Heliyon 10 (2024) e37041

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Combination of microRNA and suicide gene for targeting Glioblastoma: Inducing apoptosis and significantly suppressing tumor growth in vivo

Zahra Fekrirad [a,b], Milad Gharedaghi [a], Fatemeh Saadatpour [a], Zahra Asghari Molabashi [c], Ameneh Rezayof [d], Alireza Korourian [e], Masoud Soleimani [f], Ehsan Arefian [a,g,*]

[a] Department of Microbiology, School of Biology, College of Science, University of Tehran, Tehran, Iran
[b] Department of Biology, Faculty of Basic Sciences, Shahed University, Tehran, Iran
[c] Department of Plant Molecular Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran
[d] Neuroscience Lab, Department of Animal Biology, School of Biology, College of Science, University of Tehran, Tehran, Iran
[e] Oncopathology Research Center, Iran University of Medical Sciences, Tehran, Iran
[f] Department of Hematology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[g] Department of Stem Cells Technology and Tissue Regeneration, School of Biology, College of Science, University of Tehran, Tehran, Iran

ARTICLE INFO

Keywords:
Glioblastoma
miR-34a
Cytosine deaminase
5-Fluorocytosine
Apoptosis

ABSTRACT

Glioblastoma (GBM), a grade IV brain tumor, presents a severe challenge in treatment and eradication due to its high genetic variability and the existence of stem-like cells with self-renewal potential. Conventional therapies fall short of preventing recurrence and fail to extend the median survival of patients significantly. However, the emergence of gene therapy, which has recently obtained significant clinical outcomes, brings hope. It has the potential to be a suitable strategy for the treatment of GBM. Notably, microRNAs (miRNAs) have been noticed as critical players in the development and progress of GBM. The combined usage of hsa-miR-34a and Cytosine Deaminase (CD) suicide gene and 5-fluorocytosine (5FC) prodrug caused cytotoxicity against U87MG Glioma cells *in vitro*. The apoptosis and cell cycle arrest rates were measured by flow cytometry. The lentiviral vector generated overexpression of CD/miR-34a in the presence of 5FC significantly promoted apoptosis and caused cell cycle arrest in U87MG cells. The expression level of the *BCL2, SOX2,* and *P53* genes, target genes of hsa-miR-34a, was examined by quantitative real-time PCR. The treatment led to a substantial downregulation of *Bcl2* and *SOX2* genes while elevating the expression levels of *Caspase7* and *P53* genes compared to the scrambled control. The hsa-miR-34a hindered the proliferation of GBM cancer cells and elevated apoptosis through the P53–miR-34a–Bcl2 axis. The CD suicide gene with 5FC treatment demonstrated similar results to miR-34a in the apoptosis, cell cycle, and real-time assays. The combination of CD and miR-34a produced a synergistic effect. *In vivo,* anti-GBM efficacy evaluation in rats bearing intracranial C6 Glioma cells revealed a remarkable induction of apoptosis and a significant inhibition of tumor growth compared with the scrambled control. The simultaneous use of CD/miR-34a with 5FC almost entirely suppressed tumor growth in rat models. The combined application of hsa-miR-34a and CD suicide gene against GBM tumors led to significant induction of apoptosis in U87MG cells and a considerable reduction in tumor growth *in vivo*.

* Corresponding author. Department of Microbiology, School of Biology, College of Science, University of Tehran, Tehran, Iran.
E-mail address: arefian@ut.ac.ir (E. Arefian).

https://doi.org/10.1016/j.heliyon.2024.e37041
Received 10 March 2024; Received in revised form 12 August 2024; Accepted 22 August 2024
Available online 29 August 2024

1. Introduction

Glioblastoma (GBM), a common primary brain tumor, is highly heterogeneous at the genetic and molecular levels. This well-known feature leads to the early development of abundant subclones because of rapid mitoses. Establishing a molecular-level diagnosis and targeted therapy for these dynamic and heterogenic cell populations is challenging. The treatment efficacy could increase by targeting multiple parts and mechanisms in different subclones [1].

GBM tumors harbor stem-like cells with self-renewal ability and an undifferentiated phenotype. Cancer stem-like cells are crucial in initiating and maintaining the tumor after cytotoxic therapies. These cells contribute towards radiation resistance by increasing the DNA damage response. Multiple molecular mechanisms, including NF-κB, Notch, and PARP pathways, were altered by cancer stem-like cells to mediate the therapeutic resistance, suggesting a combination of targeted agents [2]. These unique features of GBM tumors, such as their heterogeneity, treatment resistance, and ability to maintain the tumor after therapy, contribute to the high rate of treatment failure, poor prognosis, incomplete extent of resection, tumor progression, and recurrence.

The conventional therapies used for treating GBM patients include surgery, radiotherapy, and chemotherapy, with about two years of median survival. The treatment of GBM is maximal safe surgical resection followed by radiotherapy with concomitant daily Temozolomide and a further six cycles of maintenance Temozolomide. There are no randomized clinical trials to determine the extent of surgery and specifically investigate its survival benefit in the recurrent setting [3]. Radiotherapy remains a vital modality to improve both local control and survival. This approach has some disadvantages, such as the possibility of injury to the surrounding normal tissue and low-dose delivery to tumor cells away from the radiation site, which could lead to resistance [4]. Temozolomide also could cause toxicity, especially during the adjuvant therapy period. Besides, only patients with O^6-methylguanine-DNA methyltransferase (MGMT) promoter methylation are likely to benefit more from the drug. Most patients experience recurrence within six months after standard concurrent chemoradiation and adjuvant chemotherapy [3].

Precision oncology, including novel targeted therapeutics, has the potential to provide more effective treatments for this malignant tumor. Gene therapy, which introduces growth-regulating or tumor-suppressing genes into tumors, efficiently treats GBM with many distinct features. GBM tumors rarely spread outside the brain. Therefore, modern neurosurgical techniques and imaging models enable the injection of a vector for gene delivery [5].

In clinical trials, viral vectors such as retrovirus, adenovirus, and adeno-associated virus were the initial delivery vehicles for administering Glioma gene therapy. Suicide gene therapy, commonly used in viral gene therapy, is based on converting nontoxic prodrugs into lethal active compounds [6]. Cytosine deaminase(CD) converts 5-fluorocytosine (5FC) into the cytotoxic chemotherapeutic agent 5-fluorouracil (5-FU). Then, 5-FU converts to 5-fluorouridine monophosphate (5-FUMP) and 5-fluorodeoxyuridine monophosphate (5-FdUMP). These active metabolites are incorporated into RNA and DNA, interfering with the synthesis and function of these molecules in the cell. Besides, inhibiting thymidylate synthetase by 5-FdUMP leads to the depletion of thymidine 5′-monophosphate and thymidine 5′-triphosphate affecting DNA and RNA synthesis [7]. Human cells do not contain the cytosine deaminase enzyme, so, 5FC is safe in humans at pharmacological doses. The microflora of the gut could convert a small amount of 5FC into 5-FU, leading to drug toxicity [8]. Toca 511, which delivers the Cytosine Deaminase (CD) suicide gene in conjunction with the oral prodrug Toca FC (Flucytosine), induced tumor regression in individuals with recurrent high-grade Glioma in Phase I clinical trials [9]. Vocimagene Amiretrorepvec (Toca 511) and Flucytosine (Toca FC) were administered to 403 individuals with high-grade and recurrent Glioma in a recent randomized phase II/II clinical trial. However, the results did not improve overall survival and efficacy end-points compared to the standard of care [10].

Although suicide gene therapy has been effectively applied in a variety of *in vitro* and *in vivo* studies, it has not yet reached clinical significance in cancer patients. Suicide genes could be supplemented with microRNAs (miRNAs) to enhance the efficiency of gene therapy. MiRNAs are short, non-coding RNAs capable of regulating gene expression. The glial brain tumor pathogenesis could be better discovered by analyzing the basis of miRNA roles in the cell signaling networks and cellular regulation [11]. Many miRNAs revealed dysregulated expression in GBM, and these miRNAs operate as oncogenes or tumor suppressors. The hsa-miR-34 family, exceptionally hsa-miR-34a (miR-34a), has been proven to influence and act on almost the cancer progression process. The expression of miR-34a in high-grade Gliomas is much lower than in normal brain tissue; thus, the modulation of miR-34a expression seems to inhibit brain tumor malignancy [12,13]. The third out of all GBM gene therapy trials (11.7 %) is suicide gene therapy [14]. Many previous experimental studies reported the downregulation of miR-34a in tumor tissues and cell lines of most cancer types, such as GBM [15–17]. miR-34a expression profiles of GBM tissues and stem cells obtained from Gene Expression Omnibus (GEO) datasets displayed both overexpression and downregulation of this miR compared to normal brains. In recent studies, different vectors were used to overexpress miR-34a or CD gene alone in GBM tumor cells *in vitro* and *in vivo* such as retroviral vector [18], lentiviral vector [19], mesenchymal stem cells [20,21], nanogels [22], bacterial-derived nanocells [23], and polyglycerol-based polyplex [24].

The limited information about miR-34a expression status in human GBM enhances the significance of the *in vivo* experiences. Delivery of the CD suicide gene could enhance the therapeutic efficiency of microRNA. Our previous study confirmed the effectiveness of exosome-targeted combination therapy of miR-34a and CDA/5-FC suicide gene in U87MG and U87EGFRvIII GBM cells [25]. So, We employed experimental validation to investigate the apoptotic effect of lentiviral-delivered miR-34a alone and in combination with the CD antitumor suicide gene on U87MG cells *in vitro* and murine models of GBM *in vivo*.

2. Material and methods

2.1. In silico miRNA expression analysis

We used the GEO (Gene Expression Omnibus) datasets to analyze the expression of hsa-miR-34a in GBM. The hsa-miR-34a-5p target genes were obtained from the Targetscan database and analyzed by the Enrichr online functional annotation tool to investigate the pathways in which miR gene targets are involved. The genes related to the apoptosis pathway were collected from the KEGG pathway database and checked in DIANA-TarBase v7.0 for possible target sites of hsa-miR-34a-5p. RNA22 and Targetscan databases were used to evaluate and predict the target site sequence of hsa-miR-34a in selected genes.

2.2. Cell lines

U87MG, HEK-293T, and rat C6 cell lines were purchased (Iranian Biological Resource Center). The first passage of C6 and U87MG was used, and the passage number of HEK-293T cells at the beginning of the experiments was five. DMEM (Gibco) plus 10 % heat-inactivated fetal bovine serum (Sigma), 100 units/ml penicillin (Sigma), and 0.1 mg/ml streptomycin (Sigma) were used as the cell culture media. The cell culture was carried out by incubating the cell lines in air containing 5 % CO_2 at 37 °C.

2.3. Viral vectors and reagents

The CD suicide gene and has-miR-34a-5p were synthesized and cloned in a pCDH-GFP-Puro vector to prepare the lentiviral construct containing these genes. pMD2.G and psPAX2 lentivectors were purchased from System Bioscience. 5-Fluorocytosine for *in vivo* and *in vitro* experiments were purchased from Alfa Aesar (England).

2.4. Lentivirus production and cell infection

Briefly, the plasmid carrying the CD suicide gene, CD/miR-34a, miR-34a, and the packaging plasmids (psPAX2, pMD2.G) were co-transfected into HEK 293T cells using polyethyleneimine (Polysciences, USA). The cell supernatant containing lentivirus was collected every 12 h for 72 h. The supernatants were centrifuged at 1500 rpm for 5 min at 4 °C to remove cell debris, filter sterilized (0.2 μm filters), and stored at −80 °C until transduction. Then, rat C6 cell line or U87MG Glioma cells were transduced with proper lentivirus vectors. Since all vectors were positive for Green fluorescent protein (GFP), fluorescent microscopy indicates the efficiency of transduction.

2.5. 5FC IC50 (MTT cell proliferation assay)

Transduced GBM cells (U87MG and C6) expressing the CD suicide gene were seeded onto a 96-well plate with 70 % confluency. The next day, 5FC was added to each well in triplicate at a 10–500 μg/ml concentration range. After 72h, cell viability was measured by the MTT kit (Kowsar Biotech, Iran). IC50 was calculated using the non-linear regression formula, log (inhibitor) versus normalized response-variable slope.

2.6. Apoptosis assay

GBM cells were seeded onto a 6-well plate at a confluency of 1.5×10^5 cells/well and assigned to three groups of U87MG transduced with miR-34a CD/miR-34a, CD, and the scrambled control. After overnight incubation, CD and CD/miR-34a-expressing cells were treated with 150 μg/ml of 5FC every 24 h. After 72 h, cells were prepared by Annexin V- phosphatidylethanolamine (PE) and 7-minoactinomycin D (7-AAD) apoptosis kit (BD Biosciences).

2.7. Cell-cycle assay

The cell cycle was evaluated by flow cytometry. GBM cells were seeded onto a 6-well plate (1.5×10^5 cells/well). Cells were divided into three cell groups, namely U87MG cells transduced with miR-34a, cells transduced with CD/miR-34a, cells transduced with CD, and the scrambled control. After overnight incubation, CD and CD/miR-34a-expressing cells were treated with 150 μg/ml of 5FC every 24 h. Cells were collected, washed with PBS, transferred to a new tube of cold 70 % ethanol dropwise on vortex, and retained at −20 °C for 24 h. After the removal of ethanol by centrifugation, cells were rinsed with PBS. The pellets were dissolved in a solution containing 200 μl of propidium iodide (50 μg/ml) (Sigma), RNase (1 mg/ml) (Thermo Fisher Scientific), and Triton X-100 (Sigma) and incubated for 40 min at 37 °C in the dark. The cell cycle was evaluated by flow cytometry.

2.8. Quantitative real-time RT-PCR

The total RNA contents of transduced (miR-34a, CD/miR-34a, CD, and the scrambled control) GBM cell lines (U87MG and C6) were extracted using RNX-Plus (Sinaclon, Iran). Afterward, the extracted RNA samples were converted into complementary DNA (M-MuLV enzyme, Yektatajhiz, Iran). Random hexamer primers and specific primers were used to synthesis of the cDNA and evaluate target gene

expression related to h.SNORD47, R.SNORD, and HPRT1 internal controls. The relative expression levels of the target genes were analyzed using the SYBR Green master mix (Yektatajhiz, Iran). The qRT-PCR results were analyzed using the REST 2009 software. The primer sequences are listed in Table 1.

2.9. Implantation of C6 Glioma cells

Male Wistar rats weighing 200–250 g were anesthetized (100 mg/kg Ketamine and 5 mg/kg Xylazine), and C6 Glioma cells (1×10^6 cells in 10 μl of PBS) were stereotactically implanted into the right dorsolateral striatum (anterior 0.24 mm; lateral 3.4 mm; lateral depth 3.3 mm, according to the bregma). Rats were assigned to four groups. The control group received PBS; the scrambled control group received SNORD-transduced C6 cells, the miR-34a group received miR-34a-transduced C6 cells, and the CD/miR-34a group received CD/miR-34a-transduced C6 cells with 5FC treatment groups (each group contained four rats). One day after surgery, a group of rats implanted by transduced C6 cells received subcutaneous injections of 5-FC (30 mg/kg) daily for two weeks. The dose of 5FC was calculated based on the IC50 value; the drug was injected subcutaneously into the neck to reduce the dose and increase the drug release time. Twenty-eight days after cell implantation, the animals were euthanized. The results of the *in vitro* tests revealed that the cell proliferation inhibition and apoptotic induction of CD+5FC alone were equivalent to or less than those obtained from miR-34a, so we did not conduct the *in vivo* parts of this treatment.

2.10. MRI scanning

Intracranial tumor images were obtained from a 3 T MRI scanner (Siemens, MAGNETOM Prisma). A T_2-weighted spin-echo sequence measured all experiments through the following parameters: TR = 3580, 5550, TE = 107 and 103 ms, FOV = 35×6, and 28×50 mm^2. A slice thickness of 0.8 mm without a gap was chosen to cover the entire brain lesion, and the overall scan time was 13 min. The region of interest (ROI) of tumors in all slices related to each MRI image was manually set, and the Iran National Brain Mapping Laboratory measured the total tumor volume.

2.11. Histological analysis

The brain of sacrificed animals was removed, and the tumor growth of fixed brain tissues coronally sectioned (5 μm thickness) was tested by hematoxylin and eosin (H&E) staining.

2.12. Statistical analysis

The results are presented as the mean values ± standard deviation (SD). There were three biological replicates for each experiment. The Student T-test and one-way ANOVA (followed by Tukey's post-test) algorithms were used to compare the groups, and the obtained values were analyzed by GraphPad Prism software version 8 (GraphPad-Prism Software Inc., San Diego, CA). Differences were considered significant if the *p*-value was <0.05.

3. Results

3.1. In silico miRNA expression analysis

The downregulation of miR-34a in GBM has been reported previously, and this miR is considered a tumor suppressor [15–18].

Table 1
The list of primers used in this study.

hsa-miR-34a-5p	RT Primer	5′-GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCA CTGCATACGACACAACC
	Forward	5′-AGGGTGGCAGTGTCTTAGC
H.SNORD 47	RT Primer	5′-GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCA CTGCATACGACAACCTC
	Forward	5′-ATCACTGTAAAACCGTTCCA
R.SNORD	RT Primer	5′-GTCGTATGCAGTGCAGGGTCCGAGGTATTCGCAC TGCATACGACGCTGGC
	Forward	5′-CAGCTGAGGGTTTCTTTGA
Common Reverse primer (for all miRNAs)		5′-GAGCAGGGTCCGAGGT
HPRT1	Forward	5′-CCTGGCGTCGTGATTAGTG
	Reverse	5′-TCAGTCCTGTCCATAATTAGTCC
P53	Forward	5′-GGAGTATTTGGATGACAGAAAC
	Reverse	5′-GATTACCACTGGAGTCTTC
BCL2	Forward	5′-TAAGGCGGATTTGAATCTC
	Reverse	5′-ATAATAGGGATGGGCTCAAC
Caspase 7	Forward	5′-CACGGTTCCAGGCTATTAC
	Reverse	5′-GGCAACTCTGTCATTCACC
SOX 2	Forward	5′-GGACTGAGAGAAAGAAGAGGAG
	Reverse	5′-GAAAATCAGGCGAAGAATAAT

Besides, the expression of miR-34a in GBM cell lines and tumor cells was assessed using GEO DataSets. The search results of two microarray expression profiles showed significant overexpression of miR-34a in normal neural stem cells compared to brain Glioma stem cells and in GBM tumors grade 1 compared to GBM tumor grade 4. At the same time, two other profiles contained minimal expression values and demonstrated significant down-regulation of miR-34a in normal brain tissues compared to GBM tumor tissues (Fig. 1A). The bioinformatics data could not clarify the exact function of miR-34a in GBM tumor cells as a tumor suppressor gene or an oncomir. To elucidate the biological functions of miR-34a in GBM, we further examine the obtained data from previous experimental studies and online databases by conducting *in vitro* and *in vivo* experiments. The predicted target genes of has-miR-34a-5p obtained from the Targetscan database with context scores smaller than −0.4 were evaluated by Enricher through the KEGG pathways. The top forty pathways in which has-miR-34a-5p target genes were involved, including the apoptosis pathway, were presented in Fig. 1B. The apoptosis pathway-related genes were obtained from the KEGG pathway database, which includes 136 genes. The obtained apoptosis genes were searched in DIANA-TarBase v7.0 to evaluate the hsa-miR-34a-5p target site. Forty apoptosis-related genes had the target site from which seventeen genes were validated directly (Table 2). The obtained genes were further analyzed, and one tumor suppressor gene (*P53*), one oncogene (*Bcl2*), and one Caspase7 gene as one of the genes involved in both extrinsic and intrinsic apoptotic pathways as well as the crosstalk between them were selected for expression analysis. The *Sox2* gene was selected as a transcription factor that induces the differentiation of Glioma cells into induced Glioma stem cells and performs a fundamental role in maintaining these cells [26]. The predicted has-miR-34a-5p target sites in the 3′ untranslated regions (3′ UTRs) of genes involved in apoptotic pathways are depicted in Fig. 1C.

3.2. Lentivirus production and cell infection

The transduction effectiveness of all test groups (U87MG and C6 cells) was evaluated by fluorescent microscopy (Fig. 2A).

3.3. 5FC IC50 determination

The half-maximal inhibitory concentration (IC50) of 5-fluorocytosine (5-FC) was measured against U87MG and C6 cells expressing the CD suicide gene to determine the cytotoxicity of CD against Glioma cells. The MTT test results demonstrated that 5-FC is cytotoxic to both cell lines in a dose-dependent manner. As shown in Fig. 2B, the IC50 values of 5FC against C6(CD/miR-34a) and U87MG(CD/miR-34a) were reported to be 16.53 μg/ml and 124.6 μg/ml, respectively.

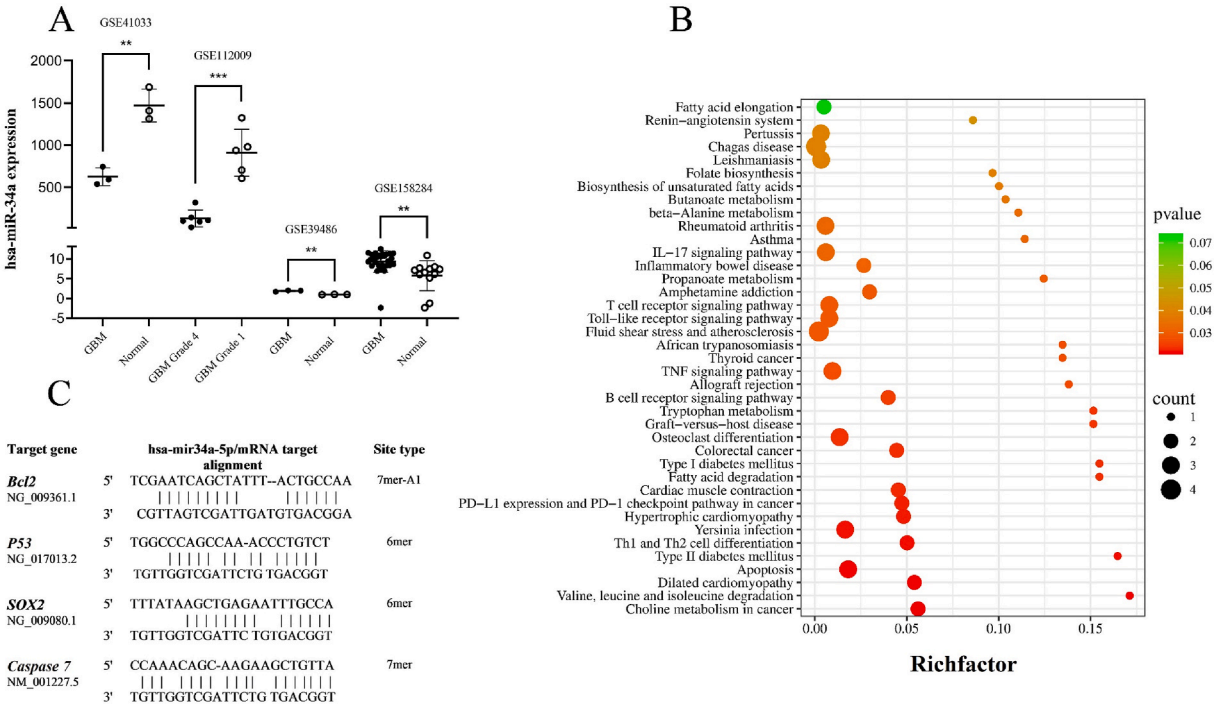

Fig. 1. A) Examination of the expression of miR-34a in Glioblastoma tumors and brain normal tissues in GEO datasets (microarray expression profiles, GSE 41033, 112009, 39486, 158284). **B)** The KEGG pathway enrichment bubble plot of hsa-miR-34a-5p target genes. The X-axis label represents the rich factor (rich factor = amount of differentially expressed genes enriched in the pathway/amount of all genes in the background gene set), and the Y-axis label represents the pathway. The size and color of the bubble represent the number of targeted genes enriched in the pathway. **C:** The proposed target sites of miR-34a in apoptosis pathway-related genes predicted by RNA22 and Targetscan databases. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
The apoptosis-related genes obtained from the KEGG pathway database, with miR-34a-5p target site according to DIANA-TarBase v7.0.

Gene Symbol	Gene Name	KEGG Entry	Method	Prediction score	Valid. type
CFLAR	CASP8 and FADD-like apoptosis regulator	[KO: K04724]	PAR-CLIP	–	Direct
CASP7	Caspase 7	[KO: K04397]	PAR-CLIP	–	Direct
PARP1	Poly(ADP-ribose) polymerase 1	[KO: K24070]	HITS-CLIP/PAR-CLIP	0.468	Direct
BCL2	BCL2 apoptosis regulator	[KO: K02161]	PAR-CLIP/Luciferase Reporter Assay/ Western Blot/qPCR	0.980	Direct
ITPR3	Inositol 1,4,5-trisphosphate receptor type 3	[KO: K04960]	HITS-CLIP	0.666	Direct
MCL1	MCL1 apoptosis regulator, BCL2 family member	[KO: K02539]	PAR-CLIP	–	Direct
ACTG1	Actin gamma 1	[KO: K05692]	PAR-CLIP	–	Direct
ACTB	Actin beta	[KO: K05692]	PAR-CLIP	–	Direct
TP53	Tumor protein P53	[KO: K04451]	PAR-CLIP/Biotin-Microarrays	0.502	Direct
PIDD1	P53-induced death domain protein 1	[KO: K10130]	PAR-CLIP	–	Direct
NFKBIA	NFKB inhibitor alpha	[KO: K04734]	PAR-CLIP	–	Direct
NFKB1	Nuclear factor kappa B subunit 1	[KO: K02580]	HITS-CLIP		Direct
PTPN13	Protein tyrosine phosphatase non-receptor type 13	[KO: K02374]	HITS-CLIP	0.476	Direct
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha	[KO: K00922]	HITS-CLIP	–	Direct
PIK3R2	Phosphoinositide-3-kinase regulatory subunit 2	[KO: K02649]	Luciferase Reporter Assay	–	Direct
RAF1	Raf-1 proto-oncogene, serine/threonine kinase	[KO: K04366]	PAR-CLIP	–	Direct
MAP2K1	Pitogen-activated protein kinase kinase 1	[KO: K04368]	Luciferase Reporter Assay	0.998	Direct

3.4. Quantitative real-time RT-PCR

The overexpression of miR-34a in U87MG and C6 cells was analyzed using qRT-PCR, and the fold increases in the expression levels were 38 and 146.5, respectively, compared to the control group (Fig. 2C). The expression levels of *P53* and *Caspase7* genes in U87MG cells transduced with lentivirus expressing miR-34a significantly increased by 1.263 and 3.123 folds, respectively, compared to the scrambled control. Besides, the mRNA levels of *SOX2* and *Bcl2* genes were almost completely inhibited. In U87MG cells transducted by the CD suicide gene and treated with 5-FC (from 48 h to 96 h after transfection), the *P53* gene was significantly increased by 9.572 folds, and the mRNA levels of *SOX2* and *Bcl2* genes were significantly inhibited. In U87MG cells expressing CD/miR-34a and treated with 5-FC (from 48 h to 96 h after transfection), the expression rates of *SOX2* and *Bcl2* genes were significantly reduced by 0.896 and 0.855 folds, respectively, when compared with the scrambled control group. Besides, the expression levels of *Caspase7* and *P53* genes were significantly increased by 2.734 and 1.294 folds, respectively, when compared with the control (Fig. 2D).

3.5. Apoptosis assay

A considerable rise in apoptosis rate was observed in U87MG cells treated with miR-34a, CD + 5FC, and CD/miR-34a + 5FC (Fig. 3A). As illustrated in Fig. 3B, the total apoptosis percentages (early and late apoptosis) of U87MG cells transduced with miR-34a, CD plus 5FC, or CD/miR-34a plus 5FC were reported to be 14.55 %, 29.05 %, and 37.5 %, respectively, compared to the scrambled control (2.6 %).

3.6. Cell-cycle assay

The number of cells in the G0+G1, S, and G2 phases was significantly different in the U87MG cell line transduced with CD/miR-34a lentivirus, which received 5-FC compared to U87MG cells transduced with scrambled lentivirus. The flow cytometry results showed an increase in the G0+G1 phase by 22.28 % and a decrease of 18.27 % and 6.81 % in the S and G2 phases, respectively. The U87MG cell line transduced with miR-34a lentivirus revealed a significant decrease in the S phase (5.07 %) compared to the U87MG cell line transduced with scrambled lentivirus. The U87MG cells expressing CD plus 5FC alone demonstrated a significant increase in the G0+G1 phase by 22.04 % and a decrease of 12.82 % in the G2 phase compared to the scrambled control (Fig. 3C and D).

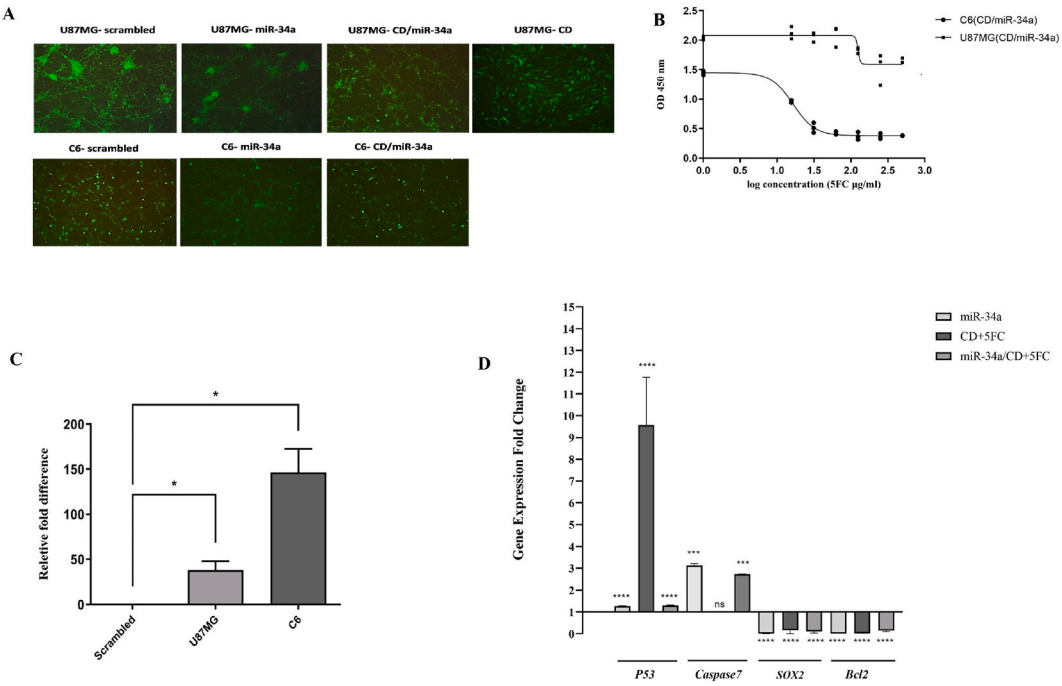

Fig. 2. **A)** The efficiency of transduction by fluorescent microscopy of GFP for miR-34a, CD, CD/miR-34a, and scrambled viruses in the U87MG and C6 cell lines. **B)** IC50 determination of 5FC against C6 and U87MG cells expressing CD. The optical density (OD) at 450 nm was measured using a microplate reader. **C)** Overexpression and relative fold change of miR-34a were confirmed by Real-Time qRT-PCR in comparison with the scrambled group. **D)** Gene expression investigated by real-time PCR in U-87MG cells 72 h after transduction with miR-34a, CD, and CD/miR-34a with 5 EC treatment and scrambled viruses. *p ≤ 0.05, ***p ≤ 0.0005, ****p ≤ 0.0001.

3.7. MRI scanning

As depicted in Fig. 4C, tumor growth was significantly inhibited in rats receiving miR-34a-expressing C6 cells compared to the control group receiving scrambled C6 cells (Fig. 4B). The CD/miR-34a-mediated inhibitory effect was significantly enhanced with 5FC prodrug treatment (Fig. 4D). The tumor volume in rats receiving miR-34a-expressing C6 cells decreased by 79.8 %, while its reduction reached 94.39 % in those receiving 5FC prodrug compared to control (scrambled C6 cells). The obtained results (Fig. 4E) demonstrated that the tumor volume was significantly lower in rats receiving CD/miR-34a-expressing C6 tumor+5FC than those only receiving miR-34a-expressing C6 cells.

3.8. Histological analysis

H&E staining indicated a typical anaplastic Glioma with increased cell number and mitotic activity in the tumor mass of the control group (scrambled C6 cells, Fig. 4F), which showed high cellularity and atypical changes in nuclei. The size of the tumor, cell number, and the atypical changes in nuclei were significantly decreased in miR-34a-transduced C6 tumor cells (Fig. 4G) compared to scrambled C6 cells. Tumor cells transduced with CD/miR-34a and treated daily with 5FC had no tumor area or, if present, a minimal area with minimal cellularity and scar-like necrotic margins (Fig. 4H).

4. Discussion

In preclinical [27–29] and clinical GBM research (NCT02015819, NCT01470794, NCT04657315), the combination of the Cytosine Deaminase (CD) suicide gene with prodrug treatment, which is capable of converting nontoxic prodrugs to toxic components in tumor cells, has displayed promising outcomes. 5-FU has been demonstrated to inhibit thymidylate synthase or to act as the false bases in RNA and DNA, causing the death of proliferating and dividing cells in tumors [30]. Besides, the tumor-suppressing capability of miR-34a is described in some cancers, including GBM. It has been reported that the miR-34a family can function downstream of the P53 pathway as tumor suppressors. miR-34a has several target oncogenes, including *c-Met*, *Notch-1*, *Notch-2*, *CDK6*, *PDGFRA* and *EGFR*. Therefore, miR-34a can potentially inhibit brain tumor malignancy [18,31]. Considering the tumor heterogeneity, Glioma stem cells, the blood-brain barrier, DNA damage repair mechanisms, and drug efflux pumps, combined therapeutic strategies have shown increasing benefits in GBM treatment. Besides, different approaches have certain strengths and weaknesses so that the combination could maximize therapeutic efficacy. The efficacy of CD suicide gene therapy against GBM [32,33] and miR34a anti-GBM ability [18,

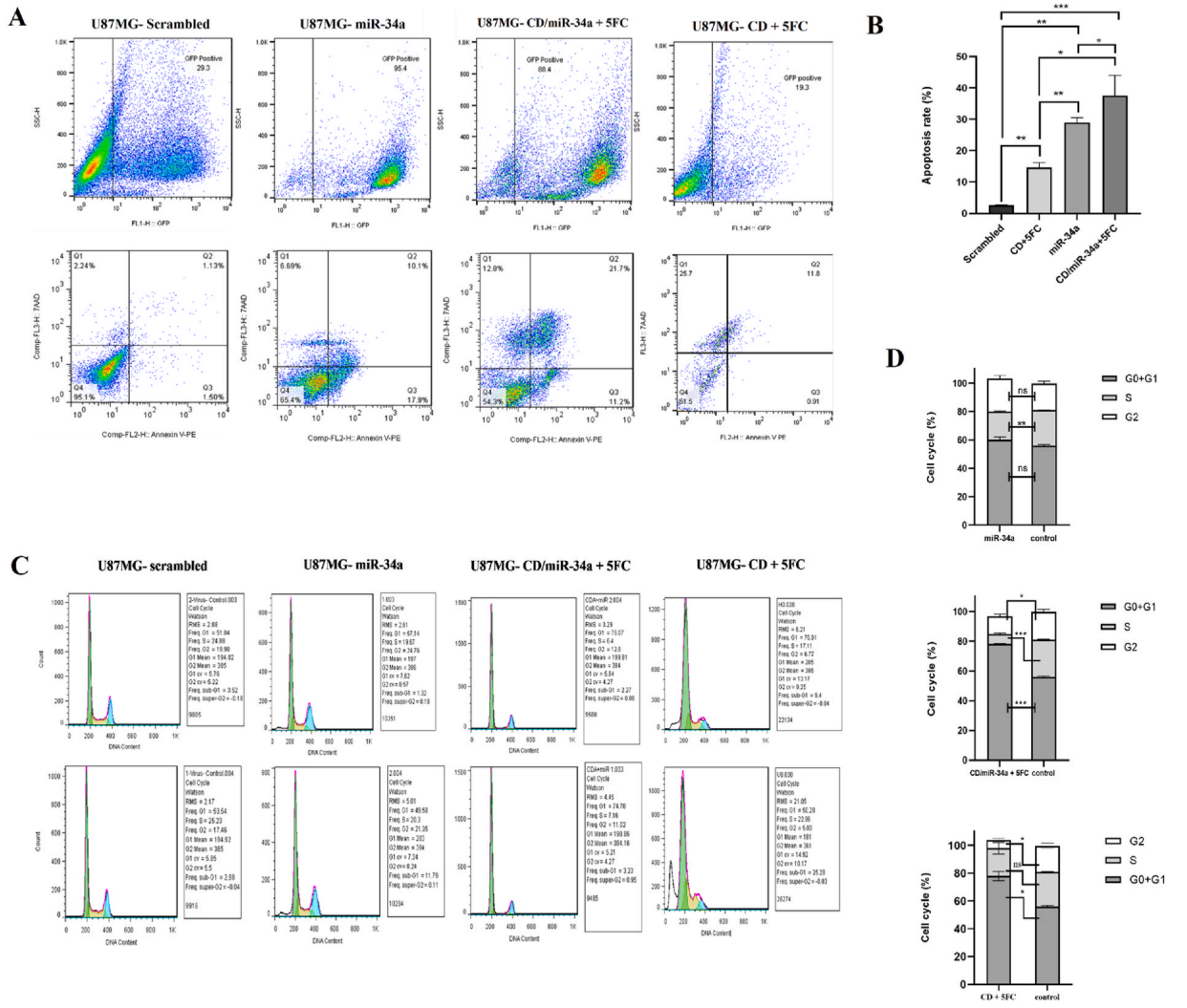

Fig. 3. A) The apoptosis rate of U87MG cells (miR-34a, CD +5FC, CD/miR-34a+5FC, and scrambled) evaluated by flow cytometry. The GFP-positive cells were subjected to Annexin (PE) and 7-AAD analysis. **B)** The total apoptosis rate of U87MG cells transduced by miR-34a, CD + 5FC, and CD/miR-34a + 5FC treatment increased significantly in comparison to scrambled control. **C)** The cell cycle diagrams of U87MG cells containing miR-34a, CD + 5FC, and CD/miR-34a with 5FC treatment compared to scrambled control. **D)** The percentage of each cell cycle phase is represented. *$p \leq 0.05$, **$p \leq 0.005$ and ***$p \leq 0.0005$, ns: non significant.

34] were reported. This study aimed to estimate the simultaneous use of miR-34a and the CD suicide gene to induce apoptosis and hinder the proliferation of GBM cells *in vitro* and *in vivo*.

The human-derived U87MG cell line was used to evaluate the impact of lentiviral transduction of miR-34a, CD, and CD/miR-34a along with 5FC prodrug treatment. After obtaining positive results of tumor inhibition in the *in vitro* experiments, the C6 cell line was intracranially administrated to rats to evaluate the *in vivo* antitumor effect of miR-34a and CD/miR-34a. An appropriate and experimental rodent Glioma model could be developed using the C6 rat glioma cell line. C6 cells could simulate human GBM when injected into the rats' brains. The implantation of C6 cells in Wistar rat brains reveals similar histological features to human GBM, including a high mitotic index and nuclear polymorphism. Besides, the immune microenvironment of C6 Gliomas resembles that of a human GBM [35].

The flow cytometry analysis demonstrated that miR-34a inhibited the proliferation while increasing apoptosis in U87MG cells in the presence or absence of the toxigenic activity of the CD suicide gene. The CD suicide gene alone also caused the same inhibition on cell proliferation and induced apoptosis in U87MG cells after 5FC treatment. Previous research on Glioma cell lines [36–38] and animal models [31] confirmed the apoptotic outcome of miR-34a overexpression on cell proliferation, survival, and invasion. Adding the CD suicide gene to miR-34a increased the apoptosis rate in U87MG cells.

Other studies also investigated the antitumor effect of CD in combination with different antitumor agents, including thymidine kinase [39,40], Temozolomide [28], and TNF-related apoptosis-inducing ligand (TRAIL) [41], all of which led to increased efficacy of

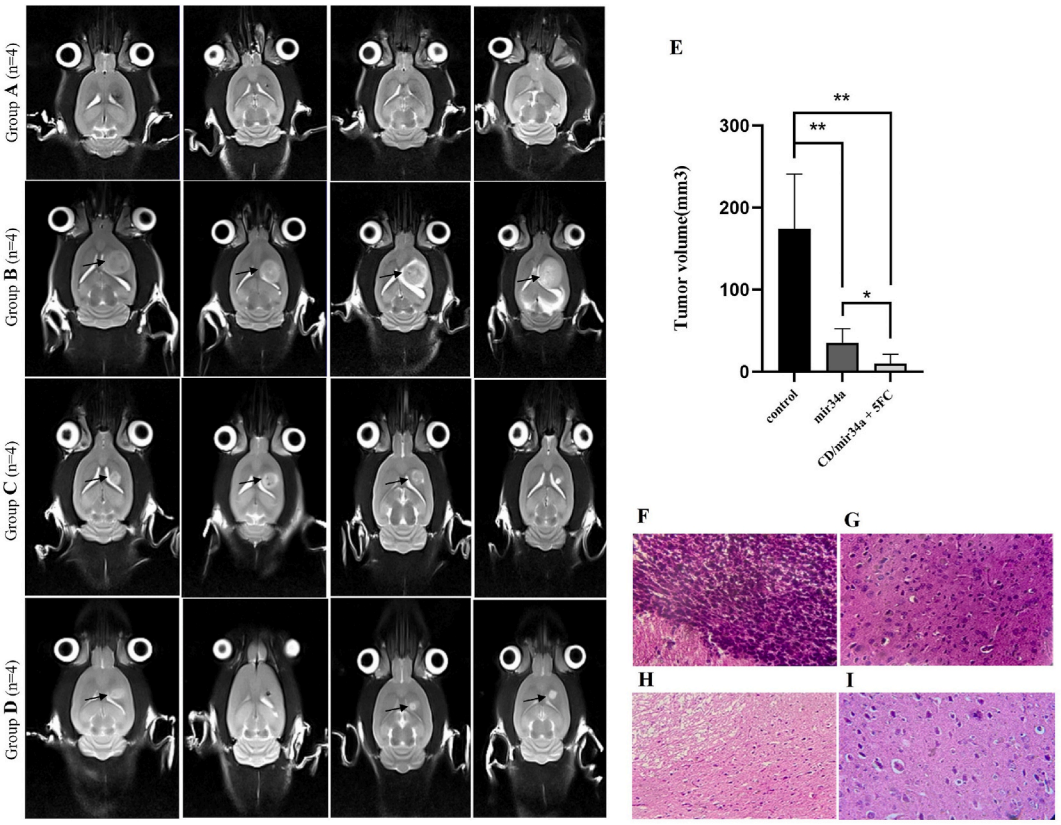

Fig. 4. The Intracranial tumor growth of glioma rat models (Tumors were indicated by arrows). **A)** Intracranial tumor growth of the PBS injection (negative control). **B)** Intracranial tumor growth of the C6 scrambled transduced injection (positive control). **C)** Intracranial tumor growth of the C6 miR-34a transduced injection. **D)** Intracranial tumor growth of the C6 CD/miR-34a transduced injection with 5FC treatment. **E)** Intracranial tumor size of miR-34a transduced C6 cells and CD/miR-34a transduced C6 cells with 5FC treatment in comparison with scrambled transduced control. **F)** H&E staining of the tumor section indicated high cellularity in the scrambled transduced C6 cells control group. **G)** The tumor size and cellularity were reduced by miR-34a transduction. **H)** CD/miR-34a+5FC induced the smallest tumor size or no tumor growth. **I)** The normal brain tissue in the negative control (PBS injection). *$p \leq 0.05$, **$p \leq 0.005$.

apoptosis induction *in vitro* and *in vivo*. To our knowledge, no study on the combined use of miRNA and the CD suicide gene against Glioma has been published. Our research is the first to describe CD/miR-34a fusion construct for inhibiting GBM tumors. Real-time PCR demonstrated that miR-34a, alone or in combination with Cytosine Deaminase, may target genes involved in apoptosis and the cell cycle of U87MG cells. The overexpression of miR-34a increased the expression level of the *P53* gene in all cell groups (miR-34a, CD+5FC, and CD/miR-34a+5FC). Despite the presence of miR-34a target site in 3'UTR of *P53*, overexpression of miRNA not only did not decrease the expression of this gene but also increased it. There is a complex interaction between the *P53* gene and miR-34a. MiR-34a positively affects *P53* transcription and protein stability by targeting multiple *P53* inhibitor genes. One identified pathway is the SIRT1(silent information regulator 1)-dependent pathway. Through binding to the 3′ UTR of SIRT1, miR-34a inhibits SIRT1 expression. The inhibition of SIRT1 increases the level of acetylated P53, which regulates the apoptosis and cell cycle. There is a positive feedback loop in which P53 induces the expression of miR-34a, which activates the *P53* gene by inhibiting *SIRT1* [42–45]. A study by Gao et al. revealed that p53 luciferase reporter activity in HCT116 cells (human colorectal carcinoma cell line) was increased by miR-34a-5p. Besides, p53 expression in primary colorectal cancer tumor tissues was positively connected with miR-34a-5p expression. Patients with p53-positive expression had a higher degree of miR-34a-5p than those with p53-negative expression [46].

B-cell lymphoma 2 (*Bcl2*) is another target gene of miR-34a. The *Bcl2* gene encodes an integral outer mitochondrial membrane protein that hinders the apoptosis pathway and cell death. MiR-34a is a tumor suppressor in U87 Glioma cells, inhibiting cell growth and triggering death via targeting *Bcl2* [37].

The *P53–miR-34a–Bcl2* axis is an accepted pathway in the stimulation of apoptosis in cancer cells. Based on the Bommer et al. report, miR-34a suppresses the luciferase reporter activity of a construct fused with the 3′-UTR of *Bcl-2* [47], and subsequent reports confirmed that miR-34a could directly target *BCL*-2 mRNA, which promotes the induction of cancer cell apoptosis [48–50]. Our results also demonstrated a significant decrease in the expression level of the *Bcl2* gene in U87MG cells (miR-34a, CD+5FC, and CD/miR-34a+5FC). Fischer et al. reported that in U87 cells, apoptosis was efficiently induced by exposure to 5-FC with a weak P53 response. 5-FC-induced cell death was independent of death receptors but accompanied by mitochondrial alterations. Also, 5-FC caused a decline in the level of Bcl-2 [51]. Our obtained data also demonstrated a significant increase in *P53* and a significant

decrease in *Bcl2* mRNA level in CD-expressing U87MG cells after 5FC treatment. In addition, the *Caspase7* gene was upregulated in U87MG cells transduced with miR-34a, which could lead to the inhibition of U87MG cell proliferation by miR-34a through the induction of caspase-dependent apoptosis [37]. Gao et al. showed that the caspase-7 protein level was increased in the miR-34a-5p transfected HCT116 cells, and miR-34a-5p gave rise to apoptosis by activating the caspase-dependent pathway [46]. MiR-34a might not directly target the *Caspase7* gene. It was found that miR-34a acts as a tumor suppressor by targeting proteins, such as SNAI1(snail), involved in apoptosis, proliferation, metastasis, and stemness. SNAI1 is a direct target of miR-34a, as confirmed by the luciferase reporter assay. SNAI1 mRNA and protein were down-regulated in ES-2 cells transfected with miR-34a mimic [52]. Aida et al. indicated that apigenin induces apoptosis through the miR-34a-5p/SNAI1 pathway in A549 cells. Down-regulation of SNAI1 mRNA by miR-34a-5p increased Caspase-3/7 activity [53]. In another study, Western blot results showed that inhibiting SNAI1 promoted the Caspase 3 and Caspase 7 levels [54]. These findings proposed that the overexpression of miR-34a decreased SNAI1 expression, leading to the cell's elevation of the Caspase 7 level.

Consistent with a previous study performed on head and neck squamous cell carcinoma stem cells [55], the overexpression of miR-34a decreased the expression level of stemness-related transcription factor *SOX2* gene in U87MG cell groups in the absence and presence of the CD suicide gene. In another study, the mRNA level of Sox2 of breast carcinoma cells exposed to cRGD-PEG/miR-34a liposomes was significantly decreased [56]. Choi et al. reported that miR-34a directly targets *Sox2*, and this gene was repressed by miR-34a in *Dicer*-deficient HCT116 cells, which was confirmed by luciferase reporter assay [57]. Sox2 is a crucial transcription factor in maintaining stemness characteristics, including pluripotency and self-renewal of cancer cells and human embryonic stem cells [55]. The proposed schematic mechanism of apoptosis induction of miR-34a and CD is represented in Fig. 5.

Previous studies suggest that miR-34a could induce cell cycle arrest [58], and this miRNA has potential target genes that regular cell cycle, including *MET*, *CDK4*, *CDK6*, *CCND1*, *CCNE2*, and *NMYC*. It has been revealed that miR-34a could significantly reduce cells in the S phase of the cell cycle and a rise in the G0/G1 phase [59]. Our flow cytometry data related to cell cycle analysis demonstrated that miR-34a, CD, and their combination significantly inhibited the cell cycle and increased the apoptosis rate.

We investigated the potential therapeutic impact of the CD and miR-34a on Glioma rat models after obtaining the *in vitro* antitumor effect mediated by the mir/suicide gene. The *in vivo* results of MRI and histopathological analyses revealed that the tumor growth in cancer cells-expressing miR-34a or CD/miR-34a plus 5FC was significantly inhibited. Cells expressing CD/miR-34a in combination with 5FC treatment had the most extensive tumor growth regression and almost total tumor suppression, consistent with an improved Glioma cell apoptotic impact *in vitro*. Moreover, there was a significant difference between the tumor inhibition rate of cells transduced with CD/miR-34a and treated with 5FC and those transduced with miR-34a. Daily administration of 5FC to rats amplifies the cytotoxic impact on tumor development. Furthermore, compared to earlier research, a lower dosage of 5FC administered subcutaneously almost entirely inhibited tumor growth in rats.

Despite promising results from both *in vitro* and *in vivo* experiments, there are some issues regarding CD/miR-34a gene therapy in patients with GBM. In this study, the C6 cell line was first transduced with CD/miR-34a and then administered to the brains of rats to induce a murine model of GBM and assess the therapeutic effect of CD/miR-34a. For the successful delivery of the miR-34a/CD suicide gene to the brain tumor cells of patients, an effective administration route is required. Recent research applied different vehicles for gene delivery of the CD suicide gene, such as viral vectors [29,60,61] and mesenchymal stem cells [28,62,63]. Using a lentiviral delivery system leads to the transduction of all kinds of cells, including tumor cells and the normal cells of the same tissue or even normal cells of other tissues exposed to the vector. Specific targeting of suicide gene therapies along with other therapeutical agents such as miRNAs will lower side effects and increase the therapeutic efficacy. The desired suicide gene expression, controlled by a tissue-specific cellular promoter, provides transcriptional targeting. This method was used against Glioma by developing a baculoviral vector containing the herpes simplex thymidine kinase gene, miR-31, miR127, and miR-143 [64]. In a similar study, HSVtk gene expression was enhanced in GBM cells by specific control of Epo enhancer responds to the core tumor low-oxygen concentration and elevates gene expression along with Nestin intron 2 (NI2), which increases gene expression in neural stem cells or GBM [65].

Combination therapy, which combines two or more therapeutic agents, is a cornerstone of cancer therapy. The combination may include radiation therapy, chemotherapy, immunotherapy, and gene therapy. We used non-coding RNA therapy with suicide gene therapy.

After the successful transport of the miR-34a/CD suicide gene, there must be an efficient way to deliver the prodrug to the tumor location. When treating patients with brain tumors with chemotherapy, some of the most significant challenges are the inability of the medicine to penetrate the blood-brain barrier (BBB) and reach the tumor, as well as the severe side effects caused by the chemicals. Gene therapy for suicidal CD prevents the toxic side effects of systemic 5-FU administration. In addition, the 5FC prodrug does not bind to plasma proteins and has excellent penetration into the BBB and cerebrospinal fluid (CSF) [66]. The patients with high-grade Glioma underwent a 4-day intracranial injection of a CD suicide gene in a phase I clinical trial, and they received oral 5-FC from 75 to 150 mg/kg/day. No toxicity and histological indications of tumorigenesis were detected [67]. The carrier dosage and/or prodrug delivery schedule are two potential strategies to improve gene therapy, both of which are critical elements in the complete elimination of tumor burden.

5. Conclusions

The present study demonstrated the efficacy of CD suicide gene/miR-34a gene therapy to induce apoptosis in Glioma cancer cells and confirmed the function of miR-34a as a tumor suppressor in GBM. The overexpression of miR-34a leads to significant apoptosis induction in GBM cells by down-regulation of *Bcl-2* gene and inhibitors of the *P53* gene. The CD suicide gene plus 5FC alone exhibited lower apoptosis inhibition to miR-34a alone. The CD/5FC treatment increased p53 transcriptional activity, triggering cell cycle arrest

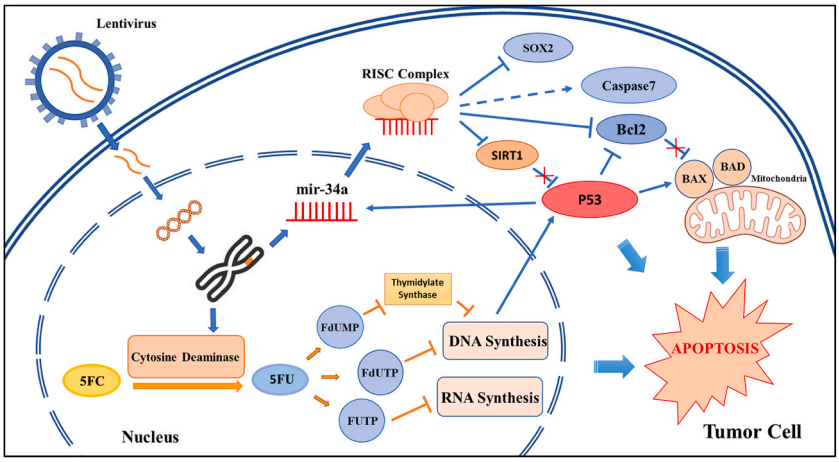

Fig. 5. A schematic representation of the mechanism of apoptosis induction in tumor cells by the simultaneous use of miR-34a and CD. The RNA coding for miR-34a and CD enters the cytoplasm of the tumor cell by the lentiviral vector and is converted into DNA through reverse transcription. The synthesized DNA arrives in the nucleus and is inserted into the chromosome by the virus integrase enzyme. After the process of transcription and translation, the produced CD enzyme causes the conversion of 5FC to 5FU. By converting to FdUMP, 5FU inhibits thymidylate synthase enzyme activity and, thus, DNA synthesis. The conversion of 5FU to FdUTP generates errors in DNA synthesis, and the conversion to FUTP causes errors in RNA synthesis. Errors and damage of DNA and RNA lead to the activation of P53. The transcribed miR-34a exits the nucleus and is placed in the RISC complex which induces a decrease in the expression of SOX2 and Bcl2 as well as an indirect increase in the expression of Caspase7. Inhibition of Bcl2 induces the mitochondrial apoptotic pathway. Also, inhibition of SIRT1 leads to increased production of P53. Through a positive feedback loop, P53 enhances the transcription of miR-34a. The increased activation and production of P53 through both miR-34a and CD pathways as well as inhibition of Bcl2, induces apoptosis in tumor cells.

and apoptosis. These two agents have a synergistic effect in apoptosis induction, and the combined treatment of the CD suicide gene and miR-34a induced a higher apoptotic effect than each treatment alone. The *in vitro* results were confirmed by *in vivo* experiments in which miR-34a alone and combined with CD significantly inhibited the tumor growth of rat animal models of GBM. Our data provide a novel and effective GBM antitumor treatment that could be achieved using CD and miR-34a gene therapy. Given that patients with GBM have an inferior prognosis, this combination treatment could increase patient survival. However, further *in vivo* and *in vitro* research would be beneficial to establish the precise technique, optimum dose, and safety of this therapeutic approach.

Data availability statement

The data utilized in this study have been archived and are accessible upon request by the corresponding author.

Ethics approval and consent to participate

The International/national guidelines for the care and use of animals were followed. Animal research was carried out according to the experimental protocol approved by the Ethics in Biomedical Research Committee (NO 942974).

CRediT authorship contribution statement

Zahra Fekrirad: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Milad Gharedaghi:** Investigation. **Fatemeh Saadatpour:** Methodology, Investigation. **Zahra Asghari Molabashi:** Investigation. **Ameneh Rezayof:** Supervision. **Alireza Korourian:** Validation, Methodology. **Masoud Soleimani:** Supervision, Conceptualization. **Ehsan Arefian:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank the Iranian National Brain Mapping Laboratory (NBML) for providing data acquisition services for this research. The authors would like to acknowledge the Iranian Council for Development of Stem Cell Sciences and Technologies and the National Network of Animal Modeling and *in vivo* Research for their financial support of this work.

References

[1] G. Hutóczki, J. Virga, Z. Birkó, A. Klekner, Novel concepts of glioblastoma therapy concerning its heterogeneity, Int. J. Mol. Sci. 22 (18) (2021) 10005.
[2] J.D. Lathia, S.C. Mack, E.E. Mulkearns-Hubert, C.L. Valentim, J.N. Rich, Cancer stem cells in glioblastoma, Genes Dev. 29 (12) (2015) 1203–1217.
[3] A.C. Tan, D.M. Ashley, G.Y. López, M. Malinzak, H.S. Friedman, M. Khasraw, Management of glioblastoma: state of the art and future directions, Ca - Cancer J. Clin. 70 (4) (2020) 299–312.
[4] D. Kwatra, A. Venugopal, S. Anant, Nanoparticles in radiation therapy: a summary of various approaches to enhance radiosensitization in cancer, Transl. Cancer Res. 2 (4) (2013) 330–342.
[5] C. Manikandan, A. Kaushik, D. Sen, Viral vector: potential therapeutic for glioblastoma multiforme, Cancer Gene Ther. 27 (5) (2020) 270–279.
[6] B. Caffery, J.S. Lee, A.A. Alexander-Bryant, Vectors for glioblastoma gene therapy: viral & non-viral delivery strategies, Nanomaterials 9 (1) (2019) 105.
[7] J. Béguin, M. Kohlhauer, E. Laloy, F. Degorce, B. Moreau, É. Quéméneur, et al., Pharmacokinetics and tolerance of repeated oral administration of 5-fluorocytosine in healthy dogs, BMC Vet. Res. 17 (1) (2021) 220.
[8] L.S.M. Sigera, D.W. Denning, Flucytosine and its clinical usage, Therapeutic advances in infectious disease 10 (2023) 20499361231161387.
[9] B.D. Philbrick, D.C. Adamson, Early clinical trials of Toca 511 and Toca FC show a promising novel treatment for recurrent malignant glioma, Expert Opin Investig 28 (3) (2019) 207–216.
[10] T.F. Cloughesy, K. Petrecca, T. Walbert, N. Butowski, M. Salacz, J. Perry, et al., Effect of vocimagene amiretrorepvec in combination with flucytosine vs standard of care on survival following tumor resection in patients with recurrent high-grade glioma: a randomized clinical trial, JAMA Oncol. 6 (12) (2020) 1939–1946.
[11] S.S. Elshaer, A.I. Abulsoud, D. Fathi, N.M. Abdelmaksoud, M.B. Zaki, H.A. El-Mahdy, et al., miRNAs role in glioblastoma pathogenesis and targeted therapy: signaling pathways interplay, Pathol. Res. Pract. (2023) 154511.
[12] S. Ghaemi, Z. Fekrirad, N. Zamani, R. Rahmani, E. Arefian, Non-coding RNAs enhance the apoptosis efficacy of therapeutic agents used for the treatment of glioblastoma multiform, J. Drug Target. (2022) 1–14.
[13] S. Li, X. Wei, J. He, Q. Cao, D. Du, X. Zhan, et al., The comprehensive landscape of miR-34a in cancer research, Cancer Metastasis Rev. 40 (3) (2021) 925–948.
[14] M. Rayati, V. Mansouri, N. Ahmadbeigi, Gene therapy in glioblastoma multiforme: can it be a role changer? Heliyon. 10 (5) (2024) e27087.
[15] E.A. Toraih, N.M. Aly, H.Y. Abdallah, S.A. Al-Qahtani, A.A. Shaalan, M.H. Hussein, et al., MicroRNA–target cross-talks: key players in glioblastoma multiforme, Tumor Biol. 39 (11) (2017) 1010428317726842.
[16] Y. Zhang, N. Cruickshanks, M. Pahuski, F. Yuan, A. Dutta, D. Schiff, et al., Noncoding RNAs in Glioblastoma, Exon Publications, 2017, pp. 95–130.
[17] M. Chen, Z. Medarova, A. Moore, Role of microRNAs in glioblastoma, Oncotarget 12 (17) (2021) 1707.
[18] D. Yin, S. Ogawa, N. Kawamata, A. Leiter, M. Ham, D. Li, et al., miR-34a functions as a tumor suppressor modulating EGFR in glioblastoma multiforme, Oncogene 32 (9) (2013) 1155–1163.
[19] R. Mi, W. Fan, J. Ji, C. Dong, G. Jin, F. Liu, The enhanced efficacy of herpes simplex virus by lentivirus mediated VP22 and cytosine deaminase gene therapy against glioma, Brain Res. 1743 (2020) 146898.
[20] Q. Li, C. Wang, L. Cai, J. Lu, Z. Zhu, C. Wang, et al., miR-34a derived from mesenchymal stem cells stimulates senescence in glioma cells by inducing DNA damage, Mol. Med. Rep. 19 (3) (2019) 1849–1857.
[21] J.H. Jung, A.A. Kim, D.-Y. Chang, Y.R. Park, H. Suh-Kim, S.-S. Kim, Three-dimensional assessment of bystander effects of mesenchymal stem cells carrying a cytosine deaminase gene on glioma cells, Am. J. Cancer Res. 5 (9) (2015) 2686.
[22] Z. Shatsberg, X. Zhang, P. Ofek, S. Malhotra, A. Krivitsky, A. Scomparin, et al., Functionalized nanogels carrying an anticancer microRNA for glioblastoma therapy, J. Contr. Release 239 (2016) 159–168.
[23] M.B. Khan, R. Ruggieri, E. Jamil, N.L. Tran, C. Gonzalez, N. Mugridge, et al., Nanocell-mediated delivery of miR-34a counteracts temozolomide resistance in glioblastoma, Mol. Med. 27 (2021) 1–17.
[24] P. Ofek, M. Calderón, F.S. Mehrabadi, A. Krivitsky, S. Ferber, G. Tiram, et al., Restoring the oncosuppressor activity of microRNA-34a in glioblastoma using a polyglycerol-based polyplex, Nanomed. Nanotechnol. Biol. Med. 12 (7) (2016) 2201–2214.
[25] R. Rahmani, J. Kiani, W.Y. Tong, M. Soleimani, N.H. Voelcker, E. Arefian, Engineered anti-EGFRvIII targeted exosomes induce apoptosis in glioblastoma multiforme, J. Drug Target. 31 (3) (2023) 310–319.
[26] L. Garros-Regulez, I. Garcia, E. Carrasco-Garcia, A. Lantero, P. Aldaz, L. Moreno-Cugnon, et al., Targeting SOX2 as a therapeutic strategy in glioblastoma, Front. Oncol. 6 (2016) 222.
[27] T.T. Huang, S. Parab, R. Burnett, O. Diago, D. Ostertag, F.M. Hofman, et al., Intravenous administration of retroviral replicating vector, Toca 511, demonstrates therapeutic efficacy in orthotopic immune-competent mouse glioma model, Hum. Gene Ther. 26 (2) (2015) 82–93.
[28] D.-Y. Chang, J.-H. Jung, A.A. Kim, S. Marasini, Y.J. Lee, S.H. Paek, et al., Combined effects of mesenchymal stem cells carrying cytosine deaminase gene with 5-fluorocytosine and temozolomide in orthotopic glioma model, Am. J. Cancer Res. 10 (5) (2020) 1429.
[29] S.-H. Chen, J.-M. Sun, B.-M. Chen, S.-C. Lin, H.-F. Chang, S. Collins, et al., Efficient prodrug activator gene therapy by retroviral replicating vectors prolongs survival in an immune-competent intracerebral glioma model, Int. J. Mol. Sci. 21 (4) (2020) 1433.
[30] G.C. Ireton, G. McDermott, M.E. Black, B.L. Stoddard, The structure of Escherichia coli cytosine deaminase, J. Mol. Biol. 315 (4) (2002) 687–697.
[31] Y. Li, F. Guessous, Y. Zhang, C. DiPierro, B. Kefas, E. Johnson, et al., MicroRNA-34a inhibits glioblastoma growth by targeting multiple oncogenes, Cancer Res. 69 (19) (2009) 7569–7576.
[32] S. Fei, X. Qi, S. Kedong, J. Guangchun, L. Jian, Q. Wei, The antitumor effect of mesenchymal stem cells transduced with a lentiviral vector expressing cytosine deaminase in a rat glioma model, J. Cancer Res. Clin. Oncol. 138 (2012) 347–357.
[33] L. Wang, Y. Yuan, S. Lin, D. Cheng, X. Wang, Q. Jiang, et al., Co-delivery of 5-fluorocytosine and cytosine deaminase into glioma cells mediated by an intracellular environment-responsive nanovesicle, Polym. Chem. 5 (15) (2014) 4542–4552.
[34] W.-B. Li, M.-W. Ma, L.-J. Dong, F. Wang, L.-X. Chen, X.-R. Li, MicroRNA-34a targets notch1 and inhibits cell proliferation in glioblastoma multiforme, Cancer Biol. Ther. 12 (6) (2011) 477–483.
[35] D. Giakoumettis, A. Kritis, N. Foroglou, C6 cell line: the gold standard in glioma research, Hippokratia 22 (3) (2018) 105.
[36] F. Guessous, Y. Zhang, A. Kofman, A. Catania, Y. Li, D. Schiff, et al., microRNA-34a is tumor suppressive in brain tumors and glioma stem cells, Cell Cycle 9 (6) (2010) 1031–1036.
[37] J. Duan, K. Zhou, X. Tang, J. Duan, L. Zhao, MicroRNA-34a inhibits cell proliferation and induces cell apoptosis of glioma cells via targeting of Bcl-2, Mol. Med. Rep. 14 (1) (2016) 432–438.
[38] S.-Z. Li, Y.-Y. Hu, J. Zhao, Y.-B. Zhao, J.-D. Sun, Y-f Yang, et al., MicroRNA-34a induces apoptosis in the human glioma cell line, A172, through enhanced ROS production and NOX2 expression, Biochem. Biophys. Res. Commun. 444 (1) (2014) 6–12.
[39] M. Lee, Y.-S. Kim, K. Lee, M. Kang, H. Shin, J.-W. Oh, et al., Novel semi-replicative retroviral vector mediated double suicide gene transfer enhances antitumor effects in patient-derived glioblastoma models, Cancers 11 (8) (2019) 1090.
[40] J. Niu, C. Xing, C. Yan, H. Liu, Y. Cui, H. Peng, et al., Lentivirus-mediated CD/TK fusion gene transfection neural stem cell therapy for C6 glioblastoma, Tumor Biol. 34 (6) (2013) 3731–3741.
[41] B. Liang, M.-L. He, C-y Chan, Y-c Chen, X.-P. Li, Y. Li, et al., The use of folate-PEG-grafted-hybranched-PEI nonviral vector for the inhibition of glioma growth in the rat, Biomaterials 30 (23–24) (2009) 4014–4020.
[42] M. Yamakuchi, M. Ferlito, C.J. Lowenstein, miR-34a repression of SIRT1 regulates apoptosis, Proc. Natl. Acad. Sci. U.S.A. 105 (36) (2008) 13421–13426.
[43] T. Ito, S. Yagi, M. Yamakuchi, MicroRNA-34a regulation of endothelial senescence, Biochem. Biophys. Res. Commun. 398 (4) (2010) 735–740.
[44] T. Zhao, J. Li, A.F. Chen, MicroRNA-34a induces endothelial progenitor cell senescence and impedes its angiogenesis via suppressing silent information regulator 1, Am. J. Physiol. Endocrinol. Metab. 299 (1) (2010) E110–E116.
[45] W. Pan, B. Chai, L. Li, Z. Lu, Z. Ma, p53/MicroRNA-34 axis in cancer and beyond, Heliyon (2023) e15155.

[46] J. Gao, N. Li, Y. Dong, S. Li, L. Xu, X. Li, et al., miR-34a-5p suppresses colorectal cancer metastasis and predicts recurrence in patients with stage II/III colorectal cancer, Oncogene 34 (31) (2015) 4142–4152.
[47] L. He, X. He, L.P. Lim, E. De Stanchina, Z. Xuan, Y. Liang, et al., A microRNA component of the p53 tumour suppressor network, Nature 447 (7148) (2007) 1130–1134.
[48] K.A. Cole, E.F. Attiyeh, Y.P. Mosse, M.J. Laquaglia, S.J. Diskin, G.M. Brodeur, et al., A functional screen identifies miR-34a as a candidate neuroblastoma tumor suppressor gene, Mol. Cancer Res. 6 (5) (2008) 735–742.
[49] Y. Fujita, K. Kojima, N. Hamada, R. Ohhashi, Y. Akao, Y. Nozawa, et al., Effects of miR-34a on cell growth and chemoresistance in prostate cancer PC3 cells, Biochem. Biophys. Res. Commun. 377 (1) (2008) 114–119.
[50] Q. Ji, X. Hao, Y. Meng, M. Zhang, J. DeSano, D. Fan, et al., Restoration of tumor suppressor miR-34 inhibits human p53-mutant gastric cancer tumorspheres, BMC Cancer 8 (1) (2008) 1–12.
[51] U. Fischer, S. Steffens, S. Frank, N.G. Rainov, K. Schulze-Osthoff, C.M. Kramm, Mechanisms of thymidine kinase/ganciclovir and cytosine deaminase/5-fluorocytosine suicide gene therapy-induced cell death in glioma cells, Oncogene 24 (7) (2005) 1231–1243.
[52] P. Dong, Y. Xiong, H. Watari, S.J. Hanley, Y. Konno, K. Ihira, et al., MiR-137 and miR-34a directly target Snail and inhibit EMT, invasion and sphere-forming ability of ovarian cancer cells, J. Exp. Clin. Cancer Res. 35 (2016) 1–9.
[53] R. Aida, K. Hagiwara, K. Okano, K. Nakata, Y. Obata, T. Yamashita, et al., miR-34a-5p might have an important role for inducing apoptosis by down-regulation of SNAI1 in apigenin-treated lung cancer cells, Mol. Biol. Rep. 48 (2021) 2291–2297.
[54] F. Zhao, J. Ke, W. Pan, H. Pan, M. Shen, Synergistic effects of ISL1 and KDM6B on non-alcoholic fatty liver disease through the regulation of SNAI1, Mol. Med. 28 (1) (2022) 12.
[55] Z. Sun, W. Hu, J. Xu, A.M. Kaufmann, A.E. Albers, MicroRNA-34a regulates epithelial-mesenchymal transition and cancer stem cell phenotype of head and neck squamous cell carcinoma in vitro, Int. J. Oncol. 47 (4) (2015) 1339–1350.
[56] F. Vakhshiteh, E. Khabazian, F. Atyabi, S.N. Ostad, Z. Madjd, R. Dinarvand, Peptide-conjugated liposomes for targeted miR-34a delivery to suppress breast cancer and cancer stem-like population, J. Drug Deliv. Sci. Technol. 57 (2020) 101687.
[57] Y.J. Choi, C.-P. Lin, J.J. Ho, X. He, N. Okada, P. Bu, et al., miR-34 miRNAs provide a barrier for somatic cell reprogramming, Nat. Cell Biol. 13 (11) (2011) 1353–1360.
[58] G.T. Bommer, I. Gerin, Y. Feng, A.J. Kaczorowski, R. Kuick, R.E. Love, et al., p53-mediated activation of miRNA34 candidate tumor-suppressor genes, Curr. Biol. 17 (15) (2007) 1298–1307.
[59] F. Chen, S.J. Hu, Effect of microRNA-34a in cell cycle, differentiation, and apoptosis: a review, J. Biochem. Mol. Toxicol. 26 (2) (2012) 79–86.
[60] D. Ostertag, K.K. Amundson, F. Lopez Espinoza, B. Martin, T. Buckley, A.P.G. Da Silva, et al., Brain tumor eradication and prolonged survival from intratumoral conversion of 5-fluorocytosine to 5-fluorouracil using a nonlytic retroviral replicating vector, Neuro Oncol. 14 (2) (2011) 145–159.
[61] L.A. Mitchell, F. Lopez Espinoza, D. Mendoza, Y. Kato, A. Inagaki, K. Hiraoka, et al., Toca 511 gene transfer and treatment with the prodrug, 5-fluorocytosine, promotes durable antitumor immunity in a mouse glioma model, Neuro Oncol. 19 (7) (2017) 930–939.
[62] M. Tibensky, J. Jakubechova, U. Altanerova, A. Pastorakova, B. Rychly, L. Baciak, et al., Gene-directed enzyme/prodrug therapy of rat brain tumor mediated by human mesenchymal stem cell suicide gene extracellular vesicles in vitro and in vivo, Cancers 14 (3) (2022) 735.
[63] G.X.E. Tu, Y.K. Ho, Z.X. Ng, K.J. Teo, T.T. Yeo, H.-P. Too, A facile and scalable in production non-viral gene engineered mesenchymal stem cells for effective suppression of temozolomide-resistant (TMZR) glioblastoma growth, Stem Cell Res. Ther. 11 (1) (2020) 1–15.
[64] C. Wu, J. Lin, M. Hong, Y. Choudhury, P. Balani, D. Leung, et al., Combinatorial control of suicide gene expression by tissue-specific promoter and microRNA regulation for cancer therapy, Mol. Ther. 17 (12) (2009) 2058–2066.
[65] B. Oh, J. Han, E. Choi, X. Tan, M. Lee, Peptide micelle-mediated delivery of tissue-specific suicide gene and combined therapy with Avastin in a glioblastoma model, J. Pharmaceut. Sci. 104 (4) (2015) 1461–1469.
[66] R. Nau, F. Sorgel, H. Eiffert, Penetration of drugs through the blood-cerebrospinal fluid/blood-brain barrier for treatment of central nervous system infections, Clin. Microbiol. Rev. 23 (4) (2010) 858–883.
[67] J. Portnow, T.W. Synold, B. Badie, R. Tirughana, S.F. Lacey, M. D'Apuzzo, et al., Neural stem cell–based anticancer gene therapy: a first-in-human study in recurrent high-grade glioma PatientsNeural stem cell–based anticancer gene therapy for gliomas, Clin. Cancer Res. 23 (12) (2017) 2951–2960.