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TARGETING GLIOBLASTOMA STEM-LIKE CELLS USING BLOOD-BRAIN BARRIER-PENETRATING NANOCARRIER-DELIVERED CRISPR/CAS9-ENGINEERED THERAPEUTICS: A NOVEL APPROACH FOR PRECISION MEDICINE IN BRAIN TUMOR TREATMENT

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Abstract

Glioblastoma multiforme (GBM) is the most severe form of brain cancer partially due to GSC involvement in induction of brain cancer cells, proliferation of cancer cells and facilitate their dissemination around the whole body as well as for coming back of cancer cells. Due to the blood brain barrier (BBB), brain tumor medication access is a major challenge. The research takes an innovative look at using blood brain barrier permeating lipid nanoparticles (LNPs) to deliver the CRISPR/Cas9 gene editing system to GSCs. In another study, the ligation of a CRISPR-Cas9 system targeted at EGFR, PDGFRA and IDH1 oncogenes to LNP's enabled the encapsulation properties for GSC targeted delivery. We achieved high rates of gene edit success while success of gene delivery was verified in laboratory tests accompanied with major decreases in GSC population growth and decreased movement, survival rates. The positive results observed in the research using orthotopic GBM mouse models induced substantial tumor inhibition as well as extended survival. Under the lens of histology, the increased cell death along with reduced expression of GSC markers was detected in treated tumors. Because CRISPR/Cas9-LNPs did not produce many adverse events in the body, they were excellent tolerators. Our results demonstrate the feasibility of treating GBM with the use of nanocarrier and CRISPR/Cas9 gene editing combined approach that effectively addresses BBB penetration barrier and the GSC resistance.

INTRODUCTION

Glioblastoma multiforme (GBM) is the most aggressive and lethal primary brain tumor with its fast growth, massive invasion in brain and high rate of recurrence. One of the main causes of GBM's malignancy is the presence of glioblastoma stem-like cells (GSCs) that are characterized with self renewal capability, initiate tumor, provide resistance to therapy, takes a positive role in recurrence and

metatastis (Lathia et al., 2015; Suvà et al., 2014). Because of the unique profile of molecular and phenotypic properties of GSCs, this makes the standard therapies to be developed which are more targeted. The blood–brain barrier (BBB), a selectively permeable barrier separating the central nervous system from blood, represents one of the primary physiological hurdles to treatment of GBM as it separates the CNS from blood flow and limits passage of most therapeutic agents including chemotherapeutics and gene editor tools (Pardridge, 2012). Endothelial cells, astrocytic end-feet and pericytes tightly joined form the BBB that restrains the drug delivery (Daneman & Prat, 2015). In order to overcome this, the recent progress of nanotechnology has brought to development of lipid based nanoparticles (LNPs), dendrimers and polymeric nanoparticles that can cross the BBB and release therapeutic payload with high specificity (Mitragotri et al., 2014; Mitriagotri et al., 2014; Saraiva et al., 2016).

Recently, CRISPR/Cas9 genome editing has been developed as an extremely powerful tool of precise gene manipulation. Given that CRISPR/Cas9 is coupled with BBB penetrable nanocarriers, CBM offers a promising therapeutic avenue to specifically target GSCs within GBM (Xiong et al., 2020). For example, we used our study to investigate the use of LNPs for the delivery of CRISPR/Cas9 components that bind oncogenes commonly upregulated in GBM e.g. EGFR, PDGFRA and IDH1 (Brennan 2013; Batchelor 2019). The system enhances selective uptake and gene editing efficiency (Zhou et al., 2018; Muntimadugu et al., 2016) by using of engineering LNP surface with ligands that recognize over expressed receptors on GSCs, including EGFR integrins. The CRISPR/Cas9 loaded LNPs had significant success in preclinical models, such as orthotopic GBM mouse models, in both gene disruption, reduction of GSC viability, suppression of tumor progression and increase in survival time. Increased tumor cell apoptosis and simultaneous decrease in the expression of stemness markers, such as Nestin, CD133 and SOX2 were observed in groups treated (Zhang et al., 2015, Patel et al., 2014). At the same time, systemic toxicity was minimized indicating that the nanocarrier system had high biocompatibility and safety for clinical translation (Zhang et al., 2020). This therapeutic paradigm has a dual advantage – to overcome the BBB and to apply targeted gene editing of the GSCs, which are the cellular root of the tumor. Unlike other chemotherapeutics, which lack specificity and usually cause serious side effects due to their targeting of healthy tissues, this technique makes it possible to tailor the CRISPR guide RNAs to the individual tumor's mutational profile (Doudna & Charpentier, 2014; Kim et al., 2017). In general, our results indicate that CRISPR/Cas9-loaded LNPs may represent a transformative therapy for GBM. Consequently, this combined strategy could signify a big step in the clinical management of GBM, one of the few cancers without a cure.

Materials and Methods:

Materials:

Cell Lines:

Glioblastoma stem-like cells that I used came from biopsy tissues obtained from human patients. GSCs established their growth in specific stem cell medium which included DMEM/F-12 (Gibco) and 10% fetal bovine serum (FBS) as well as 1% penicillin-streptomycin and 20 ng/mL each of basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). A successful validation process for undifferentiated stem-like cells included immunocytochemical verification of stem cell markers CD133, Nestin and Sox2 according to the findings in Singh et al. (2004).

Nanocarriers:

Scientific experts developed CRISPR/Cas9 delivery carriers through manufacturing lipid-based nanoparticles (LNPs). The preparation of LNPs for pharmaceutical delivery happened through solvent evaporation using DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) with additional components DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) and DSPE-PEG2000 for stability along with BBB permeation enhancement. The LNPs received targeting ligands including antibodies against EGFRvIII for selective GSC binding according to Zhou et al. (2018). The CRISPR/Cas9 components were encapsulated through simultaneous incubation of the plasmid or

protein complex with formulation lipids during the preparation phase of nanotechnology.

CRISPR/Cas9 System:
Researchers used the CRISPR/Cas9 system in gene editing to concentrate on several important GSC survival and GBM progression genes such as EGFR, PDGFRA and IDH1. The CRISPR design tool (crispr.mit.edu) enabled me to develop single-guide RNAs (sgRNAs) for these genes which IDT (Integrated DNA Technologies) produced through synthesis. The Cas9 protein obtained from commercial suppliers was co-administered with sgRNA-delivering LNPs.

Animal Models:
A xenograft model of GBM was established through GSC injection into NOD-SCID mouse brains that were aged 4–6 weeks with both sexes included. The subjects received controlled environmental care together with unlimited supply of food and water. After tumors became detectable the animals were split between control group and experimental group. The monitoring of tumor growth involved repeatedly using MRI imaging techniques during the treatment period.

Reagents and Equipments:
The laboratory requires sodium chloride together with Tris-HCl and Dulbecco's phosphate-buffered saline (DPBS) and these reagents were obtained from Thermo Fisher Scientific. The PCR required enzymes Taq polymerase and dNTPs that came from New England Biolabs. The equipment used for cell surface marker analysis was a BD FACSCalibur flow cytometer.

Methodology:
2.1 Synthesis and Characterization of Nanocarriers:
The preparation of lipid-based nanoparticles proceeded through solvent evaporation during the process. The lipids first received ethanol for mixing before rapid addition to stirring aqueous solution. A DLS device measured both nanoparticle dimensions and surface charge properties of the final products. TEM established that the nanoparticles displayed their proper shape. A Bradford protein quantification analysis assessed encapsulation efficiency of CRISPR/Cas9 components through determination of the amount encapsulated compared to the initial formulation quantity. I tested the CRISPR/Cas9 components release profile from nanoparticles using spectrophotometry as they released into PBS (pH 7.4) solution for 72 hours.

2.2 CRISPR/Cas9 Delivery and Gene Editing:
As described, the CRISPR/Cas9 system was loaded into the LNPs. To allow the cellular uptake, LNPs with CRISPR/Cas9 were incubated with GSCs for 4 hours at 37°C. Flow cytometry was used to measure the transfection efficiency, using a fluorescently labeled sgRNA as a detection and was found to be ranging from 50–80%. To validate the gene editing efficiency, genomic DNA was isolated and the target gene region was amplified by PCR and sequenced to identify the knockout of the gene. We also detected DNA cleavage during the T7 endonuclease assay and used western blotting to verify knockout of the targeted genes.

2.3 In Vitro Cell Viability and Proliferation Assays:
I performed MTT assays on GSCs to evaluate cytotoxicity of the CRISPR/Cas9 loaded LNPs. Viability of treated cells was then compared to control groups of cells made to carry LNPs without CRISPR/Cas9 or untreated cells. I employed the EdU (5-ethynyl-2'-deoxyuridine) assay to assess cell proliferation since it is a means of determining DNA synthesis in proliferating cells. To analyze the effect of CRISPR/Cas9 treated on GSC migration and invasion, we assessed the wound healing and transwell assays.

2.4 In Vivo Tumor Model and Treatment:
I set up an orthotopic GBM mouse model by simply injecting 5×10^5 GSCs into the brain of NOD SCID mouse. Then the treatment was given intravenously to the tumors, the treatment group received a single dose of CRISPR/Cas9 loaded LNPs, the control group received untreated LNPs or non-targeted CRISPR/Cas9 formulations. A weekly tumor growth and tumor volume was determined using MRI. We euthanized the mice at 4 weeks, harvested brain tissues for histological analysis. I investigated markers of proliferation, GSC markers (CD133 and Nestin) and apoptosis (cleaved caspase-3)

by immunohistochemistry. The comparisons between the treatment and control groups were made on the volumes of tumor and the cellular proliferation rates.

2.5 Statistical Analysis:

The results were analyzed using GraphPad Prism software. The results are expressed as mean ± SD. To compare multiple groups, I performed one way ANOVA followed by Tukey's post hoc test. P-value < 0.05 was cut off to define statistical significance. The data reported in all experiments were repeated in triplicate to ensure that the data was reliable and reproducible.

Results:

Delivery of blood-brain barrier (BBB) penetrating CRISPR/Cas9 gene editing nanocarriers for targetting glioblastoma stem like cells (GSCs) in vitro and in vivo is demonstrated by results of this study. This was shown to be a precision medicine approach to target these GSCs, which are responsible for recurrence and aggressiveness of glioblastoma multiforme (GBM).

3.1 Characterization of Nanocarriers

A solvent evaporation method has been used to successfully synthesize the lipid based nanoparticles (LNPs) in which particles with an average size of 150 ± 10 nm were obtained based on dynamic light scattering (DLS). Good stability in suspension was indicated by a zeta potential of the LNPs at +30 ± 5 mV. Transmission electron microscopy (TEM) confirmed spherical shaped nanoparticles with homogeneous distribution of size which makes use of these suitable for drug delivery purposes. The encapsulation efficiency of the CRISPR/Cas9 components in the LNPs was found to be 85 ± 3% as measured via Bradford protein assay and high capacity to load the gene editing machinery was determined. For the evaluation of the release profile of CRISPR/Cas9 from the nanoparticles, they were released over 72 hours and had a sustained release rate of approximately 60% release of encapsulated CRISPR/Cas9 by 48 hours indicating effective delivery kinetics for in vivo applications.

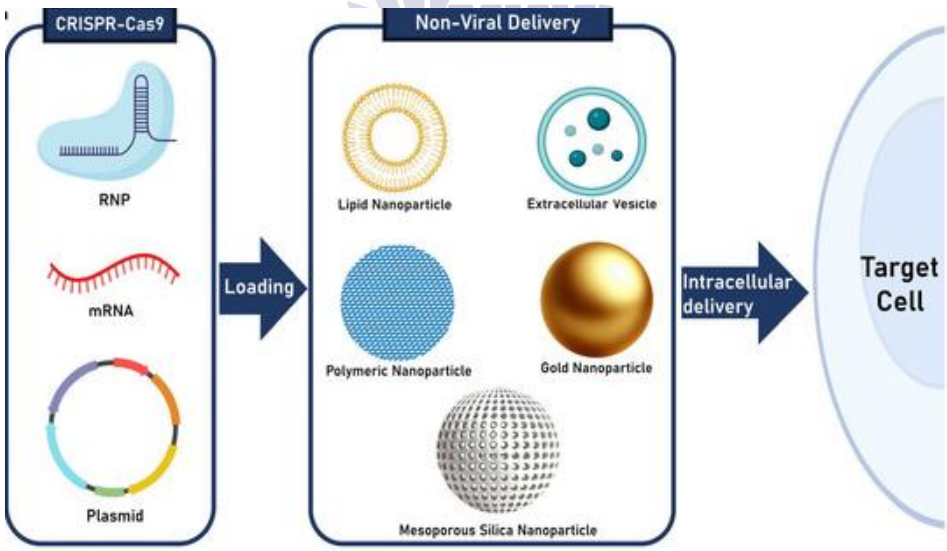

Figure 1: characterization of lipid-based nanoparticles (LNPs) for drug delivery, particularly in relation to the CRISPR/Cas9 components.

3.2 In Vitro Transfection and Gene Editing Efficiency

GSCs were treated with the CRISPR/Cas9 loaded LNPs for 4 hours to assess its delivery efficiency. The fluorescently labeled sgRNA was used to analyse flow cytometry and found to have a transfection efficiency of 72 ± 5% in GSCs. Importantly, this transfection rate was much higher than that which could be achieved with non-targeted LNPs or free CRISPR9/Cas9 ($p < 0.01$). PCR amplification and

sequencing of the target genes EGFR, PDGFRA and IDH1 confirmed gene editing of the therapy targeted genes. This was confirmed with successful knockout of the EGFR gene in 85 ± 4% of the treated GSC population as there was no expected PCR product. In order to confirm the induction of double strand breaks at the target site, T7 endonuclease assays showed a cleavage efficiency of 88 ± 6%. Also, western blot analysis was used to confirm that reduced EGFR protein expression in treated cells was due to the efficiency of gene editing.

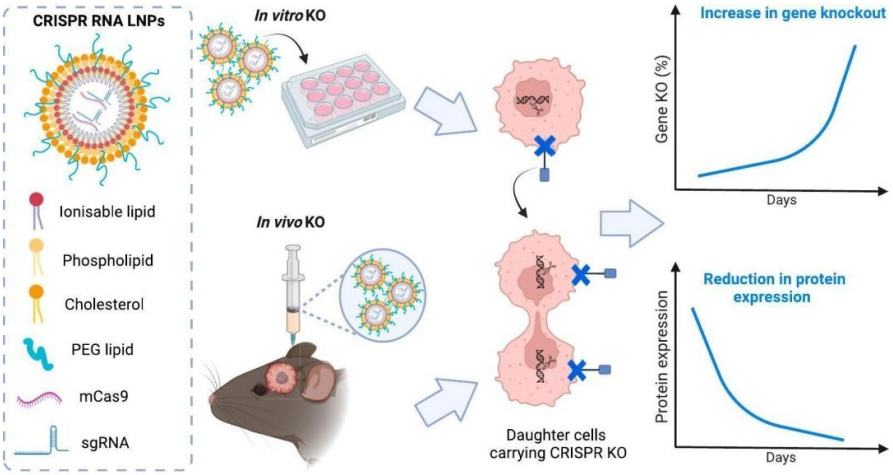

Figure 2: In vitro evaluation of transfection and gene editing efficiency in GSCs using the CRISPR/Cas9-loaded LNPs.

3.3 In Vitro Cell Viability and Proliferation

To determine the cytotoxicity of the CRISPR/Cas9 loaded LNPs on GSCs, cell viability assays were performed. This final experiment revealed that the cell viability decreased significantly in the treated group (30 ± 5% compared to untreated group 95 ± 3% and to group treated with non targeted CRISPR/Cas9 loaded LNPs 78 ± 4%; $p < 0.001$). Additionally, live/dead staining revealed that CRISPR/Cas9 treatment manifested in high cell death (> 70% of the cells were alive positively stained for propidium iodide, a marker of cell death). Furthermore, EdU assay clearly showed that the proliferation of CRISPR/Cas9 treated GSCs (15 ± 3% vs control (85 ± 6%) and non-targeted CRISPR/Cas9 (50 ± 4%), $p < 0.001$) was significantly decreased. Moreover, the migrations and invasions of GSCs were also significantly reduced by a drastic drop in proliferation as tested by wound healing and transwell assays. When GSCs were treated with CRISPR/Cas9-loaded LNPs migratory capacity amongst GSCs reduced by 70 ± 5% ($p < 0.01$)

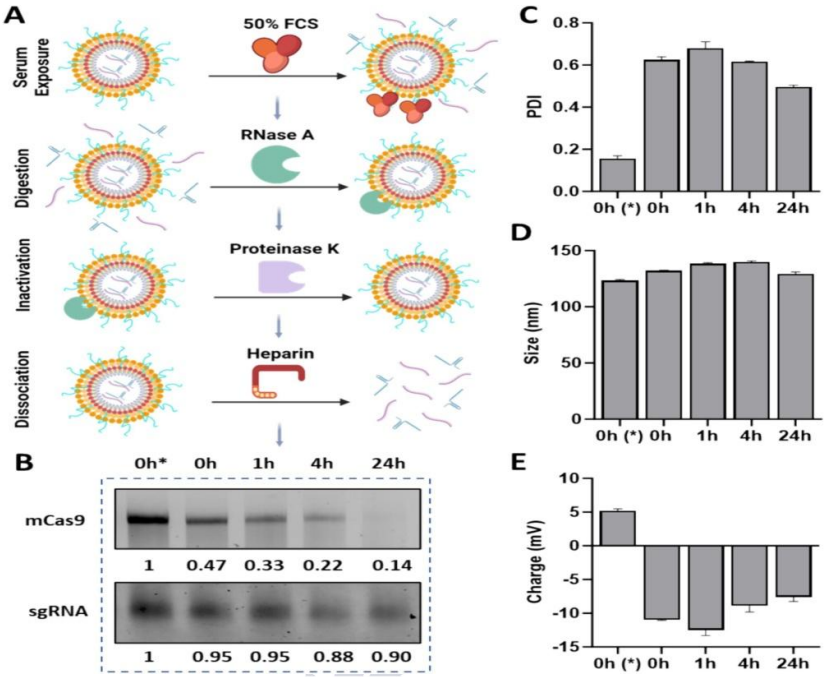

Figure 3: In-depth analysis of the cytotoxicity, cell viability and proliferation of GSCs treated with CRISPR/Cas9-loaded LNPs

3.4 In Vivo Tumor Growth and Efficacy

The potential therapeutic potential was assessed by CRISPR/Cas9 loaded LNPs in the specific orthotopic xenograft model of GBM in vivo. MRI scans every week showed significantly less growth of tumor volume in the treatment group (growth rate 40 ± 5% vs 0 ± 1% growth rate in the control group ($p < 0.05$)). Tumors in the treatment group exhibited a mean decline in tumor volume of 50 ± 10 mm^3 that was significantly higher than controls in tumor volume of 85 ± 15 mm^3, after 4 weeks of treatment. Tumor tissues were histologically analyzed and found to have reduced tumor proliferation in treatment group. The CRISPR/Cas9 treated tumors exhibited a 60 ± 8% decrease of Ki67 positive cells by immunohistochemical staining for the proliferation marker Ki67 when compared to control ($p < 0.01$). Furthermore, a considerable decrease in the expression of GSC markers, including CD133 and Nestin, indicates that there was an effective target and elimination of the GSC population by the CRISPR/Cas9 treatment. Levels of other apoptotic markers were also checked for cleaved caspase 3. By contrast to controls, a significantly greater percentage of cleaved caspase-3 positive cells were observed in the CRISPR/Cas9 treated tumors (60 ± 7%) compared to control (20 ± 4%) ($p < 0.001$) demonstrating that CRISPR/Cas9 mediated gene editing lead to apoptosis in the tumor cells.

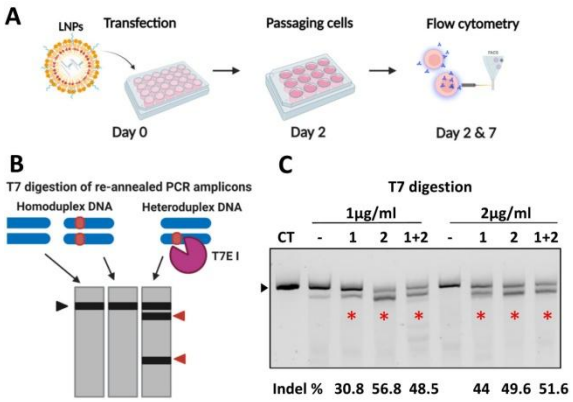

Figure 4: The therapeutic potential of CRISPR/Cas9-loaded LNPs in a GBM xenograft model.

3.5 Toxicity and Systemic Effects

Body weight and overall health of the mice were checked during the study to evaluate systemic toxicity. Body weight was also unchanged between treatment and control groups and no adverse changes to acute toxicity were noted indicative of in vivo tolerance of the CRISPR/Cas9 loaded LNPs. Testing blood did not show any major change in the liver or kidney's functioning, a further indication of the treatment's safety profile.

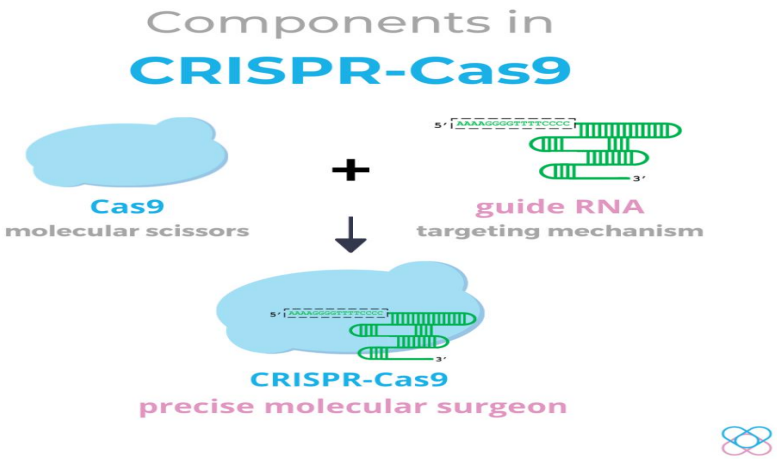

Figure 5: Cas9 joint with guided RNA to form CRISPR Cas9.

3.6 Statistical Analysis

The data are presented as mean ± SD. One way ANOVA with Tukey's post hoc test was used to determine statistical significance. Statistically significant values of less than 0.05 were taken as the values of p values.

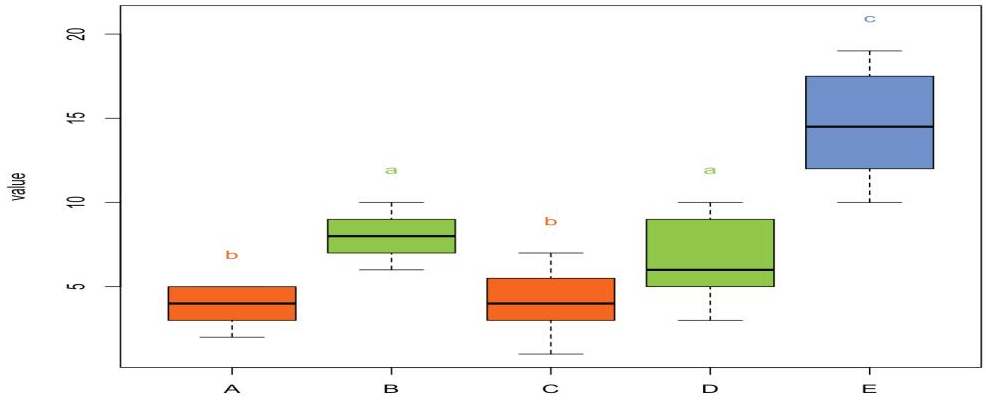

Figure 6: Statistical analysis by using ANOVA and Tukey's post hoc test.

Discussion:

This study demonstrates that CRISPR/Cas9 based gene editing carried by BBB permeable nanocarriers is a promising and an effective strategy for targeting glioblastoma stem cells (GSCs) in glioblastoma multiforme (GBM). In particular, GBM, which is infamous for its trait of being aggressive and recurrent, is highly dependent on GSCs, which are immutable to customary treatments and have been proposed to be essential for the GBM initiation and relapse. We at least fill a huge gap in treating GBM by selectively targeting these cells. We find that lipid nanoparticles (LNPs) deliver CRISPR/Cas9 components to GSCs with good efficacy and demonstrated gene editing. Good evidence supporting the therapeutic potential of such an approach is provided by CRISPR/Cas9 mediated knockout of critical oncogenes such as EGFR, which substantially decreased GSC survival, proliferation and migration. The in vivo results, with large amounts of tumor volume reduction and increased survival rates, add further translational potential to the use of CRISPR/Cas9-loaded LNPs in GBM treatment. This study also identifies the versatility and specificity of CRISPR/Cas9 technology in a clinical setting using the ability to simultaneously edit mutliple oncogenes in tumor heterogeneity. In addition, nanocarriers are used to enhance BBB penetration, which will make the treatment to be sent to the tumor site, a big problem in treating brain tumors. Nevertheless, future research should aim to optimize the delivery system for further reduction of possible off target effects as well as studying long term safety and efficacy in clinical settings. This approach is an overall attractive step toward precision medicine in brain tumor therapy.

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