HIGH SCHOOL EDITION
Journal of Student Research
Volume 10 Issue 4 (2021)

Using CRISPR-Cas9 to Target EGFRvIII and SIRP-α for Glioblastoma Treatment

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ABSTRACT

Glioblastoma (GBM) is an aggressive grade 4 brain tumor with a poor prognosis, with the 5-year survival rate remaining at a mere 5%. Standard first-line treatment consists of maximal safe resection followed by concomitant daily temozolomide with radiotherapy. However, tumor recurrence is frequent, and second-line treatments are lacking. While new therapies have been intensely studied, challenges with delivery across the blood-brain barrier (BBB) have largely hampered efforts to develop new efficacious glioblastoma treatments. The development of gene therapy like CRISPR-Cas9 to treat GBM is consequently still in the early stages. This review delves into how nanoparticles could be utilized to deliver CRISPR-Cas9 across the BBB into the tumor microenvironment and effectively edit both glioblastoma cells and tumor-associated microglia/macrophages (TAMs). This review will discuss the prevalence of the EGFRvIII mutation in GBM cells and its significant role in facilitating cell proliferation and the evasion of apoptosis, analyzing EGFRvIII as a potential target for gene knockout through CRISPR editing. Further, the immunosuppressive tumor microenvironment that characterizes GBM will be described, with an explanation of how TAMs contribute to tumorigenesis through their M2 polarization. Finally, the review will analyze the importance of the CD47/SIRP-α axis to the pro-tumorigenic nature of TAMs. It will study the possibility of disrupting SIRP-α expression in TAMs through CRISPR-Cas9 as a potential immunotherapy for GBM. Overall, this review will outline how the use of CRISPR-Cas9 to knock out EGFRvIII in GBM cells and SIRP-α in TAMs could potentially transform GBM treatment and substantially improve prognosis in patients.

Introduction

Glioblastoma

Glioblastoma (GBM) is a malignant glioma classified as a grade IV tumor for its aggressive, deadly nature (Alexander & Cloughesy, 2017; Davis, 2016; Soomro et al., 2017). It is the most common brain cancer among adults, with GBM accounting for 60% of adult brain tumors and 48% of primary malignant central nervous system tumors (Tan et al., 2020; Taylor et al., 2019).

GBM is characterized by a variety of symptoms that are largely dependent on the size and the location of the tumor in the brain (Davis, 2016). Some GBM symptoms are more subtle and harder to observe - noteworthy examples include fatigue, mood swings, behavioral changes, and mild memory loss (Alexander & Cloughesy, 2017). Meanwhile, some of the more obvious symptoms include weakness, loss of sight, speaking difficulties, headaches, and seizures, which occur in 25% of GBM patients (Alexander & Cloughesy, 2017; Davis, 2016). An increase in intracranial pressure due to tumor growth is what often accounts for the headaches as well as the focal or progressive neurological deficits experienced by patients (Davis, 2016). Headaches are quite common in patients newly diagnosed with GBM, as this symptom is indicative of a significant mass effect (Alexander & Cloughesy, 2017).

There are two subtypes of glioblastoma, which primarily differ in the state of the IDH gene: IDH-wild type, classified as primary glioblastoma, and IDH-mutant type, classified as secondary glioblastoma (Li et al., 2016; Tan et al., 2020). IDH-wild type GBM is much more common, accounting for around 90% of GBMs (Ohgaki & Kleihues, 2013; Tan et al., 2020). However, it is also seen as the more aggressive and the deadlier of the two, with a higher prevalence amongst older individuals. While primary GBMs are characterized by quick de novo progression with no evident precursor lesions, secondary GBMs have been observed to develop much more slowly, from diffuse astrocytoma and anaplastic astrocytoma (Li et al., 2016; Ohgaki & Kleihues, 2013; Tan et al., 2020). While the mutations in the two types of GBM are very distinct, the difference in tumor progression seems to be best explained by how primary GBM and secondary GBM are derived from different neural precursor cells of origin (Ohgaki & Kleihues, 2013).

Genetic predisposition is uncommon amongst GBM patients, with less than 5% of GBM patients carrying a germline alteration that predisposes them to many cancers including glioblastoma (Alexander & Cloughesy, 2017; Tan et al., 2020). Exposure to ionizing radiation has been commonly seen as the only established causative factor associated with GBM (Alexander & Cloughesy, 2017; Davis, 2016). Other potential risk factors for the disease include vinyl chloride, pesticides, smoking, petroleum refining, rubber manufacturing, cell phone use, and viruses (Alexander & Cloughesy, 2017; Davis, 2016). However, these factors are only loosely correlated to GBM diagnosis and need to be studied further (Alexander & Cloughesy, 2017; Davis, 2016).

The most common detection mechanism for glioblastoma is magnetic resonance imaging (MRI). On an MRI, GBMs typically exhibit an oddly shaped mass, with the presence of a ring-enhancing lesion and intense central necrosis (Alexander & Cloughesy, 2017; Davis, 2016). However, an issue with MRI detection is that GBM is typically observed when the tumor has already metastasized to an advanced state (Alexander & Cloughesy, 2017; Davis, 2016). Unfortunately, earlier detection mechanisms for GBM are not currently available (Davis, 2016).

Mutations in Glioblastoma

Each of the two GBM subtypes, primary glioblastoma (IDH-wild type) and secondary glioblastoma (IDH-mutant type), are associated with a distinct set of genetic mutations (Davis, 2016; Ohgaki & Kleihues, 2013; Tan et al., 2020). In primary GBM, overexpression of the epidermal growth factor receptor (EGFR) protein is quite prevalent, with 57.4% of primary GBM patients experiencing EGFR amplification in some capacity (An et al., 2018; Davis, 2016; Ohgaki & Kleihues, 2013; Tan et al., 2020). Of the many EGFR mutations observed in GBM, the EGFRvIII (variant 3) mutation is the most common EGFR genetic alteration and will be a large focus of this review as a potential therapeutic target (Taylor et al., 2019). The EGFR protein, a receptor tyrosine kinase, normally regulates cell division through a series of signaling pathways like the RAS/MAPK/ERK pathway (An et al., 2018; Eskilsson et al., 2018).

On top of EGFR, primary glioblastoma is also associated with the deletion of the phosphate and tensin homologue (PTEN) gene, loss of chromosome 10q, and mutations in the promoter of the telomerase reverse transcriptase (TERT) gene (Davis, 2016; Ohgaki & Kleihues, 2013; Tan et al., 2020).

On the other hand, secondary GBM is distinguished by mutations of the isocitrate dehydrogenase-1 (IDH-1) gene and the IDH-2 gene (Davis, 2016; Li et al., 2016; Ohgaki & Kleihues, 2013; Tan et al., 2020). Other common mutations in IDH-mutant GBM include ATRX mutations, TP53 mutations, CpG island hypermethylation leading to decreased gene expression, and the loss of chromosome 19q (Li et al., 2016; Ohgaki & Kleihues, 2013; Tan et al., 2020).

Yet, despite these genetic differences between the two GBM subtypes, three signaling pathways are typically dysfunctional in both GBMs: the p53 pathway in 87% of patients, the receptor tyrosine kinase/Ras/phosphoinositide 3- kinase signaling pathway in 88% of patients, and the retinoblastoma pathway in 78% of patients. (Davis, 2016; Taylor et al., 2019). Abnormalities in the aforementioned pathways explain the excessive cell division and the lack of apoptotic cell death in GBM (Davis, 2016).

Immunosuppressive Brain Microenvironment

The tumor microenvironment of GBM is characterized by the presence of tumor-associated microglia/macrophages (TAMs), which can encompass 30-40% of the actual GBM tumor mass (Buonfiglioli & Hambardzumyan, 2021; Grégoire et al., 2020; Morisse et al., 2018; Pires-Afonso et al., 2020). Microglia are immune cells that belong to the central nervous system as the resident macrophages of the brain, stemming from the hematopoietic precursor cells of the yolk sac in early embryogenesis (Grégoire et al., 2020; Roesch et al., 2018). In a multitude of ways, microglia help to maintain the homeostasis of the neural environment by phagocytizing apoptotic cells and supporting neurogenesis, synaptic refinement, and axonal growth (Pires-Afonso et al., 2020; Roesch et al., 2018). Furthermore, microglia play a significant role in immune surveillance and first-line defense in the brain. They can serve as antigen-presenting cells, linking the innate and adaptive immune systems (Roesch et al., 2018). Microglia can also trigger macrophage infiltration through the expression of pro-inflammatory cytokines like TNF-α and perform phagocytosis through the recognition of immunogenic antigens (Roesch et al., 2018). Bone marrow-derived macrophages (BMDMs), often deemed as peripheral macrophages in this context, arise from hematopoietic stem cells. (Buonfiglioli & Hambardzumyan, 2021; Grégoire et al., 2020; Morisse et al., 2018; Roesch et al., 2018). BMDMs can infiltrate into the brain and intermingle with the resident microglia, serving similar immune functions and expressing similar surface markers like CD11b and CD68 (Grégoire et al., 2020; Roesch et al., 2018). In this review, TAMs refer to both resident microglia as well as peripheral BMDMs found in the GBM tumor microenvironment.

Glioblastoma is associated with an immunosuppressive tumor microenvironment that is brought about by the activity of tumor-associated microglia/macrophages (Roesch et al., 2018). By suppressing anti-tumor immune responses, TAMs have been shown to promote tumorigenesis and facilitate the invasion and migration of GBM cells throughout the brain (Buonfiglioli & Hambardzumyan, 2021; Grégoire et al., 2020; Morisse et al., 2018; Roesch et al., 2018). GBM cells induce this pro-tumorigenic role by recruiting TAMs through several chemoattractants, like CCL2, CX3CL1, CSF-1, and GM-CSF (Buonfiglioli & Hambardzumyan, 2021; Morisse et al., 2018; Pires-Afonso et al., 2020; Roesch et al., 2018). Upon recruitment, the aforementioned glioma cell-derived factors trigger the upregulation of M2 markers, like CD163 and CD206, in TAMs and catalyze M2-like polarization amongst TAMs (Grégoire et al., 2020; Roesch et al., 2018). This polarization is what accounts for the anti-inflammatory activity of immune cells in the brain (Grégoire et al., 2020; Roesch et al., 2018).

It has been commonly observed that microglia and macrophages in the brain can exhibit two different phenotypes that exhibit contrasting activity. On the one hand, these immune cells can assume an M1-like phenotype (Grégoire et al., 2020; Roesch et al., 2018). In this state, microglia and macrophages serve their typical immune response functions, secreting pro-inflammatory cytokines like TNF-α, IL-1β, IL-2, and IL-12, which can help combat tumor growth (Buonfiglioli & Hambardzumyan, 2021; Grégoire et al., 2020; Roesch et al., 2018). On the other hand, microglia and macrophages can exhibit an M2-like phenotype, a state in which they secrete anti-inflammatory cytokines like Arg-1, TGF-β, IL-6, IL-10, as well as angiogenic factors like VEGF-α (Pires-Afonso et al., 2020; Roesch et al., 2018). Most TAMs in the GBM tumor microenvironment transition to the M2-like phenotype following recruitment, as mentioned previously, and ultimately play a significant role in promoting tumor growth, angiogenesis and invasion of GBM by suppressing the innate and adaptive immune systems (Grégoire et al., 2020; Morisse et al., 2018; Pires-Afonso et al., 2020; Roesch et al., 2018). Aside from inhibiting the proliferation of T cells through cytokines, TAMs in the M2-like state have even been observed to trigger apoptosis of activated T cells through Fas-FasL interaction, contributing to the ongoing immune evasion of GBM (Buonfiglioli & Hambardzumyan, 2021; Roesch et al., 2018). With M2-like polarization such a prominent feature of GBM and its progression, it is worth noting that a lower M1:M2 ratio among TAMs is correlated with worse clinical outcomes for GBM patients (Zhang et al., 2016). Overall, knowing the crucial role that the immunosuppressive tumor microenvironment plays in assisting GBM progression, it is worth considering the possibility of targeting TAMs to reorient this environment as a form of GBM treatment.

CD47/SIRP-α Signaling Axis

The CD47/SIRP-α signaling axis is central to the pro-tumorigenic properties of TAMs and the immunosuppressive tumor microenvironment commonly observed in GBM (Hu et al., 2020; Yang et al., 2019; Zhang et al., 2016).

CD47 is an integrin-associated protein encoded by a gene on chromosome 3q13 and expressed in a wide variety of cells (Hu et al., 2020). CD47 is highly expressed in GBM cells, and this upregulation of CD47 expression has been associated with a worse prognosis amongst GBM patients (Hu et al., 2020; Zhang et al., 2016). Being part of the immunoglobulin superfamily, CD47 consists of an immunoglobulin-like amino-terminal domain found in the extracellular region, 5 transmembrane domains, as well as an intracellular carboxyl-terminal tail (Hu et al., 2020; Yang et al., 2019).

Signal regulatory protein alpha (SIRP-α) is a part of the SIRP family, a group of immune receptors whose associated genes can be found on chromosome 20p13 (Barclay & Van Den Berg, 2014; Hu et al., 2020). This protein receptor has three extracellular immunoglobulin-like domains: 2 C1-set domains and an NH2-terminal V-set domain. It also has one transmembrane portion and an intracellular component including 2 typical immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that are composed of 4 tyrosine residues (Hu et al., 2020; Yang et al., 2019). While the expression of CD47 is quite wide-reaching, SIRP- α is predominantly expressed in myeloid cells like microglia and macrophages (Barclay & Van Den Berg, 2014; Hu et al., 2020; Pan et al., 2013; Yang et al., 2019). Further, with the importance of the signaling axis to GBM, it is worth pointing out that inside the SIRP family, SIRP-α has an especially high affinity for CD47 (Hu et al., 2020).

The CD47/SIRP-α pathway is initiated by the binding between the immunoglobulin-like domain of CD47 on GBM cells and the NH2-terminal V-like domain of SIRP-α on myeloid cells like microglia and macrophages. This interaction between CD47 and SIRP-α has the ability to phosphorylate the tyrosine residues in the ITIMs of SIRP-α, which can, in turn, trigger the activation of the tyrosine phosphatases SHP-1 and SHP-2. As phosphatases, these activated proteins then induce the dephosphorylation of downstream molecules in different signaling pathways, ultimately inhibiting the phagocytosis of GBM cells by microglia and macrophages. Thus, CD47 expressed on GBM cells essentially acts as a ‘do not eat me’ signal (Hu et al., 2020; Yang et al., 2019). While the downstream signaling cascades are yet to be fully understood, the suppression of myosin 11A has been observed as a likely cause of the phagocytosis inhibition that results from the signaling axis (Barclay & Van Den Berg, 2014; Yang et al., 2019). With an analysis of this mechanism, more and more evidence has suggested that the CD47/SIRP-α signaling axis plays an important role in facilitating the immunosuppressive tumor microenvironment in GBM and ultimately promoting tumorigenesis. The pathway diminishes the function of both the innate and adaptive immune systems by reducing the cytotoxicity of TAMs and potentially playing a role in M2-polarization, which can have downstream effects on T cells (Hu et al., 2020).

Current Treatments for Glioblastoma

Despite the research being conducted for new medical treatments, the prognosis for GBM patients remains extremely poor, with the 5-year survival rate still at a mere 5% and the median survival less than 2 years (Alexander & Cloughesy, 2017; Tan et al., 2020). There is a well-defined standard for GBM first-line therapy. Maximal safe resection is first utilized in attempts to reduce the tumor volume and allow for a more in-depth genetic analysis of the GBM tumor in the patient (Davis, 2016; Tan et al., 2020; Taylor et al., 2019). However, because glioblastomas are invasive and metastatic in nature, this surgical procedure is typically not curative, with the infiltration of GBM cells to different parts of the brain (Davis, 2016). Concomitant daily temozolomide with radiotherapy normally follows surgery (Alexander & Cloughesy, 2017; Davis, 2016; Tan et al., 2020; Taylor et al., 2019). After that, adjuvant chemotherapy with temozolomide is typically administered (Alexander & Cloughesy, 2017; Davis, 2016). Unfortunately, tumor recurrence following first-line therapy is common amongst GBM patients, largely due to the invasiveness of GBM,

and in some cases, because of the development of temozolomide resistance in GBM cells (Davis, 2016; Taylor et al., 2019).

However, there is no standard of care for second-line GBM therapy, and the options for treatment are quite limited for patients with recurrent tumors (Davis, 2016; Tan et al., 2020; Taylor et al., 2019). In some patients, it may be appropriate to treat with re-resection or re-irradiation (Davis, 2016; Tan et al., 2020). Chemotherapy drugs, like lomuzine, carnustine, and vincristine, may also be administered to GBM patients - even re-challenging with temozolomide is a plausible option (Davis, 2016; Tan et al., 2020). However, the benefits of chemotherapy as a second-line treatment are modest and are mainly used to improve quality of life (Davis, 2016; Tan et al., 2020). Furthermore, bevacizumab, a monoclonal antibody, is another approved second-line treatment for GBM patients that restricts tumor angiogenesis by targeting vascular endothelial growth factor in GBM cells (Davis, 2016; Tan et al., 2020). However, once again, there is no established survival benefit from bevacizumab therapy (Tan et al., 2020). Given that many of the current treatments for GBM are insufficient in improving the survival of patients and preventing tumor recurrence, it may be important to consider CRISPR-Cas9 gene therapy as a new potential treatment.

The Blood-Brain Barrier

The lack of new treatments for GBM can largely be attributed to challenges with delivery across the blood-brain barrier (BBB), as the low permeability of the BBB to different chemotherapies and immunotherapies often accounts for the failure of many promising GBM treatments in-vivo (Jena et al., 2020; S. S. Kim et al., 2015; Taylor et al., 2019). In fact, 98% of small-molecule drugs are unable to cross the BBB and enter the central nervous system (Taylor et al., 2019). Normally, the blood-brain barrier plays a crucial role in regulating the movement of molecules between the brain and the systemic circulation (Jena et al., 2020). As a highly selective diffusion barrier, the BBB ensures that nutrients important for metabolism enter the brain and that harmful toxins are left in the blood, with the presence of transmembrane proteins throughout the BBB allowing small, lipophilic molecules to diffuse through (Jena et al., 2020; S. S. Kim et al., 2015). The BBB is made up of several different types of cells: capillary endothelial cells, surrounding astrocytes, and pericytes (Taylor et al., 2019). Although the blood-brain barrier consists of several layers, the layer of specialized brain microvascular endothelial cells (BMECs) is most important to the BBB's protective role (Jena et al., 2020). BMECs form a physical barrier through the construction of tight junctions between adjacent cells, helping to restrict free access to cells inside the brain (Jena et al., 2020). These endothelial cells also interact with the cerebral neurovasculature to support homeostasis maintenance (Jena et al., 2020).

In order to combat the blood-brain barrier as an obstacle, both intracerebral injection and intentional disruption of the BBB, through the use of substances like microbubbles and hydrophilic surfactants, have been explored (Jena et al., 2020; S. S. Kim et al., 2015). In addition, the use of nanoparticles or nanomedicine, coupled with the transient disruption of the BBB, has been studied rigorously as a potential delivery mechanism for GBM treatment (Jena et al., 2020; S. S. Kim et al., 2015; Peviani et al., 2019). This will be discussed more extensively later in this paper. Overall, with the relevance of the blood-brain barrier to GBM, proper delivery mechanisms across the BBB are undoubtedly central to the success of any future prospective GBM treatments.

CRISPR-Cas9 Gene Therapy

Clustered regularly interspaced short palindromic repeats (CRISPR) are segments of genetic material originally found in bacteria and archaea, constituting many repeated, identical DNA sequences interrupted by spacer sequences (Doudna & Charpentier, 2014). Cas9 is an RNA-guided endonuclease enzyme capable of cleaving DNA at specific loci to produce a double-stranded break (Ran et al., 2013).

The CRISPR-Cas9 system was originally discovered as a component of the adaptive immune system in bacteria to confer resistance against viral infection. This mechanism in bacteria primarily happens in three steps (Doudna & Charpentier, 2014). First, upon infection, the viral genetic material that enters the bacterium is integrated

into the host genome as a spacer sequence in the CRISPR loci (Doudna & Charpentier, 2014). After that, this spacer sequence undergoes transcription into CRISPR RNA (crRNA) (Doudna & Charpentier, 2014). Finally, this crRNA assembles with Cas proteins, directing these enzymes to foreign genetic material for degradation through complementary base pairing whenever infection from the same virus occurs again (Doudna & Charpentier, 2014).

As more has been unveiled about this system, CRISPR-Cas9 technology has been explored as a new gene-editing approach, following the footsteps of zinc finger nucleases and transcription activator-like effector nucleases (Doudna & Charpentier, 2014; Ran et al., 2013). While CRISPR-Cas9 systems typically rely on the presence of a tracrRNA strand and a crRNA strand in order to function properly, single-guide RNAs (sgRNAs) have been engineered to retain the properties of both strands and serve the same purpose in CRISPR-Cas9 gene editing with more simplicity (Doudna & Charpentier, 2014; Ran et al., 2013). sgRNA can be flexibly engineered with the length of about 20 nucleotides, allowing practically any DNA sequence to be targeted for cleavage by Cas9 as long as the nucleotide sequence of the sgRNA is complementary to the target gene (Doudna & Charpentier, 2014; Ran et al., 2013). Any engineered sgRNA can simply bind to a Cas9 enzyme and guide it to the gene of interest for cleavage with high specificity (Doudna & Charpentier, 2014; Ran et al., 2013). However, it is worth noting that a short DNA sequence, the protospacer adjacent motif (PAM), must be adjacent to the target gene in order for DNA recognition to occur properly. This is because the PAM sequence is directly responsible for facilitating the binding between the Cas-9 complex and the DNA sequence of interest (Doudna & Charpentier, 2014).

Upon the introduction of a double-stranded break by Cas9 at the target gene locus, there are two possible mechanisms for DNA repair: homology-directed repair (HDR) and non-homologous end joining (NHEJ) (Doudna & Charpentier, 2014; Ran et al., 2013). As NHEJ typically results in the insertion of random mutations at the target DNA sequence, like frameshift mutations and premature stop codons, this repair pathway can facilitate gene knockout in cells (Ran et al., 2013). On the other hand, with the presence of a repair template along with the CRISPR-Cas9 complex, HDR is the pathway that takes place (Doudna & Charpentier, 2014; Ran et al., 2013). The repair template facilitates specific nucleotide modifications of the target gene through genetic recombination, allowing for gene knock-in (Doudna & Charpentier, 2014; Ran et al., 2013). However, it must be noted that HDR is much less consistent than NHEJ and happens at lower frequencies (Ran et al., 2013). Understanding the higher efficiency and reliability of NHEJ gene knockout compared to HDR gene knock-in, both of the CRISPR-Cas9 treatments discussed in this review rely on NHEJ as a means to edit the human genome.

Proposal for New GBM Treatment

As CRISPR-Cas9 gene editing strategies and research have become increasingly common in recent years, it is very plausible to consider this gene-editing mechanism for the development of novel, unparalleled glioblastoma treatments. Specifically, this review will delve into two potential ways in which CRISPR-Cas9 could be harnessed to treat GBM and improve the prognosis in patients. First, with the high prevalence of the EGFRvIII amongst GBM patients and the association of this type of EGFR amplification with worse clinical outcomes and tumor progression, CRISPR-Cas9 could be utilized to knock out the EGFRvIII gene in glioblastoma cells (Xu et al., 2017). This could ultimately help diminish excessive cell proliferation and trigger apoptosis in GBM cells. Second of all, the knockout of SIRP-α in tumor-associated microglia/macrophages through CRISPR-Cas9 will also be explored as a potential GBM immunotherapy. This treatment could help combat the immunosuppressive tumor microenvironment in GBM, which has been commonly observed as a catalyst for uncontrolled tumor growth (Maas et al., 2020). Overall, the use of CRISPR-Cas9 editing to knockout EGFRvIII and SIRP-α could translate to an efficacious GBM treatment that drastically improves the prognosis and survival of patients.

EGFR Editing

EGFRvIII Mutation in GBM

EGFR amplification and mutation can be observed in 57% of GBMs and are especially common in primary glioblastoma patients (Eskilsson et al., 2018; Xu et al., 2017). While there are several alterations of EGFR that can lead to overexpression in GBM, EGFRvIII (variant 3) is most common, with 25% GBMs housing this genetic mutation (An et al., 2018; Eskilsson et al., 2018; Gan et al., 2013; Huang et al., 2017; Xu et al., 2017). Some studies have revealed this mutation is associated with a poor prognosis amongst GBM patients (Gan et al., 2013). EGFRvIII constitutes the deletion of 801 nucleotide base pairs in exons 2-7, which leads to the removal of 267 amino acids from the extracellular domain of the protein and the formation of a new junction site with a glycine residue (An et al., 2018; Gan et al., 2013; Xu et al., 2017). With this change in amino acid sequence, the extracellular portion of EGFRvIII is truncated and no longer contains a domain to facilitate the binding of ligands; as such, this receptor protein is constitutively active in GBM cells, constantly triggering the initiation of many different signal transduction pathways (An et al., 2018; Xu et al., 2017).

Although EGFRvIII activity is weaker than ligand-activated wild-type EGFR, it has still been shown to play a significant pro-tumorigenic role in GBM, supporting cell proliferation, angiogenesis, and invasion, while also preventing apoptotic cell death (An et al., 2018; Eskilsson et al., 2018; Gan et al., 2013; Xu et al., 2017). The consequences of EGFRvIII mutation in GBM can be connected to the affected signaling pathways.

Signaling pathways affected by EGFRvIII mutations include Ras/MAPK/ERK, PI3K/Akt, and JAK/STAT (An et al., 2018; Eskilsson et al., 2018; Gan et al., 2013; Xu et al., 2017). A notable example is the aberrant PI3K/Akt pathway activation that arises from EGFRvIII (An et al., 2018; Eskilsson et al., 2018; Gan et al., 2013; Xu et al., 2017). This abnormality leads to a decrease in the levels of p27Kip, a cyclin-dependent kinase inhibitor that controls the transition from G1 phase to S phase, ultimately helping to facilitate cell cycle progression and uncontrolled cell proliferation (Eskilsson et al., 2018; Gan et al., 2013; Xu et al., 2017). This excessive induction of the PI3K/Akt pathway can lead to better chemotherapy resistance and increased cell survival.

Since the EGFRvIII mutation has not been detected in normal tissue and is a mutation that characterizes GBM cancer cells, it is a plausible therapeutic target for CRISPR editing. Because the mutation is so specific to GBM, unintended side effects with the disruption of EGFRvIII are quite unlikely (Xu et al., 2017). The potential of targeting EGFRvIII for gene knockout to treat GBM will be further analyzed in the subsequent parts of this review.

In-Vitro Studies Targeting EGFR

Several in-vitro studies concerning EGFR point to the feasibility of targeting EGFRvIII in GBM cells with CRISPR/Cas9 technology for gene knockout. An in-vitro study by Huang et al. in 2017 used LV-sgRNA to knock out exon 17 in the GBM cell lines U87 EGFRwt/vIII and LN229 EGFRwt/vIII. This study found that both EGFR and EGFRvIII expression were decreased and that the resulting cells experienced reduced levels of cell proliferation due to the inhibition of NF-κB (Huang et al., 2017). The disruption of EGFR and EGFRvIII in this in-vitro model and the consequent effects observed suggest that CRISPR-Cas9 engineering for GBM treatment is realistic.

In another in-vitro study, the co-delivery of siRNA and anti-cancer drugs was explored as a possible way to downregulate the EGFRvIII-PI3K/AKT pathway in GBM cells. The siRNA strand was engineered to target the *EGFRvIII* gene, and the drugs SAHA and Erlotinib were used to increase the efficacy of EGFRvIII pathway disruption. It was observed that the GBM cells treated with the siRNA in addition to the cancer drugs experienced substantial decreases in cell proliferation and the induction of apoptosis. This study thus suggests that reducing the expression of EGFRvIII with gene therapy could potentially have therapeutic effects in GBM patients. While this study did not analyze CRISPR-Cas9 technology, the successful use of siRNA targeting EGFRvIII points to the plausible nature of engineering sgRNA for CRISPR-Cas9 that can efficiently base pair with the EGFRvIII gene in GBM cells and facilitate endonuclease-mediated gene knockout (C. Kim et al., 2011).

In-Vivo Studies Targeting EGFR with CRISPR-Cas9

CRISPR-Cas9 has also been utilized for EGFR knockout in several in-vivo settings with mouse models. For instance, in a non-small cell lung cancer study, the adenovirus delivery of Cas9 and EGFR mutation-specific sgRNA was used in attempts to induce tumor regression in mice implanted with H1975/A549 cancer cells. Indels at the target EGFR site were induced at a frequency of 60-80%, and significant tumor growth inhibition and increased survival were observed in the adenovirus-treated xenograft mice, with 81.5% tumor size reduction compared to the control. In addition, no apparent signs of toxicity, like diarrhea or cachexia, were observed in the murine model following EGFR disruption (Koo et al., 2017). Although the characteristic EGFRvIII mutation of GBM was not targeted in this case, this study still points to the feasibility of using CRISPR-Cas9 in-vivo to reduce the expression of the EGFR gene and combat tumor cell proliferation.

In another study, CRISPR-Cas12a, another CRISPR RNA-guided endonuclease, was used to target EGFR in an A549 lung cancer xenograft model. The intratumoral delivery of an oncolytic adenovirus containing both Cas12a and CRISPR RNA was found to efficiently cleave the EGFR gene in cancer cells, inhibit excessive tumor proliferation, trigger apoptotic cell death, and ultimately facilitate complete tumor regression in treated mice (Yoon et al., 2020). While the endonuclease of this study is different from that analyzed in this review, with Cas12 generally deemed more accurate than Cas9, these results support that EGFR disruption through any means could provide therapeutic effects against cancers in general as well as GBM in particular.

It must be noted that in-vivo studies analyzing CRISPR-Cas9 knockout of EGFR are quite limited. However, both of the in-vivo studies discussed demonstrate that EGFR is a good therapeutic target for CRISPR-mediated gene knockout in the treatment of cancers and suggest that using CRISPR-Cas9 for GBM therapy could be plausible.

EGFR Targeting in Humans: Rindopepimut

Rindopepimut is a vaccine for GBM immunotherapy that exploits the EGFRvIII mutation commonly seen in glioblastoma – it consists of a 14-mer EGFRvIII peptide sequence that is conjugated to keyhole limpet hemocyanin. In regard to the mechanism of action, Rindopepimut combats GBM by inducing an immune response that specifically targets EGFRvIII-mutated cells through the actions of antigen-presenting cells as well as CD8+ and CD4+ T cells (Elsamadicy et al., 2017; Malkki, 2016).

This treatment garnered promising results in both phase I and phase II clinical trials, exhibiting clear clinical benefits with improved overall survival. In the phase II trial ‘ACTIVATE,’ it was found that 67% of the GBM patients had lost all EGFRvIII expression after receiving 3 vaccinations. Further, in another phase II trial, ‘ACT III,’ 85% of treated patients experienced heightened humoral responses against EGFRvIII, which was four times the baseline frequency (Elsamadicy et al., 2017).

Unfortunately, however, in the ‘ACT IV’ phase III clinical trial, where around 700 GBM patients were enrolled, it was found that Rindopepimut provided no survival benefit compared to the control treatment, with the median overall survival for the Rindopepimut arm 20.4 months and that of the control arm 21.1 months (Elsamadicy et al., 2017; Malkki, 2016). A plausible explanation for this disappointing outcome was patient selection. With the lack of an established EGFRvIII positive threshold, it was possible that the frequency of the EGFRvIII mutation in the treated GBM patients was quite low. This would have contributed to the lack of efficacy since Rindopepimut is specifically geared towards combatting EGFRvIII-positive cancer cells (Elsamadicy et al., 2017).

Many other phase III trials have been initiated, and these will help to further clarify whether or not Rindopepimut provides a therapeutic benefit in GBM patients. These trials will also reveal whether or not targeting EGFRvIII improves clinical outcomes in GBM patients, as suggested in many preclinical and animal studies.

Challenges with Targeting EGFR in GBM

To determine if the proposed CRISPR-Cas9 mechanisms would be efficacious, it is crucial to look into how EGFR has been targeted in GBM in the past. GBM treatments pinpointing the overexpression of this protein in glioblastoma have been commonly studied in recent years.

Small molecule tyrosine kinase inhibitors (TKIs), like erlotinib, gefitinib, afatinib, and lapatinib, have been explored as potential treatments for GBM patients, with their ability to disrupt the activation of certain signaling cascades that typically lead to tumorigenesis. Although preclinical studies of these treatments have certainly been encouraging, the efficacy of such treatments in GBM patients has been very limited, largely due to the issues concerning the penetrance of the blood-brain barrier. It has been found that the concentration of TKIs in GBM tumor tissue has generally been inadequate due to the protective role of the BBB, and this has consequently resulted in an insufficient inhibition of EGFR signaling activity in GBM cells. (An et al., 2018; Westphal et al., 2017). It is also worth noting that these TKI treatments have resulted in a plethora of side effects in patients, likely due to the lack of specificity of their inhibitory activity. TKIs have been shown to bind to receptor tyrosine kinase proteins other than EGFR and disrupt other signaling pathways (Westphal et al., 2017; Xu et al., 2017).

In addition, anti-EGFR monoclonal antibodies have also been studied as potential avenues for GBM therapy. These antibodies have typically served the function of blocking EGFR ligand binding and/or facilitating the internalization of EGFR in order to downregulate the protein receptor. Notable examples include cetuximab, nimotuzumab, and panitumumab. Although preclinical studies have suggested the promising nature of antibody treatments, they have generally failed to provide therapeutic benefits to GBM patients due to shortcomings in the intracranial and intratumoral accumulation of antibodies. It is clear that problems with crossing the blood-brain barrier have continued to hamper the development of novel, efficacious anti-EGFR antibody treatments (An et al., 2018; Westphal et al., 2017; Xu et al., 2017).

Delivery of CRISPR-Cas9 for GBM

Analyzing the pitfalls associated with many prior treatments targeting EGFR in GBM, it is quite apparent that delivery across the blood-brain barrier is inherently tied to the extent of EGFR disruption and the efficacy of GBM therapy.

The use of nanoparticles to deliver CRISPR-Cas9 across the BBB and into the glioblastoma tumor microenvironment appears to be the most convincing option to maximize efficacy. Different types of nanoparticles have been explored, including but not limited to: cationic liposomes, lipid nanoparticles (LNPs), cationic polymers, vesicles, and gold nanoparticles (Duan et al., 2021; Jena et al., 2020; Lee et al., 2018; Rosenblum et al., 2020). Compared to other delivery mechanisms, nanoparticles have been commonly studied and largely favored in the treatment of CNS diseases like glioblastoma for several reasons. It is quite easy to modify nanoparticles in terms of size, shape, composition, and degradation rate, allowing for increased specificity in targeting cells and reducing the risk of any side effects. Furthermore, nanoparticles have a small size and a surface composition that is conducive to the penetration of cell membranes and movement across the BBB (Cota-Coronado et al., 2019; Jena et al., 2020; Meneghini et al., 2021).

There are different methods of nanoparticle delivery that could be possible for GBM treatment. Passive targeting depends on the enhanced permeability and retention effect (EPR) observed in many brain tumors, including glioblastoma. The EPR effect constitutes the weakening of endothelial tissue in the BBB, stemming from defective angiogenesis of brain tumors, and this ultimately allows nanoparticles to passively disperse and accumulate in the tumor tissue. All nanoparticle treatments that have been approved for clinical use undergo this passive targeting mechanism with the EPR effect. On the other hand, active targeting entails the use of antibodies, peptides, or aptamers that can guide nanoparticles to certain receptors, like transferrin receptors, as well as antigens, allowing them to undergo receptor-mediated transcytosis across the BBB and concentrate in the tumor tissue (S. S. Kim et al., 2015). Furthermore, the disruption of the blood-brain barrier through chemical or mechanical means, with the use of things like microbubbles, MRI-guided focused ultrasound, and hyperosmotic agents, are plausible (Cota-Coronado et al., 2019; S. S. Kim et al., 2015).

Several studies have shown the effective passage of CRISPR-Cas9 into the neural environment and the consequent therapeutic benefits with nanoparticle delivery. For example, the use of lipid nanoparticles to deliver Cas9 and PLK1 sgRNA for CRISPR-Cas9 genome editing was recently explored in a study with a glioblastoma mouse model. In this study, GBM-005 Mice were injected stereotactically with CRISPR lipid nanoparticles, and it was found that the target PLK1 locus was edited with 68% frequency in the GBM tumor cells. A significant reduction in tumor growth was observed, and the median survival for the GBM-005 mice increased from 32.5 days to >48 days upon receiving the single administration CRISPR nanomedicine. The murine GBM model also experienced a 30% increase in overall survival with treatment and exhibited no real signs of immunogenicity. (Rosenblum et al., 2020). Thus, although this study concerns the PLK1 gene and not the EGFRvIII mutation, it still clearly demonstrates that using lipid nanoparticles to deliver CRISPR-Cas9 for the treatment of GBM is feasible.

In another study concerning fragile X-syndrome, delivery of CRISPR with gold nanoparticles, CRISPR-Gold, was analyzed in a fragile x mental retardation 1 knockout mouse model (FMR1). In this case, CRISPR-Gold was stereotaxically injected into the FMR1-knockout mice, with the goal of knocking out the mGluR5 gene. Upon treatment, there was a 40-50% reduction in the mRNA and protein levels of mGluR5, hinting at adequate gene knockout. The study also showed a clear therapeutic benefit, as the treated mice were rescued from the excessive digging and jumping behaviour typical of Fmr1 knockout mice upon receiving treatment(Lee et al., 2018). As such, with the results of this study, gold nanoparticles are also a plausible delivery mechanism for CRISPR-Cas9 GBM treatment targeting EGFRvIII.

Immunotherapy Approach with SIRP-α

SIRP-α in TAMs

It has been shown that the CD47/SIRP-α signaling axis plays an important role in the development of the GBM immunosuppressive tumor microenvironment, which largely promotes tumor growth and is associated with a poor prognosis in patients (Hu et al., 2020; Yang et al., 2019; Zhang et al., 2016). In order to disrupt this signaling axis, CRISPR-Cas9 could be harnessed to inhibit the expression of the SIRP-α protein in TAMs. SIRP-α is mainly expressed in myeloid cells, including microglia and macrophages, and this protein acts as an immune receptor on the cell membrane (Barclay & Van Den Berg, 2014; Hu et al., 2020; Yang et al., 2019). When CD47 on GBM cells interacts with SIRP-α on tumor-associated microglia/macrophages, the CD47/SIRP-α signaling pathway is initiated, and this induces the characteristic GBM immunosuppression that supports tumorigenesis (Hu et al., 2020; Yang et al., 2019).

In-Vitro Studies Targeting the CD47/SIRP-α Signaling Axis

Disruption of the CD47/SIRP-α signaling axis has been shown to be promising in several in-vitro studies surrounding GBM and different cancers.

Multiple in-vitro studies have looked into the use of anti-CD47 monoclonal antibodies to disrupt this signaling axis. In one glioblastoma study examining macrophages and the cell lines GBM1, GBM4 and PGBM1, it was found that upon the administration of anti-CD47 antibody treatment, phagocytosis by M1 macrophages increased by 16%. This increased phagocytic rate for this macrophage phenotype was consistent across all cell lines (Zhang et al., 2016). In another study experimenting with the GBM cell lines T387 and T3832, phagocytosis by BMDMs was also observed to increase with statistical significance in the presence of the anti-CD47 monoclonal antibody Hu5F9-G4 (Hutter et al., 2019). With studies like these, corroborating the increased phagocytosis that can result from CD47/SIRP-α signaling axis disruption, a CRISPR gene therapy targeting this signaling pathway to improve GBM progression certainly seems quite promising.

While studies surrounding GBM and the CD47/SIRP-α signaling axis have primarily analyzed checkpoint inhibitors, the expression of SIRP-α has been targeted in the study of several other cancers. Notably, the knockdown of SIRP-α, with the use of small interfering RNA and lentiviruses, was explored in an in-vitro hepatocellular

carcinoma study. In this study, it was found that compared to the control, the SIRP-α knockdown bone marrow-derived macrophages experienced significantly heightened expression of pro-inflammatory cytokines like IL1β , IL6, and TNF- α, exhibiting a switch in phenotype polarization from the tumorigenic M2-like state. Further, the SIRP-α knockdown BMDMs displayed greater survival through the activation of the Akt pathway and the NF-κB pathway (Pan et al., 2013). As such, this study demonstrates that SIRP-α knockout for GBM immunotherapy could help combat immunosuppression and yield clinical benefits since SIRP-α appears to have such an important role in the polarization of TAMs into the M2-like phenotype.

In addition, in an in-vitro osteosarcoma study, CRISPR-Cas9 was used to knockout SIRP-α in the macrophage cell line RAW264.7. To deliver the Cas9E20-ribonucleoprotein with a single guide RNA into the RAW264.7 cells, cationic arginine-coated gold nanoparticles were utilized, with a 27% editing efficiency observed. Ultimately, when the SIRP-α knockout macrophages were co-cultured with U2OS-GFP+ osteosarcoma cells, they exhibited a four-fold increase in phagocytosis of the cancer cells, compared to the unaltered RAW264.7 cells (Ray et al., 2018). Thus, once again, although this study was not focused on GBM, it still supports that the knockout of SIRP-α in tumor-associated macrophages/microglia could provide therapeutic benefits through increased phagocytosis.

In-Vivo Studies Targeting the CD47/SIRP-α Signaling Axis

Various in-vivo studies analyzing the consequences of disrupting the CD47/SIRP-α signaling axis have also been conducted with mouse models. For instance, in a study with GBM 4 and GBM 5 xenograft mouse models, treatment with anti-CD47 monoclonal antibodies was explored. In comparison to the control, it was found that the treated xenograft mouse models had more than two times the number of macrophages exhibiting the pro-inflammatory M1-phenotype and only a slightly higher count of M2-macrophages (Zhang et al., 2016).

Aside from this change in the M1-M2 macrophage ratio, a clear therapeutic benefit from the treatment was also observed in the mice, with the tumor burden greatly reduced and an apparent improvement in survival. Although the source of the M1-macrophage increase in the mice could not be definitively pinpointed, a possible cause stated in the study was the reprogramming of M2 TAMs back into an M1-like phenotype (Zhang et al., 2016). As such, this study highlights the CD47/SIRP-α signaling axis in GBM as a possible therapeutic target for CRISPR-Cas9 knockout. Disruption of this signaling mechanism could decrease the presence of M2 TAMs by altering their phenotype polarization, combat the immunosuppressive environment of GBM, and ultimately facilitate better clinical outcomes in patients.

In another study, the efficacy of the anti-CD47 monoclonal antibody, Hu5F9-G4, was analyzed with the use of T387-EBFP2-Luc xenograft mice models. Compared to the control, the frequency of GBM tumor cell phagocytosis upon anti-CD47 treatment increased with statistical significance, from 2.7% to 13.3% for tumor-associated microglia and from 1.7% to 5.2% for tumor-associated macrophages (Hutter et al., 2019). Further, it was found that upon receiving the treatment, the xenograft mice exhibited a clear survival benefit and a reduction in tumor burden, with and without the knockout of the CCR2 gene in the mice. As the CCR2 gene is essential to the infiltration of peripheral macrophages into the central nervous system and the tumor microenvironment, these results point to the vital anti-tumorigenic role played by resident microglia upon re-education with the disruption of the CD47-SIRP-α signaling axis (Hutter et al., 2019). Thus, this study clearly displays the potential therapeutic effects of an immunotherapy treatment targeting TAMs and the CD47-SIRP-α signaling axis. It also showcases the importance of effectively pinpointing microglia, in particular, with the delivery of CRISPR-Cas9 gene knockout.

It is worth mentioning that CRISPR-Cas9 gene editing as a form of GBM immunotherapy has not been examined in many in-vivo settings. However, as a subject at large, CRISPR-Cas9 immunotherapy has been explored in recent years for several diseases, including cancers (NCT02793856, NCT04417764, Stadtmauer et al., 2020) as well as HIV (NCT03164135). In fact, many human studies have pointed to the feasibility of editing immune cells in order to improve cancer patient outcomes. In one notable example of such, CRISPR-Cas9 was utilized to edit T cells for the treatment of patients with refractory cancers in a phase 1 clinical trial. Specifically, PD-1, TRAC, and TRBC in the patients' T cells were knocked out through Cas9-mediated cleavage and consequent non-homologous end

joining. With the T cells engineered ex-vivo and infused back into the patients after editing, the study found the treatment to be both safe and feasible, with off-target effects rarely observed (Stadtmauer et al., 2020). While it must be acknowledged that the ex-vivo editing strategy in this study is different from the delivery mechanism proposed, this study still helps to underscore that the proposed CRISPR-mediated SIRP-α knockout in TAMs could be a plausible treatment for GBM patients.

Delivery Method for Tumor-Associated Microglia/Macrophages

As discussed previously, the use of nanoparticles appears to be the most promising delivery mechanism for CRISPR-Cas9 GBM gene therapy due to its unique ability to move across the blood-brain barrier effectively (Cota-Coronado et al., 2019; Jena et al., 2020; Meneghini et al., 2021). However, specifically for this immunotherapy treatment concerning TAMs, there are other things to take into consideration since microglia have a distinct ability to internalize nanoparticles efficiently. Scavenger receptors, which are responsible for detecting nanoparticles as foreign material and subsequently internalizing them, are highly expressed in microglia (Meneghini et al., 2021; Peviani et al., 2019). In addition, it is believed that the phagocytic role normally played by microglia and macrophages also accounts for this internalization capability.

Thus, understanding this feature of microglia and macrophages, the nanoparticles delivering CRISPR-Cas9 to TAMs for the proposed immunotherapy can be adjusted accordingly to maximize internalization. Particularly, it will be necessary to examine the dimensions, as well as the surface charge, of the nanoparticles used since these factors have been observed to directly affect the efficiency of nanoparticle internalization by microglia and macrophages (Peviani et al., 2019). Smaller dimensions and negative surface charges, in particular, have been observed to optimize internalization (Peviani et al., 2019). Further, PEGylation on the surface of nanoparticles is something to be cautious of in developing the delivery mechanism for the proposed immunotherapy treatment; this alteration drastically reduces nanoparticle internalization through a shielding effect (Peviani et al., 2019).

Discussion

CRISPR-Cas9 GBM Treatment Proposal

Glioblastoma has continued to be a lethal disease for patients, in spite of the ongoing efforts to curate novel medical treatments. In this review, two possible CRISPR-Cas9 GBM treatments have been explored, both of which could potentially transform the prognosis and overall survival of GBM patients.

The first GBM therapy proposed is the targeting of the prevalent GBM mutation, EGFRvIII, which has been shown to play a significant role in GBM tumor proliferation. The knockout of EGFRvIII in GBM cells through CRISPR-Cas9 cleavage and non-homologous end joining could ultimately prevent aberrant pathway activation in cancer cells and greatly influence the progression of GBM tumors. In an in-vitro setting, the suppression of EGFRvIII expression with the use of siRNA, with the co-administration of anti-cancer drugs, was found to inhibit tumor cell proliferation and trigger apoptosis (C. Kim et al., 2011). Further, the immunotherapeutic drug Rindopepimut, a drug that specifically targets EGFRvIII-mutated GBM cells, has been shown to provide substantial survival benefits in both phase I and phase II clinical trials (Elsamadicy et al., 2017; Malkki, 2016). Studies like these have clearly suggested that EGFRvIII is a promising therapeutic target for GBM.

On the other hand, the second GBM therapy proposed is a CRISPR immunotherapy targeting TAMs to disrupt the CD47/SIRP-α signaling axis. This signaling pathway, which constitutes the interaction between TAMs and GBM cells, has been deemed a major facilitator of the GBM immunosuppressive tumor microenvironment. Thus, disrupting this signaling axis through the knockout of SIRP-α in TAMs with CRISPR-Cas9 could certainly help combat tumorigenesis and improve the trajectory of GBM progression. Although knocking out CD47 to disrupt the

signaling pathway could be possible, SIRP-α seems to be the better therapeutic target due to its limited expression in the human body. CD47 is highly expressed in normal red blood cells, and as such, the gene knockout of CD47 could potentially result in high toxicity, with the risk of anemia (Hu et al., 2020; Yang et al., 2019).

In an in-vitro CRISPR-Cas9 study concerning osteosarcoma, SIRP-α knockout macrophages were found to experience a four-fold increase in the phagocytosis of cancer cells (Ray et al., 2018). In addition, in an in-vivo GBM study analyzing the anti-CD47 monoclonal antibody Hu5F9-G4, treated xenograft mouse models exhibited apparent increases in survival and reductions in tumor burden (Hutter et al., 2019). With past research showing the effectiveness of inhibiting the CD47/SIRP-α signaling axis in cancers, SIRP-α knockout with CRISPR-Cas9 is certainly worth exploring further.

For both gene therapies proposed, the most hopeful delivery mechanism is the use of nanoparticles to carry CRISPR-Cas9 across the BBB and into the GBM tumor microenvironment. A xenograft GBM study highlighted in this review, in which lipid nanoparticles were harnessed to deliver CRISPR-Cas9, showcases why nanoparticles may be most effective in yielding efficacious GBM treatment (Rosenblum et al., 2020). In comparison to viral vectors, nanoparticles appear to be more effective for multiple reasons; for one, nanoparticles have advantages in packaging capacity, which may be crucial considering the large size of the Cas9 protein (Jena et al., 2020; Meneghini et al., 2021; Rosenblum et al., 2020). Further, nanoparticle delivery is also seen as the safer mechanism of the two, as the side-effects of utilizing viral vectors are still uncertain. Mutagenesis, immunogenicity, and carcinogenesis are amongst the risks that have been associated with viral delivery of CRISPR-Cas9 (Duan et al., 2021; Jena et al., 2020).

Overall, although CRISPR-Cas9 treatment for GBM has yet to be tested in human settings, there are many signs that point to the potential success of utilizing CRISPR in the contexts described for a therapeutic benefit in patients.

Future Directions for GBM Research

It is also worth discussing other GBM treatments that are being tested as well as important future directions for GBM research. At the moment, immunotherapy is something commonly studied in the development of GBM treatment. Oncolytic viral therapies, like PVSRIPO, have been shown to be promising in phase I clinical trials (Tan et al., 2020). Immune checkpoint inhibitors, like PD-1, PD-L1, and CTLA-4, have also been tested in GBM trials but have returned disappointing results, likely due to the immunosuppressive environment that characterizes GBM (Banerjee et al., 2021; Tan et al., 2020). CAR-T cell therapy, with the modification of T cells ex-vivo, has also been an emerging approach to treating GBM; however, these studies are still in their early stages (Tan et al., 2020).

Aside from immunotherapy, targeted therapies geared towards inhibiting receptor tyrosine kinases, like EGFR, EphA3, VEGF, PDGFR and MET, have also been analyzed (Tan et al., 2020; Taylor et al., 2019). While these treatments have produced mixed results in both pre-clinical studies as well as clinical trials, this is certainly a field that needs to be explored further, as the understanding of GBM pathways remains quite insufficient.

In terms of future directions, new screening methods for GBM need to be considered. As discussed previously, magnetic resonance imaging is currently the primary screening method for GBM; however, this detection often occurs at far too late a stage when GBM has already metastasized (Alexander & Cloughesy, 2017; Davis, 2016). Earlier diagnosis and treatment of GBM could potentially increase the survival of GBM patients substantially.

In regard to GBM treatment, it will be crucial to continue analyzing GBM pathways and gaining a better understanding of how such pathways can be targeted to improve clinical outcomes in patients. Researchers in the field should also look further into the immunosuppressive tumor microenvironment of GBM and consider ways in which this can be combated effectively. This component of GBM has often accounted for the failure of novel GBM treatments and has continued to be a key player in the poor prognosis of GBM patients. Furthermore, gene therapies, specifically in the realm of CRISPR-Cas9 editing, should undoubtedly be explored for GBM treatment. CRISPR-Cas9 editing is already being tested in clinical trials for several cancers other than GBM. Apart from the therapeutic targets for CRISPR analyzed in this review, this gene-editing mechanism could also be utilized to treat GBM in other ways.

CRISPR could be utilized for immunotherapy treatments like CAR-T cell therapy, used for the knockout of different tyrosine kinases, and even be utilized to knock in tumor suppressor genes commonly mutated in GBM, like p53. While the prospects of CRISPR-Cas9 certainly seem promising, only time will tell how much of a role CRISPR-Cas9 actually plays in the progress of GBM research.

Acknowledgments

I would like to thank my advisor Nicole Guilz for helping me with this project.

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