Chlorotoxin fusion protein regulates miR-374a and TNFAIP8 expression and inhibits glioma cell proliferation and promotes apoptosis

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Abstract Glioblastoma multiforme is the most common primary central nervous system malignancy, accounting for half of all intracranial primary tumors. In this study we constructed a multifunctional chlorotoxin fusion protein E-CHP that combines enhanced green fluorescent protein (E), glioma-targeting peptide chlorotoxin (C), destabilizing lipid membrane peptide riHA2 (H), and C-terminal and mouse double minute domains of p53 (P). E-CHP was expressed in *Escherichia coli* and purified by His affinity chromatography. Fluorescence microscopy observation showed that E-CHP could effectively target glioma cells; real-time quantitative PCR revealed that E-CHP increased miR-374a expression; and the dual

luciferase reporter assay showed that tumor necrosis factor alpha-induced protein (TNFAIP8) is a direct target of miR-374a. E-CHP and miR-374a inhibited the proliferation and migration of glioma cells, and Western blot analysis indicated that they suppressed TNFAIP8 expression in glioma cells and promoted the expression of caspase-3 and -8. Finally, E-CHP and miR-374a stimulated the apoptosis of glioma cells, as determined by flow cytometry analysis. These results suggest that miR-374a is a new candidate target for glioma therapy, whereas E-CHP fusion protein has the potential to be developed as a multifunctional carrier for targeted drug delivery and therapy.

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Introduction

Glioblastoma multiforme, also known as glioma, is the most common and highly malignant primary central nervous system tumor, accounting for about half of all primary intracranial tumors (Nabors et al. 2017). Owing to its invasive growth, high rate of recurrence, and resistance to radiation and drugs, surgical resection, radiotherapy, and chemotherapy are not effective in the treatment of glioma. The average survival time of glioma patients is just 12 to 14 months, and the

3-year survival rate is 3–5% (Alexander and Cloughesy 2017; Nabors et al. 2017; Tykocki and Eltayeb 2018).

Protein transduction is a promising method for delivering therapeutic proteins to glioma cells (Heitz et al. 2009; Raucher and Ryu 2015). Glioma-targeting and cell-penetrating peptides can enhance tumor anchoring and transduction efficiency. For example, RGD and F3 have been used to target integrin $\alpha_v\beta_3$ (Hu et al. 2013; Qin et al. 2014). Transcription transactivating protein (TAT) of human immunodeficiency virus 1 and polyarginine (11R) are the most widely used cell-penetrating peptides for protein delivery to cancer cells (Futaki and Nakase 2017; Gump and Dowdy 2007). Scorpion chlorotoxin (CTX), a 36-amino acid peptide, has high affinity for glioma-specific chloride channels, but not normal human cells (Cheng et al. 2014). CTX can inhibit the migration and invasion of primary glioma (Cohen-Inbar and Zaaroor 2016; Deshane et al. 2003), but has no effect on glioma cell proliferation, although the mechanistic basis for these effects remains unclear.

Micro (mi)RNAs are small, non-coding RNAs about 22 nucleotides in length that regulate the expression of oncogenes and tumor suppressor genes by targeting the 3'-untranslated region (3'-UTR) of mRNAs, thereby promoting their degradation or inhibiting their translation. It is estimated that miRNAs regulate about two-thirds of human genes (Bartel 2004). Some miRNAs are dysregulated in cancers, which could play an important role in regulating apoptosis, proliferation, and differentiation in glioma cells through targeting of tumor suppressor genes or oncogenes (Ahir et al. 2017; Jiang et al. 2017; Zhen et al. 2017). A microarray analysis carried out in our laboratory showed that CTX treatment stimulated miR-374a expression 12 folds in U87MG glioma cells (data not shown). We therefore speculated that miR-374a could be involved in the migration and invasion of glioma cells, and is thus, a potential target for glioma treatment.

Tumor necrosis factor alpha-induced protein (TNFAIP8) maintains the balance between immunity and tumorigenesis. TNFAIP8 is overexpressed in most tumor cells and can inhibit TNF to suppress cell apoptosis (Lou and Liu 2011). Tumors with high TNFAIP8 levels tend to be more malignant than those with low expression and are associated with reduced survival in patients. TNFAIP8 is also implicated in the

proliferation, migration, and invasion of tumor cells; its overexpression promotes the transition from G1 to S phase, which accelerates cell proliferation (Kumar et al. 2004; Yang et al. 2019).

We previously constructed the multifunctional fusion protein GHPc + mT, which combines glioma-targeting peptide GL1 (G) and the C terminus (Pc) and mouse double minute domain (Pm) of p53 with the destabilizing lipid membrane peptide riHA2 (H) and cell-penetrating peptide TAT (T) (Yu et al. 2016). GHPc + mT was shown to inhibit proliferation, migration, and invasion and induce apoptosis in glioma cells. In the present study, we constructed the multifunctional fusion protein E-CHP, which combines enhanced green fluorescent protein (EGFP [E]), CTX (C), H, Pc, and Pm (P), and investigated whether E-CHP shows superior targeting to glioma cells and affects miR-374a and TNFAIP8 expression and cell apoptosis.

Materials and methods

Cell culture and transfection

U87MG and U251 human glioma cells and human embryonic kidney 293T cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in Dulbecco's Modified Eagle's Medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 U/l penicillin, and 100 mg/l streptomycin at 37 °C and 5% CO₂.

miR-374a mimic, inhibitor, and negative control oligonucleotides were synthesized by GenePharma (Shanghai, China). Cell transfection was performed using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions.

Plasmid construction, expression, and purification of E-CHP fusion protein

To express the E-CHP fusion protein, the gene was amplified from the pET-CTX-HA2-Pc-Pm plasmid (previously constructed in our laboratory) using primers P1 and P2 (sequences are listed in Table S1). The PCR product was purified and digested with XhoI and SalI (Takara Bio, Dalian, China) and

inserted into the pCold-EGFP-CTX vector (previously constructed in our laboratory) digested with the same enzymes, yielding pE-CHP. The recombinant plasmid was verified by sequencing (Sangon Biotech, Shanghai, China).

Expression and purification of the fusion protein was carried out as previously described (Yu et al. 2016). Briefly, pE-CHP plasmid was transformed into *E. coli* BL21 (DE3) cells, which were then cultured in Luria–Bertani medium containing 100 mg/l ampicillin at 37 °C. When the optical density of the culture at 600 nm reached 0.5, recombinant protein expression was induced by adding isopropyl- β -D-thiogalactopyranoside at a final concentration of 1 mM overnight at 16 °C. The cells were harvested, sonicated, and centrifuged. The fusion protein was purified using a Ni²⁺-nitriloacetic acid NTA HisBind column (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Purified proteins were dialyzed against phosphate-buffered saline (PBS, pH 7.4) at 4 °C for 24 h and stored at – 80 °C.

Fluorescence microscopy

To evaluate the uptake of fusion proteins containing glioma-targeting peptide, CTX, and EGFP, U87MG cells were incubated with 10 μ M E-CHP for 4 h, then washed twice with PBS and fixed in 4% paraformaldehyde for 10 min followed by incubation with 4',6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO, USA) for 5 min. The fluorescence signal was visualized with a fluorescence microscope (IX71; Olympus, Tokyo, Japan). EGFP served as the control.

Cell viability and migration assays

Cells were seeded in 96-well plates at 2×10^3 cells per well and treated with different concentrations of E-CHP or transfected with miR-374a or negative control (NC) mimic for different periods of time. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; 20 μ l, 5 mg/ml) (Sigma-Aldrich) was added to each well, followed by incubation for 4 h at 37 °C. The reaction was terminated by adding 150 μ l dimethylsulfoxide (Sigma-Aldrich), and the optical density at 492 nm was measured on a microplate reader after 10 min of low-speed shaking.

Each sample was prepared as five replicates and the experiment was performed three times.

A transwell system (Corning Inc., Corning, NY, USA) with polycarbonate filters (8- μ m pore size) was used for the migration assay. U87MG cells were seeded at 2×10^5 cells/well in 6-well plates and incubated overnight in complete medium; they were then transfected with miR-374a, inhibitor, and NC at a concentration of 2 nM. After 24 h, the cells were digested by trypsinization and collected. Cell suspensions (2×10^4 cells/well) were seeded in 200 μ l medium with 1% FBS in the upper chambers. Medium containing 10% FBS in the lower chamber served as a chemoattractant. For E-CHP treatment, 10 μ M E-CHP was added to both the upper and lower chambers. The cells were incubated for 24 h and the chamber was washed with PBS, and the cells on the top surface of the insert were removed with a cotton swab. Cells adhering to the lower surface were fixed with methanol, stained with 0.1% crystal violet, and counted under an inverted microscope in nine random fields of view (20 $\times$ objective).

RNA extraction and quantitative real-time (qRT-PCR)

After incubation with E-CHP (10 μ M) or transfection with miR-374a mimic for 24 h, total RNA was extracted from U87MG cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions; 1 μ g of total RNA was used for cDNA synthesis with the First Strand cDNA Synthesis Kit I (Takara Bio, Otsu, Japan). qRT-PCR was performed using the One Step SYBR PrimeScript RT-PCR Kit II (Takara Bio) according to a standard protocol. U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes were used as internal controls for miR-374a and TNFAIP8, respectively. Stem-loop reverse transcription was performed for qRT-PCR detection of miR-374a. The primers 374a-RT and U6-RT were used to amplify miR-374a; primers U6-F and U6-R were used for U6; primers TNF-1 and TNF-2 were used for TNFAIP8; and primers GAPDH-1 and GAPDH-2 were used for GAPDH. The primers were synthesized by Sangon Biotech. Each sample was prepared in triplicate and gene expression levels were quantified by measuring Ct values and normalized to that of U6 or GAPDH with the $2^{-\Delta\Delta CT}$ method.

miRNA target prediction and dual-luciferase reporter assay

The seed sequence of miR-374a in the 3'-UTR of *TNFAIP8* was predicted with TargetScan (<http://www.targetscan.org>) and miRanda (<http://www.microrna.org/microrna>); the wildtype 3'-UTR fragment of the *TNFAIP8* gene was amplified by PCR from cDNA using primers TNF-3 and TNF-4. PCR products were purified and digested with Sall and SacI restriction enzymes and inserted into the pGLO vector (Promega, Madison, WI, USA) digested with the same enzymes, yielding pGLO-TNF.

To obtain pGLO-TNF-MUT, the predicted seed sequence from the 3'-UTR of *TNFAIP8* was mutated by overlap PCR using pGLO-TNF as the template. Primers TNF-mut-1 and TNF-mut-2 combined with primers TNF-3 and TNF-4 were used to generate the mutated 3'-UTR, which was inserted into the pGLO vector as described above. The recombinant plasmids were verified by agarose gel electrophoresis and were sequenced by Sangon Biotech.

293T cells were seeded in 24-well plates and cultured until they reached 80–90% confluence. The cells were co-transfected with pGLO-TNF, pGLO-TNF-MUT, or pGLO along with miR-374a mimic or NC (20 nM) using Lipofectamine 2000. The luciferase assay was performed 24 h later using the Dual Luciferase Assay Kit (Promega), according to the manufacturer's instructions. Renilla and firefly luciferase activities were measured and their ratio was calculated. Results represent the mean of at least three independent experiments.

Western blotting

After incubation with miR-374a, inhibitor, NC, or E-CHP for 24 h, total protein was extracted from U87MG cells lysed in RIPA buffer (Sigma-Aldrich) and protein expression was analyzed by Western blotting, as previously described (Yu et al. 2016). Blots were probed with rabbit polyclonal antibodies against caspase-3, -8, and TNFAIP8 (all from Bioworld, St. Louis Park, MN, USA). Mouse monoclonal anti- β -actin antibody was used as the loading control.

Detection of apoptotic cells by flow cytometry

After incubation with E-CHP (10 μ M) or transfection of miR-374a mimic for 48 h, the apoptosis of U87MG cells was evaluated using an Annexin V-Allophycocyanin kit (KeyGEN Biotech, Shanghai, China) on a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA). The cells were collected with EDTA-free trypsin and washed twice with cold PBS, then resuspended in binding buffer and stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide for 15 min. The number of annexin V-FITC-positive apoptotic cells is expressed as percentage of total number of cells counted.

Bioinformatics analysis

Oncomine Platform (www.oncomine.org), an online cancer microarray database, was used to analyze the differential expression of TNFAIP8 between glioblastomas and normal brain samples. Student's t-test was used to generate a p value. The prognostic value of TNFAIP8 mRNA expression was evaluated using Project Betastasis (www.betastasis.com), which contained gene expression data and survival information of 349 clinical glioma patients from TCGA datasets. To analyze the overall survival (OS) of patients with glioblastoma cancer, patient samples were split into two groups by median expression (high vs. low expression) and assessed by a Kaplan–Meier survival plot, with 95% confidence intervals (CI) and log-rank P value.

Statistical analysis

Prism v.7.0 software (GraphPad, San Diego, CA, USA) was used for all statistical analyses. Data are expressed as mean $\pm$ SEM. Differences between groups were analyzed with the two-tailed Student's t test, and $P < 0.05$ was considered statistically significant.

Results

Expression, purification, and glioma cell targeting of E-CHP fusion protein

The CTX-HA2-Pc-Pm fusion gene was amplified and inserted into the C terminus of EGFP to generate pE-CHP. Double enzyme digestion and sequencing confirmed the correct construction of the recombinant plasmid (data not shown). Coomassie brilliant blue staining showed that the E-CHP protein was expressed in *E. coli* and purified by affinity chromatography (Fig. 1A). The molecular weight of E-CHP was about 32 kDa. A schematic illustration of E-CHP is shown in Fig. S1.

After incubation with E-CHP for 2 h, stronger green fluorescence was observed in U87MG cells by fluorescence microscopy, compared to cells treated with the EGFP only, indicating that E-CHP has affinity

for U87MG cells due to the glioma-targeting peptide CTX and was effectively delivered to glioma cells (Fig. 1B).

E-CHP upregulates miR-374a expression in glioma cells

U87MG and U251 cells were treated with E-CHP for 24 h and total RNA was extracted for qRT-PCR analysis of miR-374a. We found that miR-374a expression was increased 2.1-fold in U87MG cells (Fig. 1C) and 3.2-fold in U251 cells (Fig. S2) by E-CHP treatment.

E-CHP and miR-374a inhibit glioma cell proliferation and migration

The effects of E-CHP and miR-374a on the proliferation and migration of glioma cells were evaluated

Fig. 1 Glioma cell targeting and upregulation of miR-374a by fusion protein E-CHP. **A** Coomassie brilliant blue staining of proteins resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis without isopropyl- β -D-thiogalactopyranoside (IPTG) induction (lane 1) and with 0.5 mM IPTG induction (lane 2), along with purified E-CHP protein (lane 3). **B** EGFP

green fluorescence in U87MG cells treated with E-CHP for 24 h; nuclei are stained with 4',6-diamidino-2-phenylindole (blue). Scale bar = 20 μ m. **C** Expression of miR-374a determined by qRT-PCR 24 h after treatment with 10 μ M E-CHP. ** $P < 0.01$. (Color figure online)

with the MTT and transwell assays, respectively. Compared to the control group, proliferation and migration were inhibited by E-CHP and miR-374a in U87MG cells in a time- and dose-dependent manner. There were significant differences between the 24- and 96-h time points at E-CHP concentrations of 5, 10, and 20 μM (Fig. 2A). At 48 and 72 h, 10 and 20 μM E-CHP showed more potent inhibition than 5 μM E-CHP. At 24 and 96 h, the concentration of E-CHP had no effect on its inhibitory effect. After treatment with miR-374a, the cell viability of U87MG cells decreased with the time and showed significant difference compared with NC at 48, 72 and 96 h (Fig. 2B). The suppression of cell proliferation by miR-374a was inversely related to miR-374a concentration, with a significant difference relative to the NC group observed at 1 nM (Fig. 2C).

The effect of miR-374a and E-CHP on the migration of glioma cells was evaluated with the transwell migration assay. Following transfection of miR-374a mimic or treatment with E-CHP, the number of migrating cells was decreased compared to the control group. In contrast, migration was unaffected in cells treated with inhibitor or transfected with the NC (Fig. 2D). Similar effects were observed in U251 cells (Fig. S3). These results suggest that miR-374a and E-CHP inhibit the migration of glioma cells.

TNFAIP8 is a target of miR-374a and is inhibited by miR-374a and E-CHP

miRNA target prediction with TargetScan and miRanda indicated that *TNFAIP8* is a potential target gene of miR-374a (Fig. 3A). To determine whether miR-374a directly binds to the 3'-UTR of *TNFAIP8*, two

Fig. 2 E-CHP and miR-374a suppress glioma cell proliferation and migration. **A** Cell viability was evaluated with the MTT assay following treatment with 5, 10 and 20 μM of E-CHP at 24, 48, 72, and 96 h. **B** Effect of miR-374a on cell viability was analyzed with the MTT assay at 24, 48, 72, and 96 h. **C** Cell

viability was evaluated with the MTT assay following transfection of 0.2, 0.4, 0.6, 0.8, and 1 nM miR-374a. **D** Effect of miR-374a on glioma cell migration, as determined with the transwell assay. * $P < 0.05$, ** $P < 0.01$

Fig. 3 TNFAIP8 is a target gene of miR-374a and is inhibited by miR-374a and E-CHP. **A** Schematic illustration of the TNFAIP8 3'-UTR in pGLO constructs. The sequences of mature miR-374a and wild-type (WT) or mutated (Mut) target sites in the 3'-UTR of TNFAIP8 are shown. **B** Luciferase activity in

seed sequences—namely, UGAUAA and CUGAUAA—were replaced with UGACGA and CUGACGA, respectively, and the dual luciferase reporter assay was performed to evaluate the regulatory interaction. miR-374a overexpression decreased the luciferase activity of the WT, but not the mutated 3'-UTR construct in 293T cells (Fig. 3A), indicating that miR-374a specifically binds the 3'-UTR of *TNFAIP8*.

The effect of miR-374a and E-CHP on *TNFAIP8* mRNA and protein expression was analyzed by qRT-PCR and Western blotting. Compared to NC-transfected cells, *TNFAIP8* mRNA and protein levels were decreased in U87MG cells overexpressing miR-374a

293T cells following transfection. **C**, **E** Effect of miR-374a and E-CHP treatment on *TNFAIP8* mRNA expression in U87MG cells, as determined by qRT-PCR. **D**, **F** *TNFAIP8* expression in U87 MG cells was analyzed by Western blotting following miR-374a transfection and E-CHP treatment. * $P < 0.05$, ** $P < 0.01$

(Fig. 3C, D). A similar inhibitory effect was observed in U87MG cells after E-CHP treatment (Fig. 3E, F). miR-374a and E-CHP also decreased *TNFAIP8* mRNA and protein expression in U251 cells (Figs. S4 and S5). These findings indicate that *TNFAIP8* is regulated by miR-374a and E-CHP.

E-CHP and miR-374a promote glioma cell apoptosis

To investigate the effect of miR-374a and E-CHP on glioma cell apoptosis, flow cytometry analysis and Western blotting were carried out 24 h after transfection or treatment. The apoptosis rates of cells treated

with miR-374a mimic and E-CHP were 19.19% and 12.86% higher, respectively, than that of the NC mimic group (Fig. 4A). Western blot analysis revealed that the levels of the apoptosis-related proteins caspase-3 and -8 were increased in U87MG glioma cells treated with miR-374a and E-CHP; the expression was upregulated and markedly higher than in the control and inhibitor groups (Fig. 4B). A similar trend was observed in U251 cells (Fig. S6). These results indicate that E-CHP and miR-374a promote glioma cell apoptosis by stimulating caspase-3/8.

Discussion

In this study, we modified the multifunctional fusion protein E-CHP using CTX for glioma cell targeting. TM601, a derivative of CTX, is currently in phase II trial as a ^{131}I label imaging reagent for the diagnosis of glioma (Cheng et al. 2014). The two short peptides derived from P53 in E-CHP have been reported to inhibit proliferation and induce apoptosis in glioma cells (Yu et al. 2016). E-CHP effectively targeted and was internalized by U87MG cells. The upregulation of

miR-374a by E-CHP suggests that miR-374a mediates the effects of E-CHP in glioma cells, which included decreased cell viability, proliferation, and migration and induction of apoptosis. The fact that CTX was not shown to inhibit glioma cell proliferation implies that the two P53 peptides in E-CHP have an additive effect.

Recent reports indicate that miR-374a plays dichotomous roles in tumor biology: it was found to function as an oncogene in osteosarcoma, breast cancer, and gastric cancer (He et al. 2015; Zhang et al. 2018), but as a tumor suppressor in colon, esophageal squamous cell, and lung cancers (Chen et al. 2019; Wu et al. 2016). Several target genes of miR-374a have been identified that are related to cell proliferation, metastasis, and sensitivity to radiotherapy. However, the role of miR-374a in glioma cells has not been previously established. The results of the dual-luciferase reporter assay demonstrated that TNFAIP8 is a direct target of miR-374a, as its mRNA and protein levels were decreased by E-CHP treatment and miR-374a transfection. Thus, miR-374a may act as a tumor suppressive miRNA in glioma cells.

Like other regulators of apoptosis, TNFAIP8 contains a death effector domain and its overexpression

Fig. 4 E-CHP and miR-374a promote glioma cell apoptosis. **A** Increased apoptosis rate in miR-374a- and E-CHP-treated U87MG cells, as demonstrated by flow cytometry analysis.

B Caspase-3/8 expression in U87 MG cells was analyzed by Western blotting following E-CHP treatment or miR-374a transfection. * $P < 0.05$

Fig. 5 TNFAIP8 expression level in glioblastoma multiforme and association with median survival time. **A** TNFAIP8 expression in TCGA dataset (10 normal vs. 542 glioblastoma specimens). TNFAIP8 was significantly upregulated in glioma

inhibits caspase-dependent apoptosis (Laliberte et al. 2010). According to data from TCGA, expression of the *TNFAIP8* gene is 2.8-fold higher in gliomas than in paracancerous tissue (Fig. 5A), and patients with elevated TNFAIP8 expression have lower median survival time (Fig. 5B). Suppression of TNFAIP8 expression inhibited tumor cell proliferation, migration, and invasion and promoted apoptosis (Niture et al. 2018; Yang et al. 2014). The anti-apoptotic effect of TNFAIP8 may be attributable to the induction of nuclear factor (NF)- κ B transcription factor. Studies have shown that in cells stimulated with TNF- α or in which NF- κ B is activated, TNFAIP8 was upregulated and pro-apoptotic caspase-8 was suppressed, resulting in the activation of caspase-3, which prevented apoptosis, and thus, indirectly promoted cell survival (Padmavathi et al. 2018; Woodward et al. 2010; You et al. 2001). In this study, treatment with E-CHP and miR-374a transfection decreased TNFAIP8 expression in glioma cells and increased their apoptosis, which be due to the increases in caspase-3 and -8 levels. Although further studies are needed to elucidate the relationship between E-CHP and miR-374a, our data indicate that miR-374a is a potential new target for glioma therapy and that E-CHP may be developed into a targeted multifunctional therapy protein.

tissue (ONCOMINE database, <https://www.oncomine.org>). **B** Elevated expression of TNFAIP8 is associated with reduced median survival time in glioma patients (Betastasis database, <http://www.betastasis.com>)

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Compliance with ethical standards

Conflict of interest All authors declare no conflict of interests.

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