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Crispr/Cas9 Delivery for Glioma Treatment: A Systematic Review

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Abstract

CRISPR/Cas9 gene editing technology represents an interesting and promising option for treating glioblastoma patients. Different in vitro studies have demonstrated the success of the gene editing process; however, the efficacy of this technology and its delivery directly into tumor cells in vivo needs to be evaluated. A systematic review of the literature was performed to identify studies using gene editing techniques at the preclinical stage in animal models to analyze their efficacy and safety in vivo. 1463 articles were identified; five articles involved the use of CRISPR/Cas9 gene editing therapy against human cells in animal models to treat glioma. Compared with unedited controls, the gene-edited cells showed reduced tumor size, prolonged survival, no damage to off-target cells and no toxic effects. However, there is high heterogeneity among the articles identified due to the different mouse strains and different delivery methods. CRISPR/Cas9 gene editing shows promising anti-cancer effect. Further reporting of in vivo studies is expected to continue, with the potential to better control for bias and reduce heterogeneity, thereby positive effects could be corroborated.

Keywords: *CRISPR/Cas9 Gene, Anti-Cancer Effect, Glioma Treatment.*

Introduction

Glioblastoma multiforme (GBM) is an extremely aggressive cancer characterized by rapid growth and high invasiveness (1). Survival is about 3 months without any treatment (2), and can be extended to 18 months with treatment (including surgery, radiotherapy and chemotherapy) (3). Given these dauting prospects, other therapeutic strategies have been explored. Immunotherapy for tumor diseases based on chimeric antigen receptors (CAR) for T cells (4), is currently being considered for the treatment of GBM. These T cells are programmed in such a way that they trigger an immune response only when they recognize tumor cells, making it possible to treat tumors without affecting surrounding cells (5). However, different tissue microenvironments lead to variations in the possible responses of tumor cells to CAR-T: the presence of cytokines, different regulatory roles of stromal cells, negative regulation exerted by tumor cells and limitations of transport to tumor sites in vivo affected the efficacy of the technique (6-8).

To improve the effectiveness and development of CARs, a gene-editing technique known as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated protein 9 (CRISPR/Cas9) has been used to establish a whole new generation of CARs, that can modify

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different tumor responses: blocking of T-cell receptors, altering apoptosis and changes in tumor growth factor beta receptor, among others (9). Since the CRISPR/Cas9 technology can edit CAR-T cells to act on tumor cells, clinical trials using this technology to treat patients with leukemias, lymphomas and myelomas are underway (10).

Although gene editing technology has reached an optimal level of technical and scientific maturity, its use in patients remains uncertain due to difficulties in achieving desired effects in expected locations (12,13). With respect to delivery media for CRISPR, electroporation, microinjection, ultrasound-propelled nanomotors, nanofluidics and lance assay nanoinjection have been tested (14). For the specific case of the CRISPR/Cas9 technology used for editing cells of human immune system, the delivery choice continues to be electroporation (15). Another strategy for in vivo delivery is the use of viral and non-viral vectors. Adeno-associated viruses are the most used vectors, because of their ability to reach target cells and low immunogenicity; however, other viruses have been used where higher carrying capacity is required (16).

Since viral vectors carry risks to the health and integrity of patients, it is important to address the non-viral vectors field. This delivery mechanism not only reduces the impact of the biological risk but also has the advantage of being easier to prepare and allowing the delivery of higher molecular weight loads (17). First, lipid nanoparticles have shown advantages in relation to biocompatibility, low toxicity, easy binding to macromolecules for loading and high endocytosis efficiency (18). Another option are polymeric nanoparticles, which have great potential for medical treatments with the gene editing technique since, due to the chemical properties of their components, high levels of selectivity and biocompatibility are achieved (19); these polymers can be designed according to the characteristics of the microenvironment and the target cells to improve stability and guarantee a more efficient intracellular transduction (20).

Nanoparticles composed of inorganic materials have also been used. Gold-based nanomaterials have been developed that allow the effective delivery due to their high chemical stability and, the possibility of modifying the physical structure and taking advantage of the optical and plasmonic properties of the metal, which have allowed remote-control systems (21). Specifically speaking about glioblastoma and the aforementioned techniques, there have been reported studies involving tumor cells implanted in mice, in vitro models that recreate the tumor microenvironment, screening studies of genes related to invasion and proliferation and transcriptional regulation studies, however, not enough in vivo studies have been performed, so the following question arises: What is the best method of transporting the CRISPR/Cas9 complex for the treatment of glioblastoma multiforme?

Methods

The present study corresponds to a systematic review of the literature in accordance with the PRISMA 2020 guidelines (11). Articles related to the CRISPR/Cas9 technique that were exclusively focused on the treatment of GBMs were reviewed. The databases Scopus (Science Direct), PubMed and EMBASE were used without limitation of publication date or any other filter and using the key words: “glioma”, “oligodendroglioma”, “oligoastrocytoma”, “glioblastoma”, “CRISPR”, “Cas9”, and their derivatives.

Eligibility Criteria

Once the search strategies were carried out in each of the databases, the criteria for including the articles (full text) to be analyzed in the present review were defined. These criteria were established based on the formulation of the PICOS question:

- Population: In vivo (animal) studies using the CRISPR/Cas9 technique.

- Intervention: Studies using the CRISPR/Cas9 technique for gene editing on the structure of CAR-T cells and measuring the impact of this editing on the behavior of glioma tumor cells.

- Comparison: Any type of control/comparison strategy included in the study (e.g., unedited CAR-T cells, placebos, other editing techniques, etc.).

- Outcomes: The primary or main outcome was reported tumor shrinkage. Secondary outcomes were survival, editing results and conditions resulting from the application of CAR-T cells.

- Study design: It was reviewed experimental studies, randomized or not, and excluded articles that were reviews, commentaries, letters to the editor, studies without control for comparison, reports or case series or any other study without intervention.

Risk of Bias and Data Analysis

Possible selection biases of the study participants, execution biases, study design biases, measurement biases, detection biases, information biases and data reporting biases were considered. To analyze the data reported in the included studies, the Odds Ratios (ORs) of the different results obtained were compared, in addition to the reported survival (in animal models) and the results of the control interventions.

Results

Identified Studies.

A total of 1463 articles were found in the databases consulted. After excluding duplicates and reviewing those relevant articles that were related to the two search terms, a total of 31 articles remained. The review process resulted in the exclusion of 26 articles because only 5 articles met all inclusion criteria. (Fig. 1)

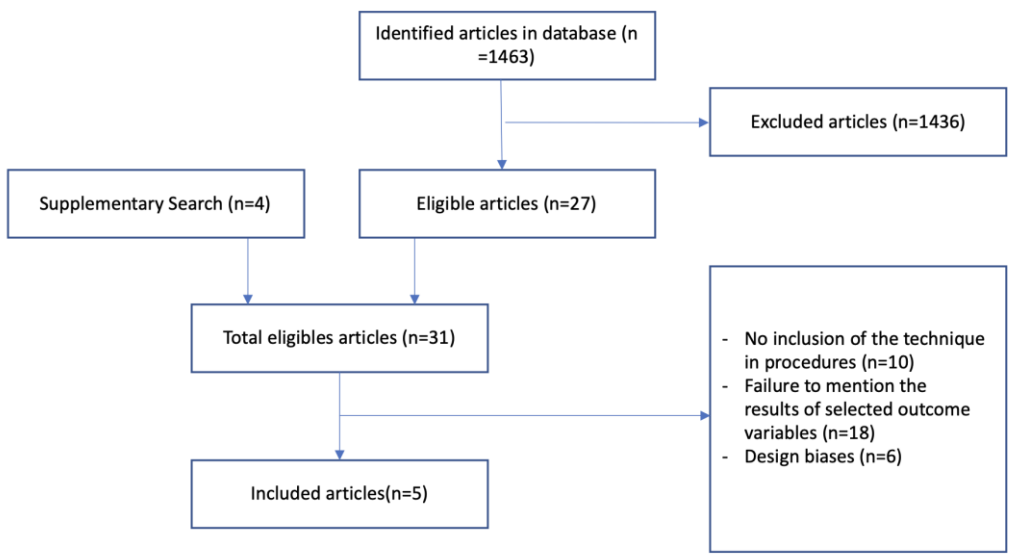

Figure 1. Flowchart of Study Articles Included for Abstraction and Analysis.

Characteristics of the Selected Studies

The results of included studies involving the implementation of CRISPR/Cas9 technology for in vivo editing of different Glioma cell lines (test animals) to assess the response to this type of therapy as a measure of mouse survival, clinical manifestations, tumor size and ease of crossing the blood-brain barrier (BBB). These studies provide complete information on the methodology used, number of animals involved, experimental conditions, and control mechanisms. The information from the 5 articles is recorded in Table 1.

Target Cells for Gene Editing and Interventional Models Used.

Four out of five studies performed editing cell called U87MG, while the remaining studies (25) used four different lineages (LN229, U251, A172, GBM8). The Ruan et al. and Zou et al. study used non-viral mechanisms to transport and release, and the other three studies used viral techniques. These studies were performed on animal models (nude mice). However, it is noteworthy that the study by Li et al. (26) was performed in athymic mice as well.

Study	**Delivery type**	**Glioma cell lines**	**in vitro assays**	**Gene edited**	
Choi et al., 2019 (22)	Viral - AAV6	U87 U87vIII U251	Activity, proliferation, and cytotoxicity	Gene knockout-in/knockout (TRAC, B2M, PDCD1)	TRAC T-cell Receptor PDCProgrammed cell death ligand 1 B2M Beta-2 microglobulin
Ruan et al., 2022 (23)	No-viral - angiopep-2 peptide decorated guanidinium and fluorine functionalized polymeric nanoparticle	U87MG U251	eficiency, permeation ability, uptake ability, targeting capacity, endosomal escape capability	Gene knokout PLK1	Oncogene polo-like kinase 1
Fatimy et al., 2017 (24)	Viral - AVV	LN229, U251, A172, GBM8	impaired the viability of all tested glioma cell lines	miR10b	Implicated in proliferation, invasion, and metastasis

Zou et al., 2022 (25)	No viral - angiopep-2–functionalized, disulfide–cross-linked nanocapsules containing Cas9 and sgRNA ANCss(Cas9/sgRNA)	U87MG GBM stem cells (GSCs) Patient-derived Cancer Stem Cell-2 (CSC-2)	Targeting eficiency BBB penetration	PLK1	Oncogene polo-like kinase 1
Li et al., 2020 (26)	Viral - AAV	U87, U251, LN18, SVG, PDX GBM	Reduced TERT transcription and TERT protein expression	TERT promoter	Reverse transcriptase - Telomeric

Table 1. Summary of the in Vitro Findings Reported in the Selected Articles.

Study	**Animal (strain)**	**In-vivo follow-up**	**Route of administration**	**Type of cell transplanted**	**Treatment description**	**Results**
Choi et al., 2019 (22)	Mice (NSG)	40 days	Tail vein	Ctrl: CART-EGFRvIII Exp: CART-EGFRvIIIΔPD-1	1X10^6 cells intravenous aplication (n=5)	- Did not demonstrate significantly prolonged survival in mice treated with EGFRvIII-specific CAR T cells systemically compared to the control
			Intraventricular		1X10^6 cells intraventricularly (n=5)	- Significantly prolonged survival in mice with EGFRvIII-

						expressing glioma, including durable, complete cures in select mice. No long-term survivors developed clinical signs of xenogeneic GVHD (graft-versus-host disease).
Ruan et al., 2022 (23)	Nude mice	50 days	Tail vein	Ctrl: - PBS - Ang-NP@RNP-gScr - NP@RNP-gPLK1 Exp: Ang-NP@RNP-gPLK1	15 μg Cas9 per mouse (on days 10, 12, 14, 16, 18 and 20 post tumor implantation) intravenous (n=5)	- No toxicity - No Off-target effects - Cellular apoptosis - GBM targeting ability suggesting the delivered RNP was accumulated mainly in the orthotopic tumor. - BBB penetration specific brain disease site targeting (Targeting capability) - 32% gene knockout and 67% protein reduction in the targeted proto-oncogene polo-like

						kinase 1 (PLK1).
Fatimy et al., 2017 (24)	Athymin Nu/nu mice	18	Intratumorally	Ctrl: empty vector Exp: miR-10b-targeting G1 miR-10b-targeting G2	(3 x 105 TU) (n=6)	- Glioma cells are addicted to miR-10b and expression of this molecule is essential for glioma viability and survival. - Reduced tumors - Rescued the body weigth - miR-10b editing prevents neoplastic - Transformation of astrocytes and selectively eradicates the transforming cells
Zou et al., 2022 (25)	mice	76	Tail vein	Ctrl: PBS ANCSS(Cas9/sgScr) Exp: ANCSS(Cas9/sgPLK1)	1.5 mg Cas9 equiv./Kg (on days 10, 12, 14, 16, 18 and 20 post tumor implantation) intravenous (n=11)	- Nontoxic - No Off-target effects increase in GBM cell apoptosis - Nanocapsules tumor accumulation and retention

						- BBB penetration - Tumor growth inhibition - Extended median survival to 68 days versus 24 or 22 days using orthotopic U87MG GBM - Induce apoptosis - Extension in median survival to 55 days, which was significantly longer than the median survival of 21 days for mice specific brain disease site targeting using GSC CSC2 (Targeting capability)
Li et al., 2020 (26)	athymic nude mice	60	intracranial	Ctrl: non-targeting CjABE Exp: −124T sgRNA-guided CjABE	m.o.i. = 100 (n=8)	Prolonged survival time, proliferation inhibition, inhibits tumour growth and prolongs overall survival.

Table 2. Summary of the in Vivo Findings Reported in the Selected Articles.

Edited Genes and Administration Path

Choi et al. (22) edited endogenous T-cell receptor (TRAC), beta-2 microglobulin (B2M) and PD-1 (PDCD1) using intraventricular in one group and tail vein in the other. Ruan et al. (23) performed an Oncogene polo-like kinase 1 (PLK1) knockout and administered it via the tail vein. Fatimy et al. (24) edited the miR10b gene and delivered it directly to tumors. Zou et al. (25) also performed a PLK1 oncogene knockout and used the blood route (tail vein). On the other hand, Li et al. (26) performed editing on Reverse Transcriptase - Telomeric promoter by intracranial administration.

Follow-up and Efficiency

Except for the study by Famtimy et al (follow-up for 18 days), most of the reviewed studies had a follow-up of more than 40 days. Choi et al. found that mice treated with EGFRvIII-specific CAR T cells via intravenous had no significant increase in survival compared to the control. In contrast to the same treatment, but with intraventricular application, survival was significantly prolonged even complete tumor remission. Ruan et al. reported no occurrences related to toxicity or other adverse effects when using the developed nanomedicine, the targeting ability could be considered sufficient to achieve higher levels of apoptosis. The ability to cross the blood-brain barrier was considered by achieving gene knockout in target cells (PLK1).

Fatimy et al. reported that Glioma cells are addicted to miR-10b, this situation is of crucial importance since it allows to define the viability of tumor cells, which leads to reduced tumor size through the eradication of astrocytes that have been transformed with miR10b loss-of-function. On the other hand, Zou et al. report as main findings that the nanomedicine technique was not toxic to mice, nor were there any effects on non-target cells. The level of accumulation of the tumor nanocapsules demonstrated the crossing of the blood-brain barrier and consequently decreased tumor growth and extended survival to an average of 68 days. Li et al. also reported increased survival from decreased tumor proliferation and growth.

Risk of Bias

Each study was analyzed according to the SYRCLE tool (27). All studies were found to have some bias, whether in presenting the results, the randomization, the mention of the level of blinding or providing information on how to ensure that the two groups are fully comparable from a pathophysiological perspective to ensure balance in terms of response to intervention.

Discussion

The evaluated gene editing technology (CRISPR/Cas9) has been shown to be effective in generating the cellular changes needed to control or even block the mechanisms of tumorigenesis and tumor growth, as well as block the immune response of these tumor cells. However, these effects have been demonstrated in in vivo experiments and are therefore at the stage of animal testing, whose main goal is to verify that the way the technology is applied in vivo is effective: the stability of the delivery method is optimal, there are ability to cross the blood-brain barrier, the final gene editing occurs only in the target cells and does not have any harmful effects or toxicity on the organism. The preclinical results presented here suggest that the in vivo applied technique is effective in terms of survival time and tumor volume reduction against glioblastoma cancer cells, although not all studies have equally confirmed these results.

Several authors have reported the effects of gene editing as a treatment option for glioblastoma patients. CRISPR/Cas9 technology is suitable for in vitro modeling of the production of

dehydrogenases, one of the major players in the development of mutations in glial cells, which alter cellular methylation levels and thereby reduce expression in mutant cell lines (28,29,30). Dehydrogenases editing using CRISPR/Cas9 technology could be used to treat patients diagnosed with glioblastoma multiforme. In fact, CRISPR/Cas9 technology produces consistent results across different types of gene editing, however, the various processes required to produce these effects in animal models (in vivo) still need to be improved, starting with achieving high efficiency of transport and delivery methods to the location of the cells being edited (31).

Different authors have reported the effects of gene editing as a management option for patients with glial cell tumors. The CRISPR/Cas9 technique is suitable for modeling in vitro the production of dehydrogenases (one of the main involved in the development of mutations in glial cells), which modifies the levels of cellular methylation, thereby decreasing the expression of mutant cell lines (28,29,30), which could be used for the treatment of patients diagnosed with glioblastoma multiforme. It is a fact that the CRISPR/Cas9 technique has achieved consistent results in different types of gene editing; however, improvements are still required in different processes necessary to replicate these effects in animal models (in vivo), starting with achieving high efficiency of the transport and delivery methods at the site where the cells being edited are located (31).

The methods of delivery to target cells used in the studies correspond to viral methods, but also to non-viral methods. It has been known for over a decade that particles associated with adenoviruses can be transported and that these viruses serve as delivery agents (32). However, the maximum load capacity of this method is 4.7kb, and due to its biological nature, it is difficult to achieve optimal efficiency of the promoters (33) and ensure that its action is only exerted for the time required to control tumor cells, without affecting other cells of the nervous system or other systems (34). The possibility of using a non-viral delivery mechanism, that could have a higher load capacity and shorter duration of action was also explored, which could improve cellular responses and reduce the risk of affecting non-target cells (35). Once it is demonstrated that this editing technology can be performed in vivo to treat glioma patients, i.e., that the delivery, release, crossing of the blood-brain barrier and effect on tumor cells is adequate; lines of research have been established aimed at advancing the preclinical and clinical phases for the application of CRISPR/Cas9 (36) because of the possible effects: Inhibition of tumor progression (37), knockout of genes involved with tumor progression mechanisms (38), increased response to chemotherapy and inhibition of tumor invasion mechanisms(39) and improvement of the degree of T-cell activity as part of the response to immunotherapy (40).

In accordance with the above, the studies discussed here in animal models allowed to confirm that gene editing using CRISPR/Cas9 technology can induce changes in the behavior of tumor cells. Choi et al., 2019 (22) study was able to show that mice expressing EGFRvIII showed higher survival and, in some cases, cure of the disease. Ruan et al., 2022 (23) and Zou et al., 2022 (25) reported interesting and promising findings from their nanomedicine experiments, including lack of toxicity, lack of effects on off-target cells, crossing the blood-brain barrier, and reduced proto-oncogene expression. Fatimy et al., 2017 (24) concluded that gene editing that blocks expression of the miR-10b molecule reduces tumor size in vivo and thus increases survival in mice, consistent with acquired knowledge from in vitro models.

Conclusion

Taken together, gene editing using CRISPR/Cas9 technology for glioma treatment that cross the blood-brain barrier can reduces tumor size, improve survival, and even cure the disease without

any toxicity or effects on off-target cells. These findings demonstrate that CRISPR/Cas9 gene editing technology is effective to perform different interventions on the different genes that favor the expression and development of tumor cells. Additional in vivo studies are expected to be reported in the future, with the potential to better control for bias and reduce heterogeneity in demonstrating beneficial effects and in conducting studies in patients.

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