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LABORATORY INVESTIGATION

Inhibition of glioma growth by a GOLPH3 siRNA-loaded cationic liposomes

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Abstract

Purpose GOLPH3 has been shown to be involved in glioma proliferation. In this study, we aimed to demonstrate that GOLPH3 can serve as a target for glioma gene therapy.

Methods During the experiment, cationic liposomes with angiopep-2 (A2-CL) were used to deliver siGOLPH3 crossing the blood–brain barrier and reaching the glioma.

Results At the cellular level, the A2-CL/siGOLPH3 could silence GOLPH3 and then effectively inhibited the proliferation of cells. In vivo experiments, using U87-GFP-Luci-bearing BALB/c mouse models, we demonstrated that A2-CL could deliver GOLPH3-siRNA specifically to glioma and effectively inhibit glioma growth.

Conclusions This study shows that GOLPH3 has great potential as a target for the gene therapy of glioma and is of great value in precise medical applications.

Keywords Glioma · GOLPH3 · RNAi · Angiopep-2 · Liposome

Introduction

Malignant gliomas are the most common of all malignant primary brain tumors [1, 2]. Despite aggressive therapy with surgery, chemotherapy, and radiation, the median survival time is only approximately 12 months [3]. RNA interference (RNAi), a new gene silencing technology that is mediated by double-stranded RNA and shuts off the expression of the corresponding gene at the post-transcriptional mRNA level, has shown great promise in gene function research and gene therapy [4–6]. Recently, RNAi-based gene silencing methods have been confirmed in humans and also show good prospects for the treatment of gliomas [7].

Golgi phosphoprotein3 (GOLPH3), also known as GPP34, GMx33, and MIDAS, encoded by the 5p13 gene with a molecular weight of about 34 KDa, is enriched at the trans-face of golgi cisternae and overexpressed in glioma [8, 9]. Gliomas with high GOLPH3 level exhibited shorter survival time, in line with the report of Zhou et al. in patients [8]. Our previous studies suggested that GOLPH3 was upregulated in gliomas, and its downregulation inhibited U87 cells proliferation both in vitro and in vivo [10]. High GOLPH3 mRNA expression has been proved as a poor prognostic factor for GBM patients by multivariate analysis.

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During our studies, we had demonstrated that siRNA792 effectively downregulated the expression of GOLPH3 in U87 cells and gliomas derived from shGOLPH3 cells were visibly smaller than those from shNC cells [11]. These results further demonstrated that GOLPH3 plays important roles in the progression of glioma. However, it remains unclear whether GOLPH3 will be potential as a therapeutic target for glioma treatment. However, their efficacy in brain is limited by its own chemical nature and the lack of transport across the blood–brain barrier (BBB) following intravenous (IV) administration [12].

Lots of cationic nanoparticles have been designed for siRNA delivery. Cation liposome (CL) is widely used owning to its high transfection efficiency and low toxicity [13, 14]. Angiopep-2, which a specific ligand for LRP-1, is used to accumulate our CL in brain tumors due to the expression LRP-1 in the BBB and human glioma cells [15–18]. In this study, we hypothesized that targeting GLOPH3 could serve as a novel therapeutic approach to inhibit the growth of GBM. Thus, to resolve these questions which mentioned above, we prepared angiopep-2 modified CLs to deliver GOLPH3-siRNA (A2-CL/siGOLPH3) for glioma treatment.

Materials and methods

Materials

1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy (polyethyleneglycol) (DSPE-PEG_{2000}), 1, 2-Dioleoyl-3-trimethylammonium-propane (DOTAP), cholesterol, dioleoylphaphetidylcholine (DOPC) were obtained from Shanghai Advanced Vehicle Technology Pharmaceutical Co. Ltd (AVT). DSPE-PEG_{2000}-PDP was brought from Xi'an ruixi Biological Technology Co; Ltd. Primary antibodies against GOLPH3 (Anti-GOLPH3 antibody ab98023) were obtained from Abcam plc., Primary antibodies against PLK1 were obtained from Cell Signaling Technology, Inc. Anti-GAPDH antibody were purchased from Bioworld Technology, Inc. 5-ethynyl-20-deoxyuridine (EdU) was brought from Ribobio (Guangzhou, China). Some sets of siRNA duplexes were listed below (siGOLPH3, siPLK1 and negative control siRNA with a scrambled sequence (siNC) from GenePharma Co. Shanghai, China; FAM-labeled siRNA (FAM-siRNA) and Cy5-siRNA from Biosyntech Co. Suzhou, China):

FAM-siRNA, siNC	antisense strand	5′-ACGUGACACGUUCGG AGAAdTdT-3′
siGOLPH3	antisense strand	5′-GUUAAGAAAUGUACG GGAATT-3′
siPLK1	antisense strand	5′-AGAUCACUCUCCUCA ACUAUU-3′

Cationic liposome formulation

CL formulations included a cationic lipid (DOTAP), DOPC, cholesterol, DSPE-PEG2000, DSPE-PEG2000-Angiopep-2 and siRNA as previously described [19]. A2-CL were prepared using ethanol injection method [20]. The normal CLs with composition of DOTAP: DOPC: Chol: DSPE-PEG2000: DSPE-PEG2000-Angiopep-2 (40: 10: 40: 9.5: 0.5, mol/mol) was added to an aqueous buffer (100 mM citrate, pH 3) with mixing to final ethanol and lipid concentrations of 30% (v/v) and 5 mg mL^{-1}.

Characterization of A2-CL/siRNA complexes

A2-CL and A2-CL/siRNA are all fresh. Malvern Zeta sizer NanoZS was used to test the nanoparticle size and zeta potential of A2-CL and A2-CL/siRNA. Transmission electron microscopy (TEM) (FEI Tecnai G2 T12) was used to observe the morphology of A2-CL/siRNA negatively stained with 2% phosphotungstate acid. The sample concentration was 1 mg mL^{-1}.

Gel retardation assay

Gel electrophoresis was carried out to test the ability of A2-CL to load siRNA. The experimental method was performed as previously described [20]. Agarose gel was prepared with 1× TAE electrophoresis buffer and 1× TAE electrophoresis buffer was added to cover the gel. Take fresh A2-CL/siRNA samples prepared with different N/P (0.2, 0.5, 1, 2 and 3) and 6× DNA loading buffer and mix 5: 1. Then Add 20 μL of sample to each sample well. The voltage was adjusted to 100 V. After approximately 10 min, the gel was placed in EB dye solution for staining for 5 min and rinsed slightly with fresh water. The result was observed on a UV transmission detector.

In vitro study

Cell cultures

The glioblastoma cell line U87 was purchased from the Shanghai Cell Bank, Chinese Academy of Sciences. U87-GFP-Luci cells were transformed with luciferase gene. U87 cells and U87-GFP-Luci cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (heat-inactivated) (FBS, Gibco). The cells were cultured

as a monolayer in a humidified atmosphere containing 5% CO_2 at 37 °C.

Intracellular uptake

5×10^4 U87 cells were inoculated in each well of 12-well plates and cultured for 24 h. Then, PBS, free FAM-siRNA and A2-CL/FAM-siRNA were added to the medium of U87 cells at concentration 1 μg mL^{-1} for 4 h. Then, cells were counterstained with DAPI and subjected to fluorescence microscope. Flow cytometric analysis was also used to evaluate these group of cells after the above processing.

Gene silencing

Quantitative real-time polymerase chain reaction (qRT-PCR)

The GOLPH3 mRNA expression levels in U87 cells were tested by qRT-PCR. Total RNA was extracted using trizol reagent (TIANGEN) according to the manufacturer's instructions, and possible DNA contamination was removed through digesting the extracted RNA with DNase I. Then, the RNA was purified again using trizol reagent and subjected to the synthesis of first-strand cDNA using a reverse transcription kit. Finally, the quantitative analysis of the cDNA was calculated by a qRT-PCR machine (750 ABI USA), and GAPDH was amplified as an interior control. All primers were synthesized by Sangon Biotech (Shanghai, People's Republic of China).

GOLPH3 sense primer: 5′-GCCTCCAGAAACGGTCCA G-3′, antisense primer: 5′-GTCAATACACCCTTTTCC ACCA-3′.

Western blot study (WB)

The ability of the siGOLPH3 delivered by A2-CL to silence the target protein was tested by WB. U87 cells (2×10^5) were seeded in a 6-well culture plate and cultured at 37 °C in 5% CO_2 for 24 h. After that the cells were treated with different siRNA NPs at a dose of 2 μg mL^{-1} siGOLPH3 for 24 h (medium was free of FBS). Thereafter, the GOLPH3 protein levels in U87 cells of different groups were analyzed by the method of WB: cultured cells (on ice) were washed twice with cold PBS and lysed. Then the lysis buffer was centrifuged at a speed of 12,000 rpm for 10 min to wipe out impurities. Quantities and equilibriums of the extracted protein samples were measured by BCA kit (Beyotime, Shanghai). Later, the proteins were transferred to polyvinylidene difluoride membrane of 0.45 mm pore size (Millipore, Billerica, MA), which were blocked with 5% milk for 1.5 h at room temperature, incubated with Anti-GOLPH3 antibody at 4 °C overnight and secondary antibodies at room temperature for 2 h. Finally, the membranes were exposed in a dark room. Band densities were quantified by using Image J Software (National Institutes of Health) and the densitometric results were shown. WB experiments were carried out in three biological replicates.

Proliferation inhibition study

EdU assay

U87 cells were seeded into 96-well plates at 1.5×10^4 cells per well. After that the cells were treated with different siRNA NPs at a dose of 2 μg mL^{-1} siGOLPH3 for 24 h (medium was free of FBS). 24 h later, the cells were exposed to 50 μM of EdU for additional 2 h at 37 °C. Then, the cells were fixed with 4% paraformaldehyde for 20 min and treated with 0.5% Triton X-100 for another 20 min at room temperature. After being washed with PBS for five times, the cells were reacted with 100 L of 1 × Apollo® reaction cocktail for 30 min. Thereafter, the DNA contents of cells were stained with 100 μL of Hoechst 33,342 (5 μg mL^{-1}) for 20 min and visualized under a fluorescent microscope (Olympus, IX71).

MTT assay

The proliferation inhibition of A2-CL/siRNA was measured by the MTT assay. 5×10^3 U87 cells were inoculated in a 96-well plate. Then different concentrations (0.5, 1.0, 1.5, 2.0, 3.0, 4.0 μg mL^{-1}) of A2-CL/siRNA were added. After 48 h, add 20 μL MTT (5 mg mL^{-1}) to each well. 4 h later, remove the liquid gently without touching the purple formazan, and dissolve the purple formazan with 150 μL DMSO. Finally, the optical density was obtained using a spectrophotometric analysis at a wavelength of 570 nm.

In vivo study

Mice

Experimental BALB/c nude mice (5 weeks old, male) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and raised in Neurosurgery Laboratory Animal Room of Xuzhou Medical University. BALB/c nude mice were used to perform biodistribution of A2-CL/Cy5-siRNA assay and the inhibition of glioma growth. All experimental personnel passed the professional skills training of experimental animals in Jiangsu Province. All experimental procedures in vivo were carried out according to the guidelines of Jiangsu Council on Animal Care.

Tumor model

Nude mice were fixed on stereotaxic instruments. Glioma cells (U87 cells) were transformed with luciferase gene

(U87-GFP-Luci). 7×10^5 U87-GFP-luci cells in 7 μL Leibovitz's L-15 medium (Gibco Company, USA) were injected into the right striatum with microsyringe. The injection time was 2 min and stay over 5 min before pulling out. After 10 days, nude mice were observed for the intensity of luci fluorescence under an in vivo imaging system to confirm the successful construction of the nude mouse tumor model.

Biodistribution experiment

On day 15, nude mice were verified to be of uniform size. And then, these mice were divided into three groups (n = 3), PBS, free Cy5-siRNA, and A2-CL/Cy5-siRNA (the dose of Cy5-siRNA is 1 mg kg^{-1}) were injected through the tail vein, respectively. After 2, 8, and 36 h injection of PBS, free Cy5-siRNA, and A2-CL/Cy5-siRNA, the mice were imaged under an in vivo imaging system under anesthesia with isoflurane. After 6 h from the completion of the injection, the nude mice were sacrificed and the brain tissue was removed and the intensity of Cy5 was observed and photographed under an in vivo imaging system. These brain tissues were made into frozen sections (20 μm thick) after fixed in 4% paraformaldehyde for 48 h and then dehydrated in gradient sucrose gradient dehydration. The nucleus was stained with DAPI and photographed by fluorescence microscope.

Antitumor experiments

In vivo fluorescence imaging, brain tissue H&E sections, and survival curves were used to evaluate the therapeutic effects of different treatment. On day 10, all tumor models were observed under in vivo imaging system. After verifying that the tumors were of uniform size, these mice were randomly divided into five groups (n = 7). Afterwards, on days 12, 14, 16, and 18, each group was injected via tail vein with PBS, A2-CL/siNC, free siGOLPH3, A2-CL/siGOLPH3, and A2-CL/siPLK1, respectively (the dose of Cy5-siRNA is 1 mg kg^{-1}). On day 25 and 40, nude mice were photographed under in vivo imaging system and analyzed for fluorescence values. At day 47, 1 nude mouse from each group was randomly selected to kill, the brain tissue was removed and the largest section of the tumor was selected for paraffin sections and H&E stained. These sections were taken using EVOS FL Auto (Life Technologies, America). Throughout the in vivo experiment, we observed the daily survival of the nude mice and recorded the weight every 2 days.

Immunofluorescence

Brain sections were deparaffinized and rehydrated, and 5% bovine serum albumin (BSA; Solarbio, Beijing, China) was used to block non-specific protein binding at room temperature for 2 h. Slides were incubated with Anti-GOLPH3 antibody (1: 200) overnight at 4 °C. Goat anti-rabbit IgG-AlexaFluor647 (1: 200) secondary antibodies were added and incubated in the dark for 30 min at 37 °C. Images were captured by fluorescence microscope. Identical exposure times and light intensities were applied to all images.

Organ safety evaluation

After the above treatment,1 nude mouse from each group was randomly selected for organ safety evaluation. At day 47, these mice were killed and the major organs were collected and made into H&E stained paraffin sections. These sections were taken by EVOS FL Auto.

Statistical analysis

The statistical analysis was performed using the GraphPad Prism 7 software package. All of the data are presented as the means ± SD of three independent experiments. Comparisons of the mean values between the control and treated groups were performed using student's t test. A Kaplan–Meier survival curve and the log-rank test were used for the in vivo survival analysis. *$P < 0.05$ were considered statistically significant.

Results

Preparation and characterization of A2-CL/siRNA

CLs were prepared using the ethanol injection method as previously reported [20]. As shown in Fig. 1a, A2-CL had a strong ability to bind siRNA, forming a complete complex at a N/P ratio of 2: 1 with no free siRNA in the gel. TEM images revealed that A2-CL/siRNA were liposomes with about 80 nm and well dispersed (Fig. 1b). The CLs were 69.2 nm in diameter and siRNA encapsulate A2-CL/siRNA were 88.0 nm in diameter (Fig. 1c, d). After encapsulation of siRNA by A2-CL, the zeta potential of A2-CL was changed from 31.2 mV to 9.54 mV (Fig. 1e, f).

In vitro cellular uptake study

Cellular uptake was monitored after treating the U87 cells with PBS, free FAM-siRNA and A2-CL/FAM-siRNA for 6 h. As shown in Fig. 2a, under fluorescence microscopy, the A2-CL/FAM-siRNA treated U87 cells showed much higher green fluorescence than free FAM-siRNA treated cells, which suggested that the A2-CL improved the intracellular uptake of siRNA. The above results were further confirmed

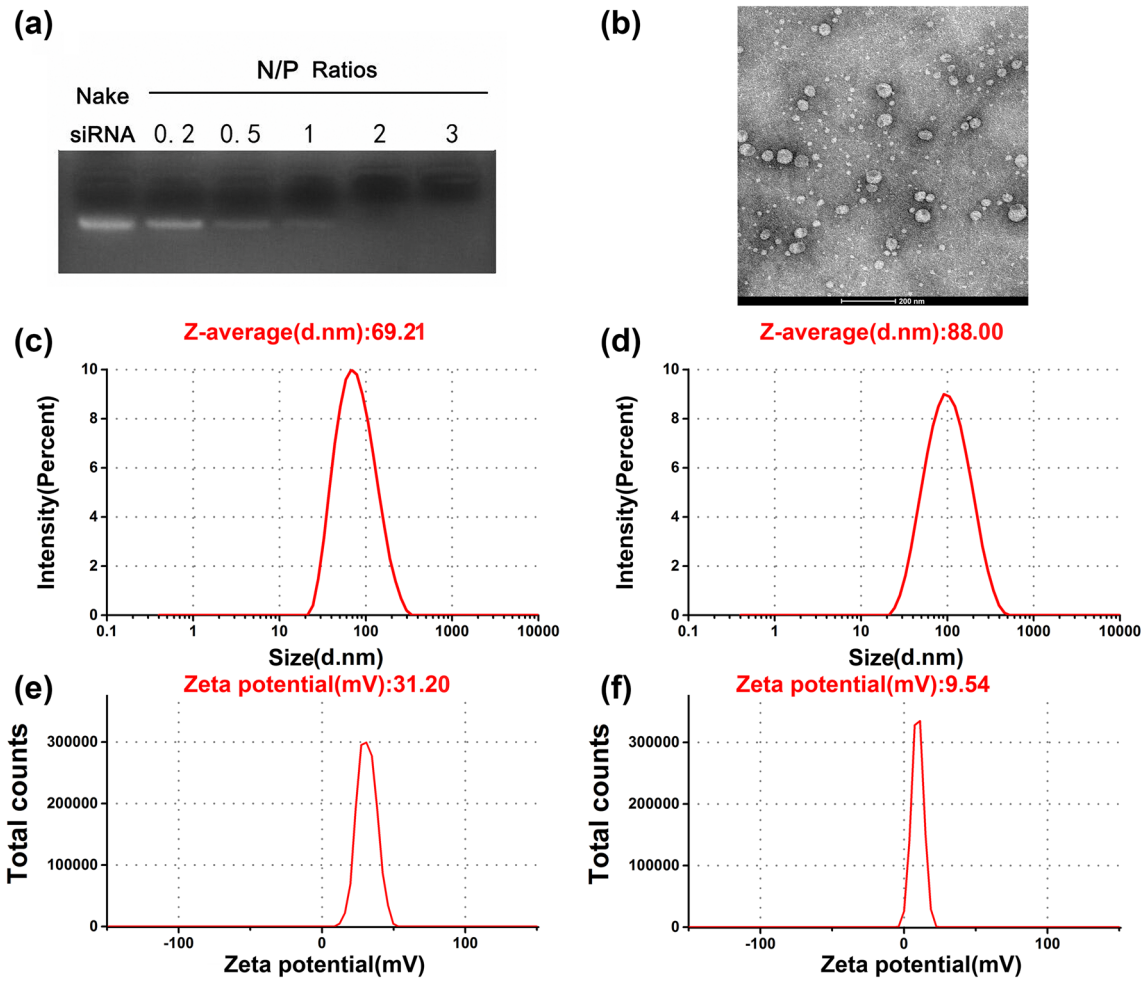


Fig. 1 Morphology and properties of A2-CL. **a** Gel retardation assay of A2-CL. **b** TEM image of A2-CL/siRNA. **c** The nanoparticle size of A2-CL. **d** The nanoparticle size of A2-CL/siRNA at N/P = 2: 1. **e** The zeta potential of A2-CL. **f** The zeta potential of A2-CL/siRNA at N/P = 2: 1

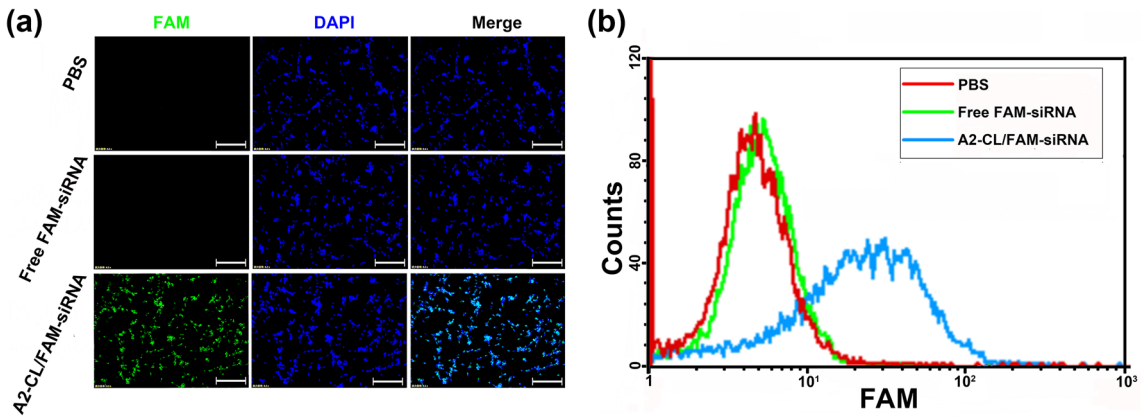


Fig. 2 The intro cellular uptake essay of A2-CL/FAM-siRNA by U87 cells. **a** U87 cells after treatment with PBS, free FAM-siRNA, A2-CL/FAM-siRNA. FAM (green) and cell nuclei (blue) (scale bars: 200 μm). **b** U87 cells with each treatment analyzed by flow cytometric

by the flow cytometry (Fig. 2b). The free siRNA was less entered into cells, due to the negative charges of siRNA.

Gene silencing and proliferation inhibition capability

To evaluate the biological activities of siGOLPH3 delivery by A2-CL, the level of GOLPH3 mRNA and the GOLPH3 protein were evaluated. As shown in Fig. 3a, the GOLPH3 in U87 cells was silenced by siGOLPH3 in targeted A2-CL, leading to ~ 71.8% knockdown of GOLPH3 mRNA. In contrast, the cells showed no effect with treatment PBS, A2-CL/siNC and free siGOLPH3. We also examined the GOLPH3 protein expression in U87 cells by WB. Coinciding with the results of qRT-PCR, A2-LP/siGOLPH3 exhibited significantly silencing of GOLPH3 protein expression (68.3%). Meanwhile, A2-CL/siPLK1 also show significant gene silence (68.3%) and protein expression silence (51.5%) ability. The above results indicated that A2-CL/siGOLPH3 contained strong gene silencing ability. Polo-like kinase 1 (PLK1), a highly conserved ser/threonine kinase [19], is highly expressed in glioma tissues and has been proved to be a therapeutic approach to kill GBM. The use of siPLK1 as a target for RNAi therapy has been effective in some studies [7, 20]. Therefore, in experiment below, we used it as a positive control to better explore the therapeutic effect of siGOLPH3.

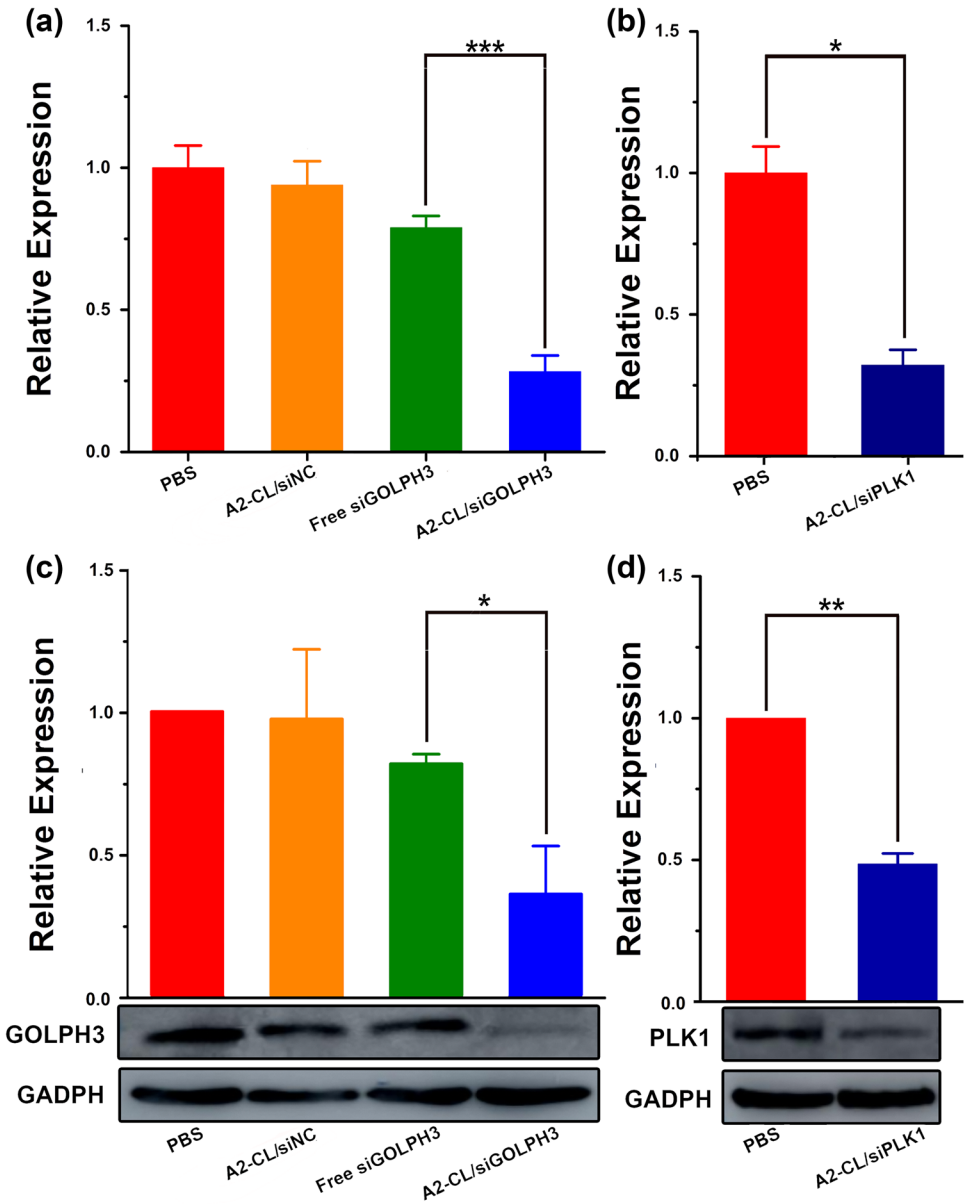


Fig. 3 The expression of GOLPH3 and PLK1 in U87 cells by A2-CL/siRNA. **a** The expression of GOLPH3 mRNA in U87 cells after treatment by RT-qPCR. **b** The expression of PLK1 mRNA in U87 cells after treatment by RT-qPCR. **c** The expression of GOLPH3 protein in U87 cells after treatment by WB. **d** The expression of PLK1 protein in U87 cells after treatment by WB. The concentration of siRNA was 2 μg mL^{-1}, as mean ± SD, n = 3, *p < 0.05, **p < 0.01, ***p < 0.001

Inhibitory effect on cell proliferation by A2-CL/siGOLPH3

The proliferation inhibition of A2-CL/siGOLPH3 was estimated using EdU and MTT assay. EdU assay revealed that the group of cells treated by A2-CL/siGOLPH3 had significantly less percentage of EdU-positive cells than the group separately treated by PBS, A2-CL/nonsense siRNA and free siGOLPH3 (Fig. 4a, b). These results showed that A2-CL/siGOLPH3 could reduce the U87 cells proliferation. Next, we estimated the proliferation inhibition of siGOLPH3 loaded by A2-CL using MTT assay. As shown in Fig. 4c, that U87 cells showed significant inhibition of cell proliferation after A2-CL/siGOLPH3 treatment verified by MTT assays. Cell proliferation inhibition rate reached to 47% at the siRNA concentration 2 μg mL^{-1}.

Glioma distribution of A2-CL/Cy5-siRNA

The results of in vivo imaging and frozen sections confirmed our hypothesis that A2-CL/siRNA can cross BBB and target glioma cells due to the function of angiopep-2 modified A2-CL/siRNA. As shown in Fig. 5a, there still was Cy5-siRNA fluorescence in glioma after 36 h injection of A2-CL/Cy5-siRNA. Meanwhile, we found that the Cy5-siRNA fluorescence of glioma treated with A2-CL/Cy5-siRNA was significantly higher than those of mice injected with free Cy5-siRNA, which indicating that A2-CL/siRNA could carry more siRNA to the tumor area effectively without being degraded (Fig. 5a–c). After that, we made the mouse brain tissue into frozen sections to observe the distribution of Cy5-siRNA by fluorescence microscope. As shown in Fig. 5d, with DAPI-stained cell nuclei, in the brain tissue of mice injected with A2-CL/Cy5-siRNA, it was observed that the red fluorescence of Cy5-siRNA overlaps with the green fluorescence of the

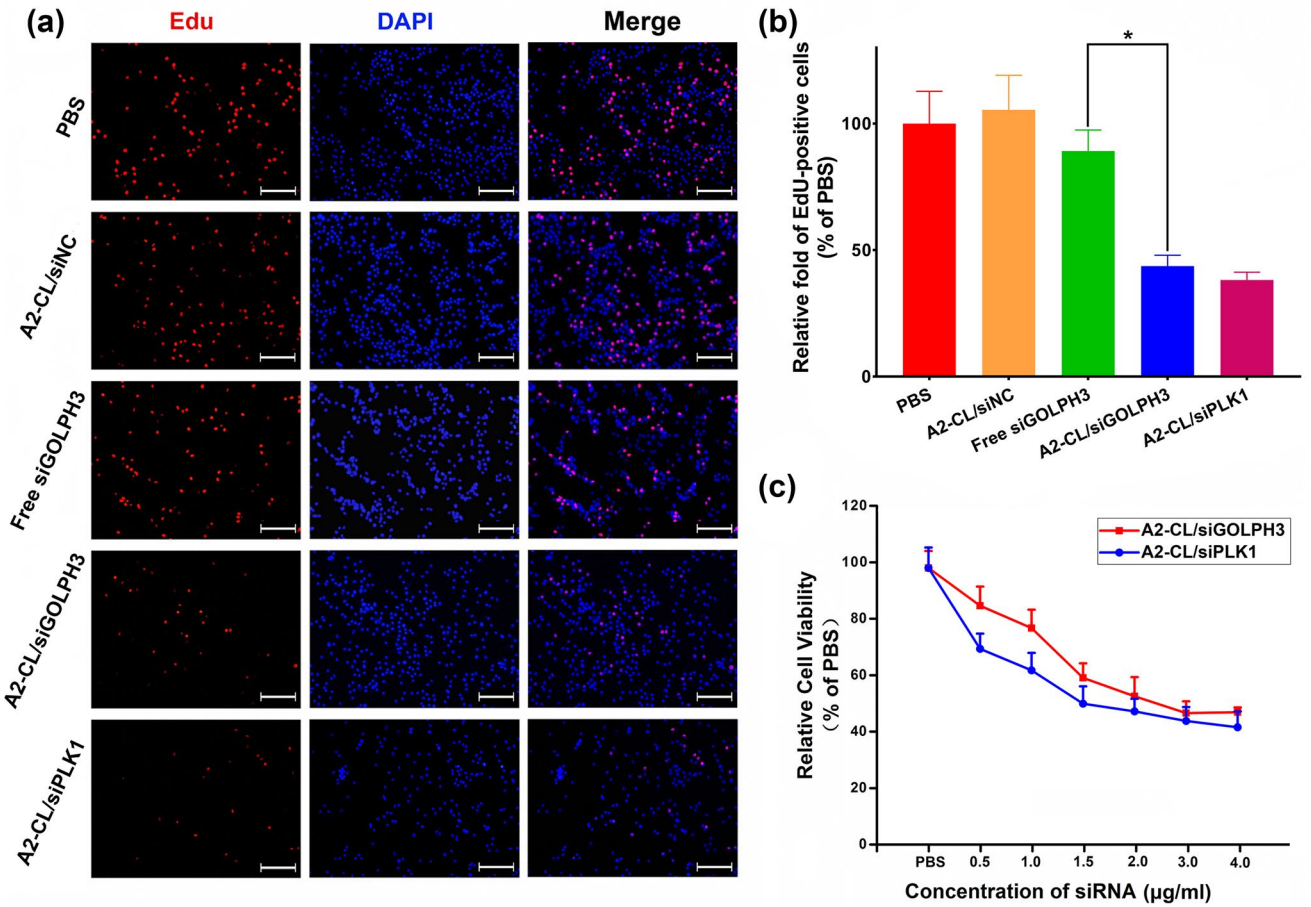
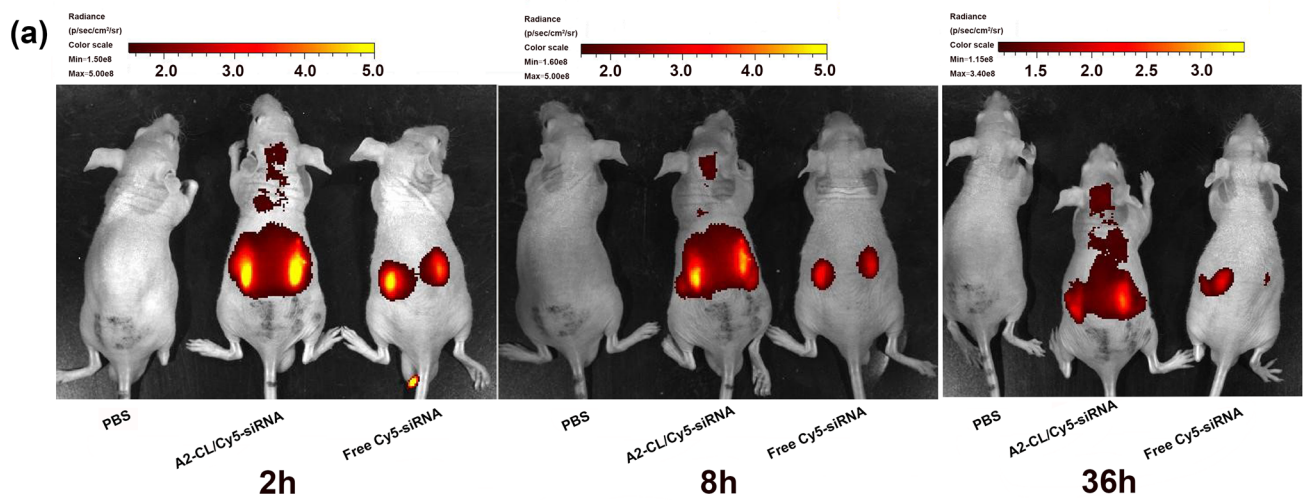


Fig. 4 The assay of proliferation inhibition of U87 cells by A2-CL/siRNA. **a** EdU assay observed under fluorescence microscope in U87 cells transfected with PBS, A2-CL/siNC, free siGOLPH3, A2-CL/siGOLPH3 and A2-CL/siPLK1 respectively, incubation for 48 h (scale bars = 200 μm). **b** Quantification assay of Edu assay results in (**a**). **c** MTT assay in U87 cells transfected with A2-CL/siGOLPH3 and A2-CL/siPLK1 respectively, incubation at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 μg mL^{-1}, as mean ± SD, n = 6, *p < 0.05


(a)
Radiance (p/sec/cm²/sr)
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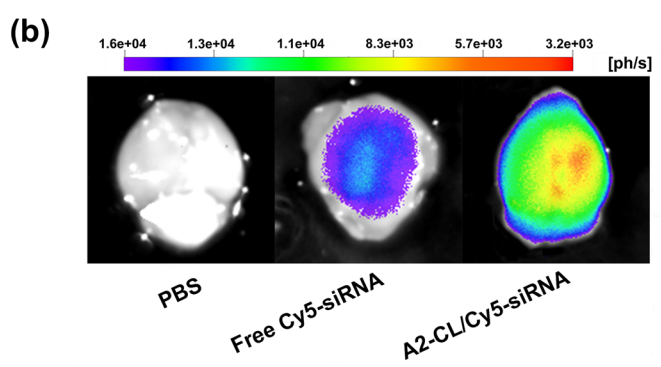
Max=5.00e8
2.0 3.0 4.0 5.0
Min=1.60e8
Max=5.00e8
Min=1.15e8
Max=3.40e8
1.5 2.0 2.5 3.0
PBS
A2-CL/Cy5-siRNA
Free Cy5-siRNA
2h
8h
36h


(b)
1.6e+04 1.3e+04 1.1e+04 8.3e+03 5.7e+03 3.2e+03
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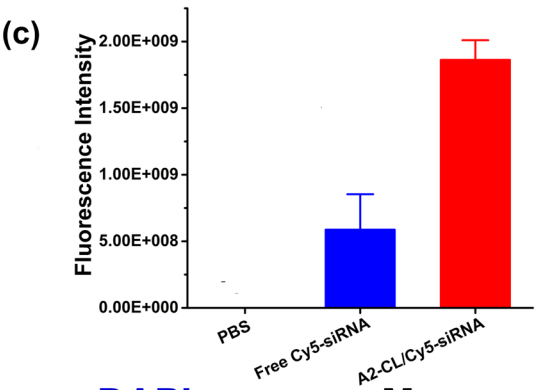
PBS
Free Cy5-siRNA
A2-CL/Cy5-siRNA


(c)
Fluorescence Intensity
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1.00E+009
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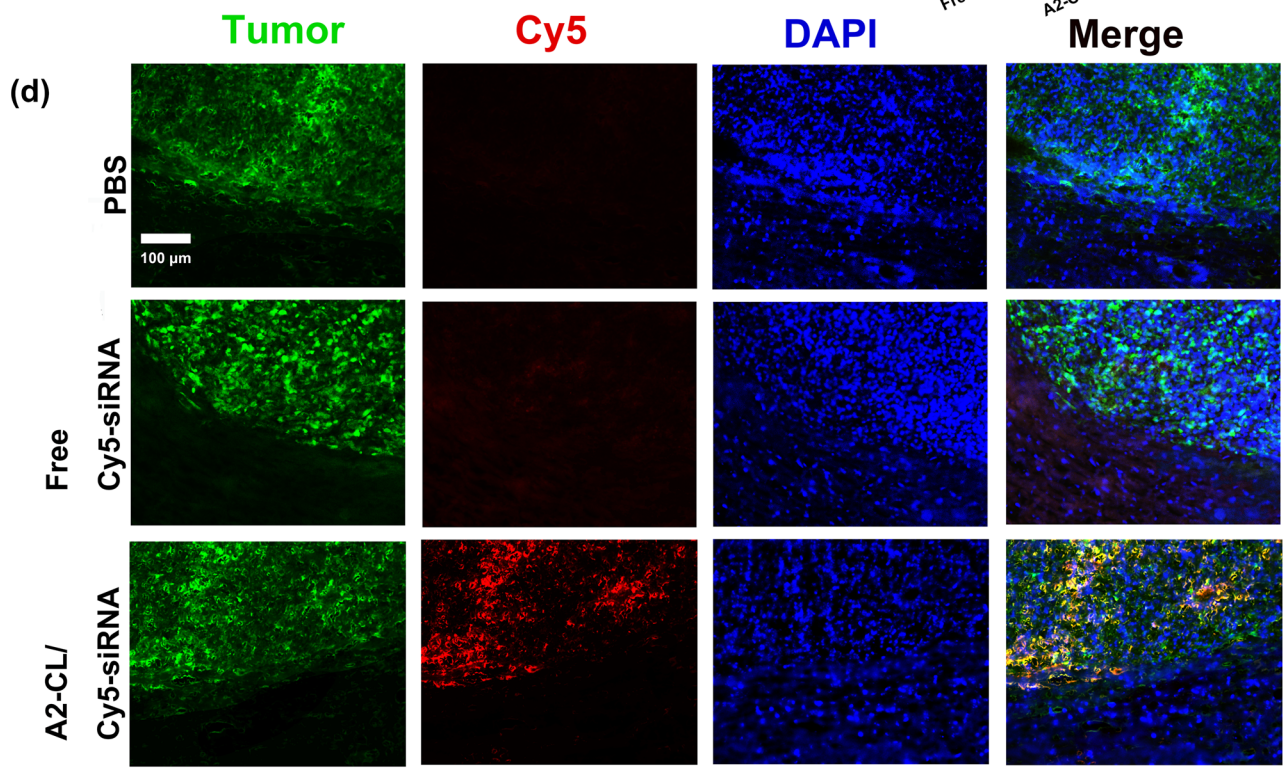
PBS
Free Cy5-siRNA
A2-CL/Cy5-siRNA


(d)
Tumor
Cy5
DAPI
Merge
PBS
Free Cy5-siRNA
A2-CL/ Cy5-siRNA
100 μm

◂**Fig. 5** Distribution of Cy5-siRNA in U87-GFP-Luci-bearing nude mice after tail vein injection. **a** Body distribution of Cy5 fluorescence at 2 h, 8 h and 36 h. **b** The fluorescence images of Cy5-siRNA showed by brain of each group under in vivo imaging system at 6 h. **c** Quantification of Cy5 fluorescence signal of each group at 6 h. **d** Fluorescence images of frozen sections of brain tissue from each group. Cy5-siRNA (red), tumor (green), and cell nuclei (blue). (Scale bar: 100 μm)

glioma. On contrast, the free Cy5-siRNA group showed only few Cy5-siRNA fluorescence distribution in glioma. All these above demonstrated that A2-CL/siRNA could cross the BBB and deliver the siRNA into glioma in vivo and provided the basis for our next validation of the effect of siGOLPH3 in vivo.

Anti-glioma study of A2-CL/GOLPH3-siRNA

Finally, we examined the impact of GLOPH3 knockdown in orthotopic U87-GFP-Luci bearing brain tumor. 10 days later, these mice were randomly divided into five groups and injected PBS, A2-CL/siNC, free siGOLPH3, A2-CL/siGOLPH3 and A2-CL/siPLK1 through the tail vein at days 12, 14, 16 and 18, respectively. On days 10, 25 and 40, we monitored intracranial tumor fluorescence to assess tumor growth. As shown in Fig. 6b, the tumors in PBS, free siGOLPH3, A2-CL/siNC group injection mice grew rapidly. By day 40, the tumor injection with PBS, A2-CL/siNC, and free siGOLPH3 were 36.4-fold, 38.3-fold, and 31.4-fold higher than at day 10, respectively, as determined by bioluminescence measurement. The tumor growth rate was 16.6-fold higher than at day 10 in A2-CL/siGOLPH3 group (Fig. 6b, c).

The body weight change and median survival times were monitored to estimate antitumor efficiency. As shown in Fig. 6d, the weight of mice with treatment of A2-CL/siGOLPH3 slowly decreased, while all other groups lost weight rapidly. The Kaplan–Meier survival curves of mice was observed, too. The median survival time for the mice injection with A2-CL/siGOLPH3 was 63.5 days, longer than that of the PBS (54 days), A2-CL/siNC (56 days), and free siGOLPH3 (57 days) treatments (Fig. 6e). As shown in Fig. 6f, at 47 days after glioma transplantation, the transplanted gliomas in A2-CL/siGOLPH3-treated mice were visibly smaller than in the PBS, A2-CL/siNC, and free siGOLPH3- treated mice. Based on H&E staining of coronal sections of mouse brains, the smallest glioma volumes were observed in A2-CL/siGOLPH3-treated mice. As a result of immunofluorescence, it was found that the expression of GOLPH3 in the experimental group was significantly inhibited. This confirms the gene silencing effect of A2-CL/siGOLPH3 on in vivo glioma.

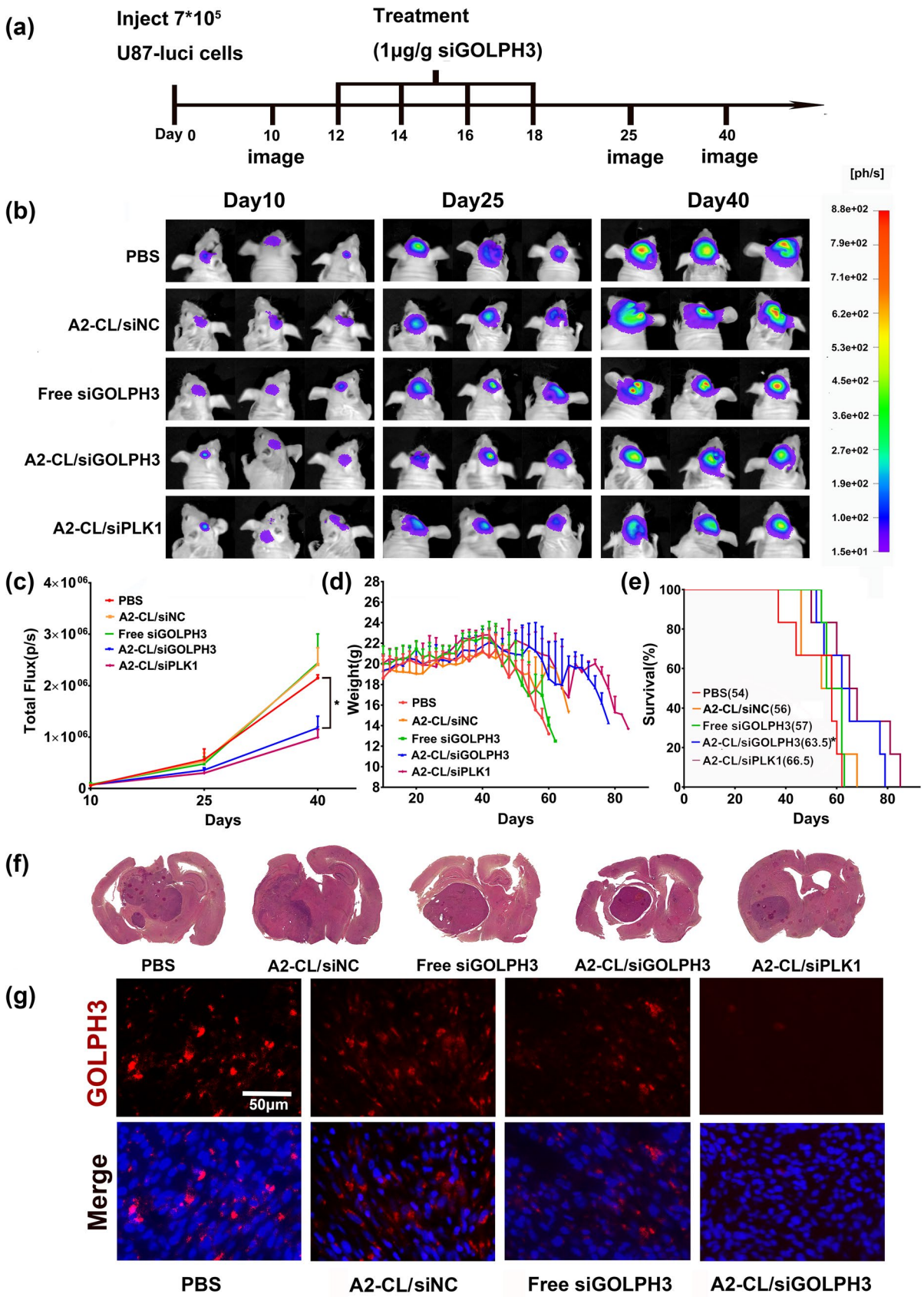
Discussion

GOLPH3, a highly conserved protein enriched at the trans-face of golgi cisternae, is associated with worse prognosis of gliomas [8, 11]. In our previous study, we have found that GOLPH3 inhibits glioma cell growth by promoting endocytosis and degradation of epidermal growth factor receptor (EGFR), thereby inhibiting PI3K-Akt-mTOR signaling, and plays a role in glioma proliferation [21, 22]. In this experiment, we attempted to demonstrate that GOLPH3 could serve as a target for the treatment of glioma by siGOLPH3 delivered by CLs [23]. The effect of silencing GOLPH3 on the proliferation of glioma were tested in vitro and in vivo in this study.

The obstacle to the use of RNAi in the treatment of gliomas lies in its own chemical nature and the existence of the BBB [24, 25]. siRNAs are hydrophilic and negative-charged, which prohibit them from crossing biological membranes, and are prone to rapid enzymatic degradation in vivo [26]. Therefore, in this study, CLs were chosen to deliver siGOLPH3. CLs could prevent the siRNA from being degraded and increased in siRNA uptake by tumor cells. The ability of A2-CL/siRNA to penetrate BBB and enter into glioma is essential for the siGOLPH3-therapy efficacy. Therefore, we chose angiopep-2 as a targeting peptide. Angiopep-2 is a specific ligand for LRP-1 and can cross BBB and target glioma cells that express LRP-1. In our experiment, the diameter of A2-CL/siGOLPH3 was 88 nm and zeta potential was 9.54 mV analysis with dynamic light scattering (Fig. 1d, f). These nanosized and zeta potential suggested that A2-CL/siGOLPH3 could enter into glioma. The experimental process also proved that the CLs equipped with angiopep-2 successfully delivered siRNA to the glioma in vitro and in vivo, which laid the foundation for our experiments.

In the preliminary experiment, GOLPH3 was highly expressed in U87 cells [11, 27]. Thus, A2-CL/siGLOPH3 was transfected in U87 cells and the expression of GOLPH3 in U87 cells were detected by RT-PCR and WB. As shown in Fig. 3a, b, A2-CL/siGLOPH3 effectively suppressed the expression of GOLPH3. Our previous studies suggested that downregulated the GLOPH3 expression could inhibit glioma cell proliferation in vitro [11]. EdU incorporation assay and MTT assay were used to test the glioma cell proliferation in vitro. As shown in Fig. 3a–c, GOLPH3 downregulation inhibited glioma cell proliferation.

Whether, downregulated the GLOPH3 expression could inhibit glioma growth in vivo. First, the orthotopic U87-GFP-Luci tumor-bearing nude mice were developed. Subsequently, we conducted in vivo experiments to deliver A2-CL/siGOLPH3 by tail vein administration


(a)
Inject 7*10^5
U87-luci cells
Treatment
(1μg/g siGOLPH3)
Day 0
10
12
14
16
18
25
40
image
image
image
(b)
Day10
Day25
Day40
[ph/s]
8.8e+02
7.9e+02
7.1e+02
6.2e+02
5.3e+02
4.5e+02
3.6e+02
2.7e+02
1.9e+02
1.0e+02
1.5e+01
PBS
A2-CL/siNC
Free siGOLPH3
A2-CL/siGOLPH3
A2-CL/siPLK1
(c)
Total Flux(p/s)
Days
PBS
A2-CL/siNC
Free siGOLPH3
A2-CL/siGOLPH3
A2-CL/siPLK1
(d)
Weight(g)
Days
(e)
Survival(%)
Days
PBS(54)
A2-CL/siNC(56)
Free siGOLPH3(57)
A2-CL/siGOLPH3(63.5)*
A2-CL/siPLK1(66.5)
(f)
PBS
A2-CL/siNC
Free siGOLPH3
A2-CL/siGOLPH3
A2-CL/siPLK1
(g)
GOLPH3
Merge
50μm
PBS
A2-CL/siNC
Free siGOLPH3
A2-CL/siGOLPH3

◂**Fig. 6** Tumor proliferation assay in the U87-GFP-Luci glioma nude mouse model. **a** Tail vein injection of PBS, A2-CL/siNC, free siGOLPH3, A2-CL/siGOLPH3 and A2-CL/siPLK1 on days 12, 14, 16 and 18. siRNA (the dose of Cy5-siRNA is 1 mg kg^{-1}) **b** Bioluminescent signal of U87-GFP-Luci tumor model mice of each groups after treatment. n = 3. **c** Quantification of the tumor bioluminescence signal (n = 4). **d** Curve of mice weight (n = 6). **e** Kaplan–Meier survival curve for the mice (n = 6). **f** Paraffin sections of HE-stained tumor tissue, as mean ± SD, *p < 0.05. **g** Immunofluorescence of brain sections of each groups after treatment

after establishing a reliable model of glioma. Because the orthotopic implantation glioma model is closer to the actual tumor environment in the human body, it is better to pre-lay the foundation for the drug to enter the clinical research phase. In vivo experiments also showed that the treatment of GOLPH3 as a target to achieve the expected inhibition of glioma cell growth and increased the survival of tumor-bearing nude mice. At the same time, we found that siGOLPH3 did not work as well as siPLK1 in comparison with the mature gene target PLK1, but there was no significant difference in some proliferation inhibition experiments. These prove that it is feasible and potential to use GOLPH3 as a target for the treatment of glioma. During our experiments, A2-CL/siGOLPH3 delivery did not significantly affect other tissues and organs. No nude mice died of liposomal or other side effects. With the mature liposome synthesis technology, our experiment is safe.

Taken together, we delivered siGOLPH3 using CLs to demonstrate that GOLPH3 can be used as a target for the treatment of gliomas. This study provides another alternative safe and effective treatment strategy for the treatment of glioma. However, the specific clinical effects and possible mechanisms involved also need further verification.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent Informed consent was obtained from all individual participants included in the study.

Research involving human or animal rights This article does not contain any studies with human participants performed by any of the authors. This article contains studies with animals performed by authors and all applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

References

1. Wen PY, Kesari S (2008) Malignant gliomas in adults. N Engl J Med 359:492–507
2. Sukhdeo K, Hambardzumyan D, Rich JN (2011) Glioma development: where did it all go wrong? Cell 146:187–188
3. Jain RK, Di TE, Duda DG, Loeffler JS, Sorensen AG, Batchelor TT (2007) Angiogenesis in brain tumours. Nat Rev Neurosci 8:610–622
4. Bumcrot D, Manoharan M, Koteliansky V, Sah DWY (2006) RNAi therapeutics: a potential new class of pharmaceutical drugs. Nat Chem Biol 2:711–719
5. Rietwyk S, Peer D (2017) Next-generation lipids in RNA interference therapeutics. ACS Nano 11:7572–7586
6. Dowdy SF (2017) Overcoming cellular barriers for RNA therapeutics. Nat Biotechnol 35:222–229
7. Cohen ZR, Ramishetti S, Peshesyaloz N, Goldsmith M, Wohl A, Zibly Z, Peer D (2015) Localized RNAi therapeutics of chemoresistant grade IV glioma using hyaluronan-grafted lipid-based nanoparticles. ACS Nano 9:1581–1591
8. Zhou J, Xu T, Qin R, Yan Y, Chen C, Chen Y, Yu H, Xia C, Lu Y, Ding X (2012) Overexpression of golgi phosphoprotein-3 (GOLPH3) in glioblastoma multiforme is associated with worse prognosis. J Neurooncol 110:195–203
9. Buschman MD, Rahajeng J, Field SJ (2015) GOLPH3 links the golgi, DNA damage, and cancer. Can Res 75:624–627
10. Scott KL, Kabbarah O, Liang MC, Ivanova E, Anagnostou V, Wu J, Dhakal S, Wu M, Chen S, Feinberg T (2009) GOLPH3 modulates mTOR signalling and rapamycin sensitivity in cancer. Nature 459:1085–1090
11. Zhou X, Xie S, Wu S, Qi Y, Wang Z, Zhang H, Dong L, Wang X, Yu D, Liu G (2017) Golgi phosphoprotein 3 promotes glioma progression via inhibiting Rab5-mediated endocytosis and degradation of epidermal growth factor receptor. Neuro Oncol 19(12):1628–1639
12. Boado RJ (2007) Blood–brain barrier transport of non-viral gene and RNAi therapeutics. Pharm Res 24:1772–1787
13. Podesta JE, Al-Jamal KT, Herrero MA, Tian B, Ali-Boucetta H, Hegde V, Bianco A, Prato M, Kostarelos K (2010) Antitumor activity and prolonged survival by carbon-nanotube-mediated therapeutic siRNA silencing in a human lung xenograft model. Small 5:1176–1185
14. Lv H, Zhang S, Wang B, Cui S, Yan J (2006) Toxicity of cationic lipids and cationic polymers in gene delivery. J Control Release 114:100–109
15. Oller-Salvia B, Sanchez-Navarro M, Giralt E, Teixido M (2016) ChemInform abstract: blood—brain barrier shuttle peptides: an emerging paradigm for brain delivery. Chem Soc Rev 47:4690–4707
16. Cho CF, Wolfe JM, Fadzen CM, Calligaris D, Hornburg K, Chiocca EA, Agar NYR, Pentelute BL, Lawler SE (2017) Blood-brain-barrier spheroids as an in vitro screening platform for brain-penetrating agents. Nat Commun 8:15623
17. Yang ZZ, Li JQ, Wang ZZ, Dong DW, Qi XR (2014) Tumor-targeting dual peptides-modified cationic liposomes for delivery of siRNA and docetaxel to gliomas. Biomaterials 35:5226–5239
18. Mckinley KL, Cheeseman IM (2014) Polo-like kinase 1 licenses CENP-A deposition at centromeres. Cell 158:397–411
19. Gao H, Zhang S, Cao S, Yang Z, Pang Z, Jiang X (2014) Angiopep-2 and activatable cell-penetrating peptide dual-functionalized

nanoparticles for systemic glioma-targeting delivery. Mol Pharm 11:2755–2763
20. Semple SC, Akinc A, Chen J, Sandhu AP, Mui BL, Cho CK, Sah DWY, Stebbing D, Crosley EJ, Yaworski E (2010) Rational design of cationic lipids for siRNA delivery. Nat Biotechnol 28:172–176
21. Liu H, Qiao C, Yang J, Weng J, Zhang X (2014) Self-assembling doxorubicin-prodrug nanoparticles as siRNA drug delivery system for cancer treatment: in vitro and in vivo. J Mater Chem B 2:5910–5924
22. Shukla A, Dong K, Sharma M, Magidson V, Loncarek J (2015) Plk1 relieves centriole block to reduplication by promoting daughter centriole maturation. Nat Commun 6:8077
23. Zhou X, Zhan W, Bian W, Hua L, Shi Q, Xie S, Yang D, Li Y, Zhang X, Liu G (2013) GOLPH3 regulates the migration and invasion of glioma cells though RhoA. Biochem Biophys Res Commun 433:338–344
24. Zhang X, Ding Z, Mo J, Sang B, Shi Q, Hu J, Xie S, Zhan W, Lu D, Yang M (2015) GOLPH3 promotes glioblastoma cell migration and invasion via the mTOR-YB1 pathway in vitro. Mol Carcinog 54:1252–1263
25. Abbott N, Ronnback L, Hansson E (2006) Astrocyte-endothelial interactions at the blood-brain barrier. Nat Rev Neurosci 7:41–53
26. Hobbs SK, Monsky WL, Yuan F, Roberts WG, Griffith L, Torchilin VP, Jain RK (1998) Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment. Proc Natl Acad Sci USA 95:4607–4612
27. Miller TE, Liau BB, Wallace LC, Morton AR, Qi X, Dixit D, Factor DC, Kim LJY, Morrow JJ, Wu Q (2017) Transcription elongation factors represent in vivo cancer dependencies in glioblastoma. Nature 547:355–359