a generally uniform regimen. According to the Stupp protocol, maximal safe resection is followed by radiotherapy and chemotherapy containing temozolomide. Temozolomide exercises cytotoxicity against GBM cells by creating O6-methylguanine lesions, which lead to DNA fragmen-
expression of multidrug resistance protein 1.96 CSCs also have reported that resistance to conventional antican-
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dation and disruption of DNA replication. The resulting effects include tumor suppression and apoptosis of tumor cells.135 Response to temozolomide, however, is not consistent in all patients. Long-term GBM survivors have been shown to harbor tumors with reduced or absent expression of methyleneguanine-DNA methyltransferase (MGMT).32 MGMT removes the methyl groups added by temozolomide, thereby preventing GBM cell death. Hence, with decreased expression of MGMT, because of methylation of its promoter, the therapeutic efficacy of temozolomide is enhanced.89

That patient survival times differ according to MGMT status underscores the significance of understanding the diversity of tumor biology. Such an understanding can be of immense importance with regard to CSCs because activity of MGMT has been shown to be 32- to 56-fold higher in CD133-positive than in CD133-negative cells, leading to increased resistance to temozolomide.21,24 Others have reported that resistance to conventional anticancer drugs, such as doxorubicin, etoposide, carboplatin, and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), is stronger in glioma CSCs than in non-CSCs because of enhanced expression of multidrug resistance protein 1.36 CSCs also upregulate mRNA of Fas-associated death domain–like antiapoptotic molecules Bcl-2 and Bcl-X.42,108,118 Higher expression of BCRP1 (a drug-resistant gene) and antiapoptosis proteins and inhibitors also confer a protective advantage to CSCs.21

In addition, the fact that a higher proportion of CD133-positive glioma cells survive, relative to most other tumor cells, points to their increased resistance to ionizing radiotherapy.120 By preferentially activating the DNA damage checkpoint in response to radiation, CSCs are able to repair radiation-induced DNA damage more effectively than are CD133-negative tumor cells. With exposure to conventional radiation, CD133-positive cells also exhibit enhanced activation of 3 key mediators of cell cycle checkpoint proteins: rad17, chk1, and chk2.9,10,16 If exposed to specific inhibitors of the chk1 and chk2 check-

### TABLE 1: Commonly used glioma CSC markers

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point proteins, CSCs become more radiosensitive, akin to CD133-negative tumor cells.21

Because of their inherent resistance to established therapeutic measures, CSCs are valuable targets for the development of specific treatment modalities for improving the overall response of GBMs.200 Increasing work is now uncovering the unique biological processes of glioma CSCs. Supplementing CSC-specific treatments with existing approaches may increase the effectiveness of overall treatment. We will further review some of the strategies being used to target glioma CSCs.

**CSC-Specific Pathways and Receptors**

Therapeutic targeting of CSC pathways and receptors responsible for tumor proliferation, maintenance, and drug resistance is of considerable interest. The notch signaling pathway is one of the major targets for CSC-specific therapies. Notch ligands, receptors, and targets have been found in a wide variety of neoplasms, including but not limited to lung, breast, cervix, renal, pancreas, medullo-blastoma, and GBM.5,27,62,69,99,105,107,112,155 In addition, in many of these tumors, increased notch activity has been shown to promote tumor growth, and blockade of the pathway inhibits proliferation. In the context of the CNS, the notch signaling pathway regulates neural stem cells in the brain, and high activity of the notch pathway has been demonstrated in glioma CSCs.40 Targeting the notch pathway by gamma-secretase inhibitors depletes glioma CSCs through reduced proliferation and increased apoptosis associated with decreased protein kinase B (Akt) and signal transducer and activator of transcription 3 (STAT3) phosphorylation.34

Notch signaling may also play a major role in linking CSC renewal and angiogenesis in GBMs. Using a 3D organotypic explant system of surgical GBM specimens, Hovinga et al. inhibited notch signaling and reported not only decreased proliferation and self-renewal of tumor cells but also decreased endothelial cells.62 A more recent study suggested that the brain microvascular endothelial cells are the source of notch ligands that lead to CSC sus-
tenance and renewal.157 Currently, notch signaling pathway inhibitor RO4929097 is being tested in clinical trials for recurrent and progressive GBMs (NCT01122901 at clinicaltrials.gov).

The hedgehog pathway plays an essential role in the development of the cerebellum.26,146 This pathway has also been identified as being important in the pathogenesis of gliomas. In addition, Gli is a component of the sonic hedgehog signaling pathway and is amplified in gliomas.169 Hedgehog signaling, has been shown to deplete CSCs in GBM.11 Similarly, Clement et al. reported that interference of hedgehog signaling through lentivirus-mediated silencing resulted in decreased self-renewal and tumori-
genicity of CSCs.21 SANT-1 inhibition of the hedgehog signaling pathway is one of the major targets for CSC-specific therapies. Notch ligands, receptors, and targets have been used to target glioma CSCs.

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genicity of CSCs.21 SANT-1 inhibition of the hedgehog pathway has also been shown to reduce proliferation of CSCs.31 Furthermore, there is increasing evidence of an intimate relationship between hedgehog signaling and endo-
thelial migration.150 Vismodegib (GDC-0449) is a small molecule antagonist of the hedgehog pathway30 and is cur-
Currently being tested in a clinical trial for recurrent GBM (NCT00980343 at clinicaltrials.gov).

Because inflammation is thought to be an integral part of the pathophysiology of GBM, Sharma et al. investigated the role of cyclooxygenase-2 in glioma CSCs. They reported that administration of celecoxib significantly reduced self-renewal capacity and increased apoptosis of glioma CSCs. Celecoxib has also been shown to reduce radioresistance in glioma CSCs, leading to reduced tumor growth and increased mean rates of survival among mice.

Glioma CSCs also produce high levels of vascular endothelial growth factor, which induces endothelial cell migration. However, treatment of CD133-positive GBM cells with bevacizumab blocks the tumor cells’ ability to stimulate endothelial cell migration and initiate tumors in vivo. Calabrese et al. demonstrated that treatment of GBMs with bevacizumab depleted tumor blood vessels, significantly reduced the number of CSCs, and decreased the growth rate of the tumors. Because of the efficacy of antiangiogenic agents as determined in preclinical studies, they have been tested in clinical trials. Unfortunately, bevacizumab has failed to show improvement in the overall survival times for patients with newly diagnosed GBM. Similarly, cediranib, either as monotherapy or in combination with lomustine, did not prolong progression-free survival times for patients with recurrent GBM more than lomustine alone.

Amplification and mutation of receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR), are other common genetic alterations that occur in GBMs. Recent studies have determined the presence of a constitutively active EGFR mutant (EGFRvIII) in glioma CSCs. This genetic alteration pathway potentiates tumor growth and heterogeneity through interleukin-6–mediated notch signaling and Src family kinase–dependent phosphorylation of DOCK180 (dedicator of cytokinesis). Clinical trials investigating the efficacy of EGFR inhibitors in GBM, however, have yielded disappointing results so far.

Aberrant Wnt signaling is molecularly linked to many human cancers, including colorectal, breast, ovarian, hepatocellular carcinoma, neuroectodermal, and glioma. Dysregulation of the Wnt pathway in glioma CSCs has also been reported. Other similar targets being investigated for glioma CSC treatment include the homeobox family, phosphatase and tensin, telomerase, efflux transporters, and micro-RNA.

Chemotherapy Sensitizers

Compared with other tumor cells, CSCs are more refractory to chemotherapy, and resistance to temozolomide remains a significant hurdle in the treatment of GBMs. To circumvent this issue, investigators have reported several ways to potentiate the cytotoxicity of chemotherapeutic agents. These include cell-cycle checkpoint abrogation and depletion in the expression of antiapoptosis proteins and DNA repair enzymes. A molecular chaperone, 90-kD heat shock protein, has recently been associated with increased resistance to chemotherapy and is expressed at 2–10-fold higher levels in tumors than in normal tissues. Consequently, drugs that inhibit this protein expression potentiate the cytotoxicity of chemotherapeutic agents in human glioma cell lines.

GPI 15427, a novel poly(adenosine diphosphate–ribose) polymerase–1 inhibitor, has been tested to show that its systemic administration shortly before temozolomide administration significantly increases the life span of tumor-bearing mice. Oral administration of GPI 15427 also effectively increases sensitivity to temozolomide. More recently, the effect of secreted frizzled-related protein 4, a Wnt signaling antagonist, was shown to sensitize glioma CSCs to doxorubicin and cisplatin. Similarly, another study used a proteasome inhibitor, bortezomib, and revealed that combination therapies based on bortezomib and bevacizumab offered an increased benefit when the 2 agents were used in combination.

Xu et al. targeted CD44, which is upregulated in GBM and is a CSC marker, and reported that its depletion impeded the growth of GBMs and sensitized the tumor cells to cytotoxic drugs in vivo. Tyrosine kinase inhibitors have also been examined for sensitization of the tumor. Wachsberger et al. investigated the chemotherapy sensitization efficacy of cediranib (a potent receptor tyrosine kinase inhibitor) and found that it enhanced the effectiveness of temozolomide.

One of the most commonly used chemotherapeutic agents in the treatment of GBM is BCNU, but it often fails to eradicate CSCs. Recent work has uncovered an overexpression of multiple ion channel genes that are related to drug efflux. When a chloride channel blocker, 4,4ʹ-diisothiocyanostilbene-2,2ʹ-disulfonic acid, is used in combination with BCNU, the effect of BCNU synergistically increases.

Radiation Sensitizers

For developing approaches to sensitize CSCs, a concentrated effort has been made to understand the biology of CSC radioresistance. Because transforming growth factor–β (TGFβ) is a known modifier of radiation responses, a TGF-β receptor type I kinase inhibitor, LY2109761, has been used in combination with radiotherapy to increase the radiosensitivity in glioma cell lines, including CSCs. Similarly, LY364947, another small-molecule TGFβ receptor type I kinase inhibitor, improved response when administered before radiotherapy. A TGFβ inhibitor, is being evaluated in an ongoing clinical trial (NCT01220271 at clinicaltrials.gov).

EGFR activation has also been implicated in the radioresistance of many cancers, including gliomas. Georger et al. used gefitinib (a tyrosine kinase inhibitor) in EGFR-amplified gliomas and reported a positive trend toward superior antitumor activity when combined therapy (gefitinib plus radiation) was administered. On further investigation, Kang et al. found that gefitinib enhanced radiosensitivity by reducing activation of EGFR-Akt and expression of DNA-dependent protein kinase catalytic subunit. ZD1839 (Iressa) is another selective EGFR tyrosine kinase inhibitor that demonstrates a significant ra-
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diosensitizing effect in GBMs. In the clinical domain, patients who enrolled in a Phase I/II study of radiation therapy concurrent with gefitinib therapy for newly diagnosed GBMs showed good drug tolerance but no survival benefits. Other tyrosine kinase inhibitors investigated as radiosensitizers for GBM are erlotinib and vandetanib.

STAT3 is a member of a family of DNA-binding molecules, and the aberrant activity of the Janus kinase 2/STAT3 pathway is associated with glioma CSCs. Inhibition of this pathway leads to decreased proliferation of glioma CSCs. Yang et al. reported that resveratrol (inhibitor of the STAT3 axis) significantly improved the survival rate among patients in their xenotransplant model, in part by synergistically enhancing the radiosensitivity of CSCs.

Valproic acid is a commonly prescribed antiepileptic drug used for the management of seizures in brain tumor patients. Valproic acid is also an effective inhibitor of histone deacetylase and is involved in modulating chromatin structure and gene expression. Valproic acid increases chemosensitivity to temozolomide and enhances the effect of radiotherapy in glioma cell lines. Pretreatment with N-acetylcysteine can partially recover the apoptotic effect of temozolomide/valproic acid combination treatment.

Another approach to making glioma CSCs more radiosensitive is to inhibit the DNA damage responses that follow radiotherapy. A dual phosphoinositide 3-kinase/mTOR inhibitor, NVP-BEZ235, inhibits two central DNA damage response kinases: DNA-dependent protein kinase catalytic subunit and ataxia telangiectasia mutated. By doing so, NVP-BEZ235 potentiates the damage caused by ionizing radiation in glioma cells. Some of the other targets that reportedly increase the radiosensitivity of CSCs include polo-like kinase 1, cyclin-dependent kinase 6, insulin-like growth factor–1 receptor, DNA binding protein–1, and miR-210.

CSC Differentiation Agents

Differentiation-inducing agents can also be used to decrease the number of CSCs and make a tumor more susceptible to therapy. One of the first agents to be used as a differentiating agent for GBM CSCs was bone morphogenetic protein–4. Bone morphogenetic proteins play a differentiating role in the adult brainstem cell niche and increase the acquisition of astrogial differentiation. Piccirillo et al. demonstrated that bone morphogenetic proteins trigger the Smad signaling cascade in GBM cells, leading to a decrease in the clonogenic ability and number of CD133-positive glioma cells.

The oncogene BMI1, which regulates gene expression by modifying chromatin organization, is highly expressed in CD133-positive cells. Recent research has demonstrated the role of BMI1 in the self-renewal of CD133-positive tumor cells. Not surprisingly, knockdown of this gene by use of short-hairpin RNA–expressing lentiviruses has been shown to inhibit the clonogenic potential of CD133-positive cells in vitro and in vivo.

Metformin, a first-line drug for treatment of Type 2 diabetes, was recently reported to possess antiancancer properties affecting the survival of CSCs in breast cancer models. Würth et al. investigated the effect of metformin on glioma cells and reported CSC-specific inhibition of the Akt-dependent cell survival pathway affecting self-renewal mechanisms. Metformin is being tested for the treatment of GBM (NCT02149459 and NCT01430351 at clinicaltrials.gov).

Induction of autophagy has also been promoted for differentiation in glioma CSCs. Drugs such as rapamycin and curcumin trigger the differentiation cascade by activating autophagy. Other differentiating targets include girdin (an actin-binding protein) and the vaniloid-2 cation channel. Cannabinoids and sorafenib have also been documented to induce glioma CSC differentiation and deplete the CSC population.

Immunotherapy

Another area of significant interest is the stimulation of host responses to selectively eradicate CSCs without affecting normal tissue. Systemic immunotherapy that uses dendritic cells has been shown to induce a potent antglioma response. Dendritic cells are effective antigen-presenting cells that have the ability to prime naïve T cells. Although the number of dendritic cells in circulation is very low, various methods can be used to increase them in vitro with sources such as the bone marrow, cord blood, and peripheral blood.

Additionally, the ability of dendritic cells to cross the blood-brain barrier and enter into perivascular and parenchymal spaces makes them very suitable for glioma immunotherapy. Certainly, early clinical trials of dendritic cell vaccines demonstrated strong systemic and intracranial T-cell responses and robust infiltration of tumor with T cells. Glioma CSCs also demonstrate exquisite susceptibility to T cell–mediated immunity. By using tumor specimens, Avril et al. demonstrated that glioma CSCs were more sensitive to lysis mediated by natural killer and T cells than were corresponding serum-cultured GBM cells obtained from the same initial tumor specimen.

T cells can also be modified to target CSC surface markers. Ahmed et al. generated T cells specific for EGFR2 by using gene transfer from GBM patients. They demonstrated potent antitumor activity against autologous EGFR2-positive tumors, including their putative stem cells. More recent evidence also suggests that dendritic cell–mediated immunotherapy can be enhanced via elimination of regulatory T cells.

Passive immunotherapy that uses monoclonal antibodies or Fc-fusion proteins has similarly shown great potential. One of the GBM receptors studied in this regard is interleukin-13 subunit alpha-2; several studies have revealed the ability of interleukin 13–zetakine–redirected T cells to cause regression of GBM and CSCs. Nanoparticles have also been used to deliver monoclonal antibodies to glioma CSCs. Wang et al. evaluated single-walled carbon nanotubes conjugated with CD133 monoclonal antibodies and found effective selective targeting of CD133-positive cells along with eradication with photothermalysis.
Virotherapy and Gene Therapy

Among the emerging therapeutic options targeting CSCs, virotherapy has shown noteworthy promise in terms of glioma CSCs. Fueyo et al. constructed a tumor-selective adenovirus, Delta-24, that carried a 24-bp deletion in the early region 1A, responsible for binding retinoblastoma protein. In vivo and in vitro results from their study demonstrated a potent lytic effect on glioma cells. A second-generation Delta-24 virus (Delta24-hyCD) also exhibited significant chemosensitization and glioma control when combined with 5-fluorocytosine.

In another study, a combination of adenoviral virotherapy and temozolomide chemotherapy demonstrated significant overexpression of autophagy markers, acidic vesicular organelles, and light-chain-3 proteins in vitro. In vivo studies showed that survival rates were significantly higher when combination therapy was used.

Gene-silencing techniques can also be used for a better understanding of the role of certain genes in the biology of CSCs and can be exploited for therapeutic purposes. Bao et al. investigated the role of a neuronal cell adhesion molecule, L1CAM, in glioma CSCs by using lentiviral-mediated short-hairpin RNA interference. They reported disrupted neurosphere formation, induced apoptosis, and inhibited growth of glioma CSCs. Similarly, Wang et al. investigated the significance of c-Myc expression in glioma CSCs by using short-hairpin RNA interference and demonstrated that decreased expression of the target decreased proliferation and survival of CSCs.

Another innovative approach that has shown considerable potential is the nanoparticle delivery platform for gene therapy. By using a systemically administered nanoparticle carrying the p53 gene, Kim et al. targeted GBM cells, including CSCs. They reported that the combination of the nanoparticle-carrying p53 and temozolomide increased the antitumor efficacy of temozolomide and enhanced survival benefits in a mouse model of highly temozolomide-resistant GBM.

Conclusions

CSCs play an integral role in the growth and development of GBMs. Although current treatment modalities might be able to eradicate most of the tumor mass, the CSCs are mostly left unperturbed, which accounts for the high rates of recurrence and resistance to therapy. Ongoing research to elucidate the unique properties of CSCs has led to the development of targeted therapeutics for elimination of the CSC population. Supplementation of traditional therapies with CSC-directed agents might improve the overall therapeutic response of GBMs.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: both authors. Acquisition of data: both authors. Analysis and interpretation of data: both authors. Drafting the article: both authors. Critically revising the article: both authors. Reviewed submitted version of manuscript: both authors. Approved the final version of the manuscript on behalf of both authors: Ehtesham. Study supervision: Ehtesham.

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