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Brain-targeted delivery of CRISPR/Cas9 mediated glioblastoma therapeutics

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Abstract. Glioblastoma multiforme (GBM) is a life-threatening malignant tumor of the central nervous system, for which there is currently no effective treatment. Its low survival rate has been interpreted as a result of its high proliferation rate, resistance to apoptosis, and the ability to create a microenvironment conducive to tumor growth. Recently, a powerful and accurate gene-editing tool, Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-Associated Protein 9 (CRISPR/Cas9), has high potential in various scientific fields. Such technologies can manipulate cellular mechanisms and defective genes that lead to the progression of many serious diseases such as cancer. One of the major barriers to the application of this technique is the development of a delivery method to diffuse CRISPR/Cas9 efficiently and accurately to the target location in brain. Most existing delivery methods are failed to be translated into clinical result due to the lack of promising safety and efficiency. Thus, I will introduce several strategies here that can be used potentially for CRISPR-Cas9 system delivery in GBM treatment including the principle, advantages, limitations, and latest developments of these systems. This review is composed to provide a concise summary for future researchers to understand the current challenges and approaches in CRISPR/Cas9 mediated GBM therapeutics delivery.

Keywords*:* glioblastoma, CRISPR, Cas9, drug delivery.

1. Background

1.1. Glioblastoma multiforme

Glioblastoma multiforme (GBM) is a WHO degree IV glioma brain tumor that is derived from neural stem cells (NSCs), NSC-derived astrocytes, and oligodendrocyte precursor cells (OPCs). According to CBTRUS statistical report, it is the most common primary malignant tumor that derives from the brain and other central nervous system (CNS), with only a 6.9 % of five-year survival rate [1]. Many hallmarks were discovered that contribute to its aggressiveness and lethality, including the presence of the self-renewing and multipotent tumor-initiating GBM stem cell (GSC) [2], sustained proliferative signals, programmed cell death escape, building block recycle mechanism, and immunosuppressive microenvironment, etc. [3]. There are limited treatments that can be applied in clinics nowadays and can hardly eliminate symptoms or prolong the lifespan of patients efficiently. The traditional treatment is usually surgery; however, many image-guided tools have been developed to provide more precise resection, a thorough removal of the tumor is hindered due to the high infiltrative nature of GBM. Thus, the surgery is usually adjuvanted with other treatments like cytotoxic chemotherapy, anti-angiogenic

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chemotherapy, radiation therapy, etc., which are usually accompanied by intense side effects and may lead to a therapy resistance and tumor recurrence. To overcome this long-standing challenge, many novel treatments are emerging, for example, immune therapy and gene therapy.

1.2. Clustered regularly interspaced short palindromic repeats

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) was first been discovered in 1987 by Nakata group while they studied the E.Coli iap sequence [4].This type of repeats has then been found to be present throughout prokaryote species. Mojica group recognized and summarized the gene sequence of this family in 2000 [5] and proposed its function as a part of the prokaryotic adaptive immune system [6].In the following years, many scientists have studied this mechanism to prove the strong potential of CRISPR technology in the biomedical field. Among all CRISPER associated systems, *S. pyogenes derived Cas9* is the most studied one up to today. CRISPR Cas9 can achieve gene gain- or loss-of-function easily by just two components, a single guided RNA (sgRNA) and a DNA endonuclease Cas9. Once the system has been introduced to the cell, the sgRNA first recognizes and binds the target sequence. This process recruits the Cas9 protein, which is able to create a double strain break (DSB) at the targeted sequence. This dsDNA breakage then triggers non-homologous end joining (NHEJ) or homology-directed repair (HDR) in the organism. The outcome can be different as we engineer the Cas9 protein or manipulate the repair pathway. The versatility of the system allows it to be utilized in a wide range of applications, for instance, gene therapy for brain cancer. While zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have already made gene editing feasible, CRISPR revolutionizes this process due to its low cost, simplicity, and efficiency. However, a safe, accurate, and efficient delivery approach with enough packing capability and BBB permeability is required for people to apply this set of powerful tools in brain targeted gene therapy. Here, two categories of mainstream delivery strategies for CRISPR-Cas9 mediated GBM therapy are described, viral vector and non-viral vector.

2. Viral vector

Viral vector is a category of common molecular biology tool that can be used to deliver the gene of interest to cultured cells or living organisms. They have been studied for glioma gene therapy over the past 25 years and some viral based GBM gene therapy has already been used in clinic, for example the Novel GADD34-expressing Oncolytic HSV-1 [7]. Viral vectors can achieve highly efficient cell membrane and transduction, target specific cells, express transgenes stably in short or long term, and be manufactured on large scale with lower cost compare to novel non-virus vectors [8]. The three frequently used and widely approved by regulators viral vectors, adeno-associated viruses (AAVs), adenoviral vectors (AdVs), and lentiviral vectors (LVs), are considered to be strong candidates for CRISPR delivery [9]. Although viral vectors have many advantages, only AAVs are currently approved to have strong potential in brain targeted CRISPR delivery due to their ability of traversing blood-brain barrier (BBB) after administration and delivering genes to the CNS [10]. AdVs, on the other hand, are generally considered impenetrable to the BBB [11].

Atchison and colleagues first separated AAVs during preparations of a simian adenovirus in 1965 [12]. This is a small, non-enveloped and single-stranded dependoparvovirus as it lacks of essential genes for replication and gene expression [13]. What's important is that it's considered non-pathogenic, which makes it a safe vector for gene therapy. Scientists have then developed recombinant AAVs (rAAVs) by deleting viral coding sequence and only keeping T-shaped inverted terminal repeats (ITRs). This modification further reduced its immunogenicity and cytotoxicity, and enlarged its packaging capability up to 5.0kb [14]. Due to the aforementioned advantages, AAV has been applied successfully in the first approved [15] and many following gene therapy treatments.

Different AAV strains specifically bind with different category of cell surface glycan receptors. The capsids of AAVs interact with the glycans and initiate the infection. AAV2, AAV3, and AAV13 bind heparan sulfate proteoglycan (HSPG), AAV1, AAV4, AAV5, and AAV6 bind sialic acid (SIA), AAV9 binds terminal N-linked galactose (GAL), and Bovine AAV (bAAV) binds sialic acid which linked to

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specific gangliosides with assistant of chitotriose for infection [16,17]. This specificity of binding then determines their transduction result. For instance, study shows that the direct injection of AAV2 selectively leads to neuronal transduction, not astrocytes or microglia transduction [18][18], which is likely due to the larger HSPGs expression on the surface of the neuron. On the other hand, SIA binding AAVs, like AAV1 and AAV5 showed capability on both neuronal and some glial transduction in animal CNS; AAV9 has also approved to be able to target neuronal and glial, with neonatal neurons and adult astrocytes transduction expressing preferentially [19-21].

AAVs could be delivered in different approaches, for example, direct administration, including intra-cerebrospinal fluid (CSF) administration and intra-parenchymal administration, and systematic administration, which also called intravenous administration. CSF administration has been emerging as a way of gene therapy delivery that bypasses BBB and reaches the brain tissue [22]. CSF locates in the subarachnoid space, cerebral ventricles, cisterna magna and openings under the cerebellum (foramena). Experiments also confirmed its ability to flow from the ventricles throughout the parenchyma towards the subarachnoid space which enbales to distribute ICV-infused gene therapy throughout the CNS [23]. Systemic administration, on the other hand, is an administration via bloodstream which minimize the invasion and decrease the difficulty of clinical application. When using such approach, mannitol can be co-infused intra-artierialy to create transient openning of BBB without causing any permanent damage [24].

Although AAV showed potential on delivering the CRISPR system to many targets tissue types via bypassing BBB, only limited studies showed the feasibility of AAV-delivered CRISPR/Cas9 mediated GBM treatment, mainly by combining it with existing treatment. For instance, Choi et al. reported that CRISPR-Cas9 disruption of programmed cell death ligand 1 (PD-1) via AAV6 vector enhances activity of universal EGFRvIII CAR T cells in a preclinical model of human glioblastoma. Although CAR T-cell therapies has already been used in clinic for hematological malignancies treatment, it did not perform remarkably in GBM treatment, partially due to its high and sustained PD-1 expression in cancerous environment. Authors used AAV6 capsuled CRISPR-Cas9 to engineer the CAR T cell, disrupted the endogenous T-cell receptor (TRAC), beta-2 microglobulin (B2M) and PD-1 (PDCD1) gene and created CART-EGFRvIIIΔPD-1 cells that are resistant to PD-1 inhibition. The result shows that CART-EGFRvIIIΔPD-1 cells have better efficiency on U87vIII infected mice than that of CART-EGFRvIII cells in a long run. In addition, when two type of CAR T cells and control solution were administrated into the ventricular system, CART-EGFRvIIIΔPD-1 cells led to significantly prolonger survival time in mice and 40% of mice in CART-EGFRvIIIΔPD-1 cells group were cured from EGFRvIII-expressing glioma [25].

Whereas, upon today, no study clearly indicates the feasibility of the solely-AAV-based CRISPR/Cas9 delivery method. First, its safety issue needs to be considered cautiously while developing AAV related delivery method. Reports indicate that integration events among recombinant AAV does occur, although rare, and increase the risk of oncogenicity and genotoxicity [26]. Additionally, AAV's genome size restricts its maximal transgene capacity to be around 5kb, and many CRISPR-Cas9 system have larger size, especially when the ribonucleoprotein (RNP) form or other large transgene cassettes are applied to achieve higher efficiency. Other than CRISPR-Cas9, combining system like novel Cas proteins with a smaller size or split Cas9 proteins with AAV might be the future direction of study. In conclusion, the major issue in design and commercialize AAV-based CRISPR/Cas9 delivery method is to find a balance between potency, immunogenicity, and manufacturing cost.

3. Non-viral vector

Another category of the delivery system is non-viral vector, and in cancer gene therapy delivery the extracellular vesicle (EV) is one representative. Extracellular vesicles (EVs) are one of the essential intercellular communication approaches, transferring proteins, lipid, and genetic material across cell membrane, and be taken up directly by neighbor cells, or cell at distant sites though biofluids. Three common categories of EVs are microvesicles (MVs), exosomes, and apoptotic bodies [27,28]. As these EVs play different roles and function in different manners, they also show diverse potentials in the

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delivery of CRISPR/Cas9 mediated glioblastoma therapeutics. However, rarely could they bypass BBB to reach the target site in brain tumor.

In 2017, Zeming Chen and colleagues reported a synesthetic liposome-templated hydrogel nanoparticles that has achieved efficient delivery of RNP complexes of Cas9 protein and sgRNA for PLK1 inhibition in mouse flank tumor model. This delivery system is composed by a polyethyleneimine (PEI) hydrogel core that encapsules the engineered RNP and a cationic 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP) lipid shell. To ensure the treatment feasibility, a various number of engineering strategies were tested and approved. (1) The crosslinking structure of cyclodextrin (CD)-engrafted PEI (PEI-CD) and adamantine (AD)-engrafted PEI (PEI-AD) via CD-AD interaction overcomes the low encapsulation efficiency of traditional DOTAP liposome delivery system, maintains the protein activity and enhances the packaging and delivery. (2) The use of mHph3 ligands then ensure a high level (1.3 times more efficient than Lip2k) transfection of human brain cancer U87 cell. These mHph3-conjugated, DOTAP liposome templated hydrogel nanoparticles, aka. LHNPs, released 91.5%of DNA and 85.2% of protein over 3 days in a controlled pace and were engulfed by U87 cells with efficiency up to 100%. (3) The minicircle DNA technology and RNP cargo formation (Cas9 protein combined with candidate sgRNAs synthesized by chemical modification) were applied and a 79.3% and 80.2% cell growth inhibition in U87 cells and GS5 cells were reached, respectively. (4) On the top of that, another important design, surface conjugation of internalizing RGD (iRGD), was inspired from Sugahara and colleagues' study which published early in 2007. Their study shows that while traditional RGD peptide only deliver tumor drug to the blood vessel and surrounding area, the tumor-homing peptide iRGD (CRGDK/RGPD/EC) can bound tumor vessels and dive deep into the extravascular tumor parenchyma [29]. Zeming Chen and colleague reported that the iRGD conjugated LHNPs has increased the concentration of nanoparticles within the in vivo tumor cells 2.6-fold compared to the mice treated with the non-iRGD-conjugated LHNPs. Meanwhile, this treatment via Cas 9/minicircle-sgPLK1-2 loaded iRGD conjugated LHNPs demonstrated great performance in inhibiting tumor growth in the sample mice by eliminating the PLK1 expression, which originally contribute to the anti-apoptosis capability of GBM. As result, they observed that the mice in experimental group had an average tumor volume that is 23.5% of those in control group. (5) Last but not least, the application of Lexican in brain tumor aimed BBB penetration is tested. And the result did show a 2.1 times nanoparticle accumulation difference between the Lexican+ iRGD included and the iRGD included only LHNPs treatment. This treatment with Lexican+ iRGD enabled the median survival time of diseased mice to increase from 29 days to 40 days, which is a significant breakthrough in GBM and made the CRISPR/Cas9 -LHNPs mediated GBM therapeutics a realistic choice in lab for the first time [30].

More recently, in 2022, Jun Liu and colleagues have further confirmed the feasibility of the ribonucleoprotein (RNP) complexes encapsulated LHNPs delivery system by applying it in a ZNF117 targeted GMB treatment. They proved that the down regulation of ZNF117 eliminates the glioblastoma stem cells (GSC) differentiation towards the oligodendroglial lineage and, therefore, controls the tumor resistance and recurrence. As Chen and colleagues' delivery system, they also conjugated iRGD on the surface of nanoparticles and involved the BBB opening helper molecule, Lexiscan, to penetrate BBB transiently and allow for autocatalytic brain tumor targeting mechanism. This treatment has then been tested in vivo on PS30-derived mouse xenografts via injection of 1 mg of NPs (sgRNA equivalent dose of 0.2 ug) intravenously three times a week for three consecutive weeks. The result shows that the mice treated with this engineered ZNF117 targeted and RNP loaded LHNPs has significantly longer median survival time and, simultaneously, are more sensitive to temozolomide (TMZ) indicating a possibility of effective combination treatment [31].

In 2022, Weimin Ruan and colleagues applied a different strategy that targets proto-oncogene polo-like kinase 1 (PLK1) in U87MG. They combined the positive guanidinated polymers that can stabilize the nanoparticle by displaying electrostatic interaction and hydrogen bond with siRNA or proteins [32-43], and fluoropolymers that can enhance protein encapsulation and stabilization to ensure the integrity of the nanoparticle in the complex charge-rich circulatory system. At the same time, Ang-NP@RNP is formulated as a cargo since angiopep-2 peptide can recognize LRP1 receptors of BBB and glioma cells,

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which hypothetically improve the performance of treatment on blood circulation time, BBB penetration, tumor accumulation, gene editing efficiency, suppression of tumor growth and medium survival time of mice infected by human orthotopic GBM. Their experiment on Balb/c mice via tail vain injection indicates that Ang-NP@RNP and NP@RNP treatment exhibit a similar elimination half-life time, 40.4. and 43.0 min, which is much longer than that of un-engineered free RNP, 11.7 min. Moreover, the fluorescence imaging system (IVIS) result shows that the decorated Ang-NP@RNP nanocomplexes lead to prolong stable time (24 h) and stronger accumulation in glioblastoma (up to 12.9% of injection does, 1.8- and 5.5-fold higher than that achieved by NP@RNP or free RNP respectively) and mainly in orthotopic tumor comparing to free RNP. Regarding to the overall effectiveness, glioblastoma xenograft mouse model treated with Ang-NP@RNP-gPLK1 has a median survival time of 40 dyas, which is significantly longer than the ones treated with NP@RNP-gPLK1 (27 days), Ang-NP@RNP-gScr (22 days) or PBS (18 days). Additionally, all qualitative and quantitative analyses reveal that Ang-NP@RNP-gPLK1 therapeutics increases the tumor cell apoptosis and lower the tumor proliferation remarkably in a safe way [35]. Though evidence is lack to show that these therapeutics can kill the cancer cells thoroughly in bulk tumors and no perfect solution for the side effect caused by off-target editing so far, the synthesis of CRISPR/Cas9 -LHNPs mediated GBM therapeutics and approval of its efficiency in vivo is a remarkable improvement.

4. Conclusion

Heretofore, the effectiveness of CRISPR/Cas9 mediated GBM treatment delivered by viral and non-viral vectors have both been discussed. Among all vectors, AAV and polymeric NPs are most studied and successful cases are emerging. Since these studies utilize different experiment and analysis methods, there is no head-to-head comparison between these delivery systems. The data and information that mentioned and cited in this article can be used as a reference to see what's been known currently. There is still a long way to go before CRISPR/Cas9 mediate glioblastoma therapeutics can be utilized clinically to treat GBM. Even though we have this powerful tool- CRISPR/Cas9- many obstacles are remained unsolved, like the triangle of potency, immunogenicity, and manufacturing cost in AAV based delivery system design and the unclear delivery efficiency in bulk parenchymal tumors and off-target issue in polymeric NP based design. Above mentioned nodus might be studied and overcame in the near future, and by then, the CRISPR/Cas9 based GBM gene therapy will be one step closer to the clinic.

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