Glioma targeting peptide in combination with the P53 C terminus inhibits glioma cell proliferation in vitro

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Abstract Glioma is a prevalent malignant primary brain tumor in adults, the treatment for which remains a challenge due to its high infiltration and recurrence. Hence, treatments that lead to the suppression of glioma cell migration and invasion may be used in addition to surgery to increase the therapeutic outcome. In this study, we aimed to construct a multifunctional protein that would exert an effect on glioma cell proliferation and migration. The protein is named GL1-P53C-11R and it consists of the glioma-targeting peptide GL1 (G), the P53 C terminus (Pc) and the cell-penetrating peptide arginine (R). GL1-P53C-R was expressed with the fusion protein ZZ and immunofluorescence analysis showed effective delivery of the fused ZZ-GL1-P53C-R protein represented as ZZ-GPcR. The ZZ-GPcR exhibited an inhibitory effect on the proliferation, migration and invasion of U87ΔEGFR cells. Western blotting results indicated

that it caused significant changes in the expression levels of cell cycle and apoptotic proteins. Flow cytometric analysis showed increase apoptosis. Our findings suggest that the P53C in the fusion protein ZZ-GPcR can enter into glioma cells to exert its inhibitory effect.

Keywords Glioma · Targeting peptide · P53 C terminus · Cell proliferation · Apoptosis

Introduction

Glioblastoma multiforme (GBM) is one of the most malignant brain tumors, making up approximately 50% of all primary intracranial tumors (Clark et al. 2016). However, at present, treatment mainly consists of surgical resection combined with standard radiation therapy and chemotherapy. Due to its highly aggressive growth and invasion, glioma has an exceptionally high rate of recurrence despite intensive multi-modal treatments and many patients succumb to the disease with an average survival period of barely one year following standard therapy (Emdad et al. 2012; Appin et al. 2013).

Cell-penetrating peptides (CCPs), also referred to as protein-transduction domains (PTDs), are a class of peptides that are able to cross the cellular membrane and deliver macromolecules such as proteins, nucleic acids and nanoparticles safely into cells (Fonseca et al.

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2009; Chugh et al. 2010; Bolhassani et al. 2017). Intracellular delivery is necessary for many therapeutic molecules with targets inside the cell. Owing to their reduced bioavailability in vivo, many promising drug candidates are not developed to their full therapeutic potential (Dinca et al. 2016). The intracellular delivery of CPPs has also been used for the therapeutic control of cellular behavior (Kamei et al. 2016). TAT and polyarginine are the most widely used CPPs for the delivery of cargo into cells (Heitz et al. 2009; Krautwald et al. 2016). Recent studies showed that CPPs have the potential to improve the intestinal absorption of peptide and protein drugs when used in the non-covalent drug-CPP approach (Kamei et al. 2016). CPPs have also been used to treat immunological diseases through the delivery of immunomodulatory molecules in vivo (Lim et al. 2016).

However, the lack of cell specificity may limit the application of CPPs in vivo. In combination with glioma targeting peptides, their tumor targeting ability could be enhanced. GL1 is a 12 amino acid peptide that was demonstrated to target glioma cells with high specificity (Ho et al. 2010). As a key tumor suppressor protein with numerous growth inhibitory functions, P53 undergoes various post-translational modifications that may result in either activation or repression of its activity (Uo et al. 2007; Chen et al. 2016). A short peptide derived from the P53 C-terminal lysine-rich regulatory domain (amino acids 361–382) modulates the DNA binding activity of wild-type P53 and induces apoptosis in human tumor cell lines of different origins expressing mutant or wild-type P53 protein (Araki et al. 2010; Hamard et al. 2012).

We previously combined GL1, TAT, riHA2 (NH2-terminal 20 amino acid peptide of the influenza virus haemagglutinin-2 protein) with the P53 C terminus and MDM2 (mouse double minute) binding domain (Yu et al. 2016). The MDM2 binding domain of P53 is involved in cell cycle arrest, DNA repair and apoptosis (Yamada et al. 2012). The fusion protein was able to induce glioma cell apoptosis and to inhibit glioma cell proliferation. In this study, we aimed to optimize the functional part of the fusion protein. We therefore fused the Pc with glioma targeting peptide (GL1) and polyarginine (R). The GPcR was then fused with ZZ (antibody affinity motif of protein A) to form ZZ-GPcR, and its effect on glioma cell proliferation and migration was evaluated.

Materials and methods

Cell culture

The human malignant glioma cell lines paU87 and U87ΔEGFR (which expresses epidermal growth factor receptor variant type III, EGFRvIII) were kindly donated by Professor Hideki Matsui of Okayama University (Okayama, Japan). The cells were maintained in Dulbecco's modified Eagle medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen Gibco, Carlsbad, CA, USA) and 1% penicillin–streptomycin (Sangon biotech, Shanghai, China). All cells were cultured in a 37 °C incubator with 5% CO₂.

Plasmid construction, expression and purification of fusion proteins

To express ZZ-GPcR, the gene was amplified from the plasmid pET-ZZ-GL1-riHA2-P53C-11R (pET-ZZ-GHPcR, previously constructed in our laboratory, Yu et al. 2016) using reverse PCR. The primer sequences were 5'-CCGGGATCCGGGGAGCAGGGCTCACTCAG-3' and 5'-CCGGGATCCGAGCCTCCAGTCAGGGTTCGGTGG-3' (underlined sequences indicate the BamHI site). The PCR product was purified and digested with BamHI. The digested product was self-ligated with T₄ DNA ligase to form the prokaryotic expression vector pET-ZZ-GL1-P53 C-11R (pET-ZZ-GPcR). To express GL1-EGFP-R, two phosphorylated oligonucleotides (5'-AATTCGCGCCGCAGGAGACGACGGCGACG GCGAAGAAGGA-3' and 5'-AGCTTCCTTCTTCGCCGTCGCCGT CGTCTCCTGCG GCGCG-3') were annealed and inserted into the EcoRI and Hind III digested pET-GL1-EGFP, resulting in pET-GL1-EGFP-R. All recombinant plasmids were identified by electrophoresis on a 1% agarose gel following digestion with the above restriction enzymes and further subjected to sequencing (Sangon Biotech). The plasmids pET-EGFP, pET-GL1-EGFP and pET-ZZ used for the expression of EGFP, GL1-EGFP and ZZ, respectively, were constructed in our previous study (Yu et al. 2016).

The expression and purification of the fusion proteins were performed as previously described (Feng et al. 2007). Briefly, expression plasmids were transformed into *Escherichia coli* (*E. coli*) BL21 (DE3) and cultured overnight in Luria–Bertani

medium containing 100 µg/ml ampicillin at 37 °C. When the optical density of the culture at 600 nm reached 0.4–0.6, the protein expression was induced by adding isopropyl-*b*-D-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM and cultured for 6–8 h at 30 °C. *E. coli* cells were then collected by centrifugation and subjected to sonication. The proteins were purified using a Ni²⁺-HisBind column (Thermo Scientific, Waltham, MA, USA) and analyzed by SDS-PAGE. The purified proteins were dialyzed against PBS at 4 °C for 24 h and stored at –80 °C until use. Luria–Bertani medium, ampicillin and IPTG were all purchased from Sangon biotech.

Uptake analysis of proteins containing GL1 and polyarginine

To evaluate the specificity and uptake of the fusion proteins containing GL1 and polyarginine, cells were incubated with 2 µM EGFP, GL1-EGFP and GL1-EGFP-11R for 2 h. They were then washed twice with PBS and fixed in 4% paraformaldehyde for 10 min, followed by incubation with DAPI (Life Technologies, Carlsbad, CA, USA) for 5 min. To demonstrate the intracellular delivery of the ZZ-GPcR, U87ΔEGFR cells were incubated with 5 µM protein using the ZZ as control, after 2 h incubation, immunofluorescence analysis was carried out using anti-His mouse monoclonal antibody (mAb, TransGen Biotech, Beijing, China) as the primary antibody, Alexa Fluor 594-labelled mouse IgG purchased from Life Technologies (Willow Creek, OR, USA) was used as the second antibody. Washing, fixing and nucleus dying were performed as above. Fluorescence signal was visualized by confocal laser microscopy (Leica, Nussloch, Germany).

Cell viability assay

U87ΔEGFR cells were seeded in 96-well plates at a density of 2000 cells/well and incubated with 5 µM ZZ-GPcR and ZZ (as negative control) for 24, 48, 72 and 96 h, and cell viability was analyzed using the Cell Counting Kit 8 assay (Dojindo, Tokyo, Japan). The effect of protein concentration on cell viability was investigated by incubating the cells with proteins at varying concentrations of 1, 2 and 5 µM for 96 h. Following incubation for the indicated time at 37 °C, 10 µl CCK-8 was added to each well and incubated for

1–2 h. The absorbance value was measured at 450 nm with a microplate reader and was plotted against the incubation time.

Colony formation assay

U87ΔEGFR cells incubated with 5 µM ZZ-GPcR for 72 h were seeded in six-well culture plates at 300 cells/well ($n = 3$ per group). Following incubation for 10 days at 37 °C, the cells were fixed with methanol and stained with 2% Giemsa solution. After washing twice with PBS, the numbers of colonies with more than 50 surviving cells were counted under an inverted microscope. The colony formation efficiency was calculated as follows: number of colonies/number of cells inoculated $\times 100\%$. Data are expressed as the mean of three experiments for each group.

Cell migration and invasion assays

For the cell migration assay, 2×10^4 U87ΔEGFR cells were seeded in 200 µl medium with 1% FBS in the upper chambers of a 24-well transwell plate containing polycarbonate filters with 8 µm pores (Corning Inc., Corning, NY, USA). In the lower chambers, 500 µl medium containing 10% FBS was added as the chemoattractant. To the upper and lower chambers, ZZ-GPcR was added to a final concentration of 5 µM. After the cells were incubated for 24 h at 37 °C in a 5% CO₂ atmosphere, 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 Giemsa solution and counted under an inverted microscope in nine random fields of view (40 $\times$ objective). The procedure for the cell invasion assay was similar to that of the migration assay, except that the transwell membranes were coated with Matrigel (BD Biosciences, San Jose, CA, USA) at 1:3 dilution and incubated for 30 min at 37 °C. Cells adhering to the lower surface were counted in the same way as those for the cell migration assay.

Western blotting

For recombinant proteins identification, *E. coli* cell lysates with or without IPTG induction were separated by SDS-PAGE and transferred to a PVDF membrane, and then an anti-His mouse mAb was used as first

antibody. For protein level analysis in the glioma cell line U87ΔEGFR treated with a final concentration of 5 μM ZZ-GPcR for 72 h, cells were lysed in RIPA buffer (Sigma, St. Louis, MO, USA) and the protein concentration in the cell lysates was quantified using a BCA protein assay kit (Multisciences, Hangzhou, China). Equal concentrations of proteins were separated by SDS-PAGE and transferred to a PVDF membrane. Blots were incubated with the following primary antibodies against: Bcl-2, Bax, E2F-1, phospho-Rb, p21 and β-actin (all rabbit polyclonal); CDK4 (mouse monoclonal); and p16 (goat polyclonal). All primary antibodies were purchased from Bioworld Technology, Inc. (St. Louis Park, MN, USA). After incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies, positive bands were visualized by employing an enhanced chemiluminescence method (Thermo Scientific, Waltham, MA, USA) on a ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, USA).

Apoptosis assay

After incubating with ZZ-GPcR for 72 h, U87ΔEGFR cells were washed twice in cold PBS and resuspended in cold binding buffer, then treated with annexin V-FITC and propidium iodide for 10 min. Apoptosis was evaluated using a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The number of annexin V-FITC-positive apoptotic cells was expressed as percentage of total number of cells counted.

Statistical analysis

All experiments were repeated three times. Data are shown as mean ± SD and were analyzed using the Student's *t* test. The level of significance was taken at $p < 0.05$.

Results

Expression and purification of recombinant proteins

By inverse PCR, ZZ-GPcR was successfully amplified and was confirmed by double enzyme digestion with Nde I and Xho I, which showed the correct

construction (data not shown). Sequencing analysis verified the absence of any mutations of the recombinant plasmids pET-ZZ-GPcR and pET-GL1-EGFP-R. ZZ-GPcR (22.9 kDa) and GL1-EGFP-R (33.6 kDa) were expressed, purified and identified by Coomassie Brilliant Blue staining (Fig. 1a, b). Western blotting analysis confirmed the size of the expressed proteins (Fig. 1c, d).

Glioma targeting activity of GL1, polyarginine-containing peptide

After incubation with GL1-EGFP-R for 2 h, fluorescence in U87Δ EGFR cells that overexpressed EGFRvIII could be clearly observed by confocal microscopy. But in paU87 cells which expressed less EGFR, the signal intensity was lower than that in U87ΔEGFR cells. Compared with GL1-EGFP-R treated cells, the fluorescence signals in cells treated with EGFP without the targeting peptide and the penetrating peptide, and GL1-EGFP containing only

Fig. 1 Expression, purification and identification of the fusion proteins. Coomassie Brilliant Blue staining (**a**, **b**) and Western blotting (**c**, **d**) of ZZ-GPcR and GL1-EGFP-R. Lane 1, ZZ-GPcR (**a**, **c**) and GL1-EGFP-R (**b**, **d**) protein expression without IPTG induction; lane 2, ZZ-GPcR (**a**, **c**) and GL1-EGFP-R (**b**, **d**) protein expression with 0.5 mM IPTG induction; lane 3, purified ZZ-GPcR (**a**) and GL1-EGFP-R (**b**) were used as reference

the targeting peptide were weaker, indicating the high affinity of GL1 to U87ΔEGFR and the transmembrane transport ability of polyarginine to glioma cells (Fig. 2a). Compared with ZZ treated cells, stronger immunofluorescence signal in U87ΔEGFR cells further proved that ZZ-GPcR could be effectively delivered into glioma cells (Fig. 2b).

Effect of ZZ-GPcR on glioma cell proliferation and colony formation

As shown in Fig. 3a, ZZ-GPcR exhibits a stronger inhibitory effect on U87ΔEGFR than the ZZ control does. Cell proliferation was suppressed from day 2,

with an IC_{50} of 4 μ M. Inhibition by ZZ-GPcR was dose-dependent and reached a level of 52% at 5 μ M on day 4 for U87ΔEGFR (Fig. 3b).

Colony formation analysis showed that the fusion protein ZZ-GPcR could significantly inhibit U87ΔEGFR colony formation as compared to the ZZ control (Fig. 3c).

Effect of ZZ-GPcR on cell migration and invasion

To evaluate the effect of the fusion protein ZZ-GPcR on the migration and invasion potential of the U87ΔEGFR cell line, transwell assays were carried out. Compared to the ZZ control, migration (Fig. 4a,

Fig. 2 Analysis of the targeting specificity with confocal microscopy.

a U87ΔEGFR and paU87 cells were incubated with 2 μ M EGFP, GL1-EGFP and GL1-EGFP-R for 2 h and EGFP fluorescence in cells treated with fusion proteins was visualized. Bar = 100 μ m.
b U87ΔEGFR cells was incubated with 5 μ M ZZ-GPcR and ZZ for 2 h, followed by the treatment with anti-His mouse mAb as the primary antibody and Alexa Fluor 594-labelled anti-mouse IgG as the secondary antibody, the immunofluorescence signals were visualized using a confocal laser microscope. Bar = 50 μ m

Fig. 3 Effect of ZZ-GPcR on glioma cell proliferation and colony formation.

a U87ΔEGFR cells were incubated with the fusion protein ZZ-GPcR (5 μM) for the indicated time and viability was assessed. **b** U87Δ EGFR cells were incubated with the fusion protein ZZ-GPcR at different concentrations for 96 h and viability was assessed. **c** Cells were incubated with 5 μM ZZ-GPcR for 72 h and seeded in six-well culture plates for colony formation assay for 10 days. ZZ was used as a control. **d** Quantitative analysis of colony formation. * $P < 0.05$, ** $P < 0.01$

b) and invasion (Fig. 4c, d) were effectively inhibited by ZZ-GPcR in U87ΔEGFR cells.

Expression of cell cycle related proteins

The expression levels of apoptosis and cell cycle related proteins were altered in the U87ΔEGFR cell line following treatment with ZZ-GPcR, as observed by Western blotting. ZZ-GPcR caused a visible down-regulation in the level of Bcl-2 while it increased the expression of Bax compared to the ZZ control (Fig. 5a).

Compared to the control, treatment with ZZ-GPcR decreased the expression of CDK4, phospho-Rb and E2F-1. Meanwhile, the up-regulation of p21 and p16 was observed (Fig. 5b).

ZZ-GPcR induced apoptosis

ZZ-GPcR induced apoptosis of U87ΔEGFR after 72 h treatment, which was mainly observed at the early apoptosis time point, by flow cytometric analysis. Increase in levels of early and late apoptosis of ZZ-GPcR-treated cells relative to ZZ-treated controls was 16.44 and 5.97% in U87ΔEGFR cells, respectively (Fig. 5c).

Discussion

Most malignant gliomas remain resistant to nearly all aggressive treatment strategies employed. The 5-year survival rate for patients with malignant glioma is less than 10% (Riemenschneider et al. 2010). Till now, there has been no effective method to remove the cancerous tissue entirely without causing damage to the normal brain and thereby avoiding recurrence. Thus, a better understanding of the molecular changes in these malignant cells would help to improve therapy. Recently, targeted therapy in glioblastoma has been demonstrated to provide alternative ways for an effective and personalized therapy (Weathers and Gilbert 2017). EGFR, VEGF, P53, PTEN and other biomarkers have been well investigated in glioma and used as targets for therapy (McNamara et al. 2013). Similarly, for glioma stem cells, targeting CD133 is a potential therapeutic strategy for the elimination of the cancer stem population (Cho et al. 2017).

As a common molecular hallmark of glioma, EGFR promotes a pro-proliferative signal (Fischer and Aldape 2010). The EGFR variant III (EGFR vIII), the most prominent mutated receptor tyrosine kinase receptor, occurs in approximately 50–60% of glioma patients whose tumors show amplification of the wild

Fig. 4 Effect of ZZ-GPcR on glioma cell migration and invasion. **a** Migration of U87ΔEGFR cells treated with the ZZ-GPcR fusion protein, as determined by transwell migration assay. **b** Quantitative analysis of the results in (a). **c** Invasion of

U87ΔEGFR cells treated with the ZZ-GPcR fusion protein as determined by matrigel assay. **d** Quantitative analysis of the results in (c). * $P < 0.05$, ** $P < 0.01$

Fig. 5 Effect of ZZ-GPcR on apoptosis and cell cycle-related protein expression. U87ΔEGFR cells were incubated with ZZ-GPcR (5 μ M) and ZZ (control) for 72 h and apoptosis and cell cycle-related protein expressions were evaluated by Western blotting with β -actin being used as a loading control (**a**, **b**), while apoptosis was evaluated by flow cytometry (**c**)

type EGFR (Gan et al. 2009). We found that GL1 fusion proteins had a higher affinity for the U87ΔEGFR cells that overexpressed EGFR VIII than for the paU87 cells with less EGFR expression (Fig. 1c). Although the membrane receptor of the glioma targeting peptide GL1 has not been investigated, the mutant EGFR may be the potential target of GL1. We also observed a weaker fluorescence signal when EGFP was fused with only GL1 as compared to GL1-EGFP-R. In order to improve the P53C delivery efficiency, in an earlier study, we added a cell-penetrating peptide TAT to the fusion proteins. The combination of the glioma targeting peptide and CPPs showed superior activity in the inhibition of glioma cell proliferation and migration (Yu et al. 2016).

As one of the three defined intrinsically disordered regions (IDR), the P53 C terminus is relatively short and unfolded. The C-terminal domain (CTD) has been shown to participate in every aspect of P53 performance as a transcription factor. Previous studies have shown that the C-terminal region of P53 modulates the susceptibility of P53 to MDM2-mediated degradation (Wang et al. 2009; Park et al. 2016). We had reported that the peptides derived from the P53 C terminus, and the sequence corresponding to the N-terminal MDM2-binding site of P53, can suppress GBM proliferation (Yu et al. 2016). The multifunctional nature of the CTD is likely to depend on its IDR, which possesses a unique amino acid composition that dictates both the intrinsic flexibility and the potential presence of regions known as molecular recognition features (Laptenko et al. 2016).

Our study shows that the fusion protein ZZ-GPcR had a significant inhibitory effect on U87ΔEGFR cell proliferation and migration as compared to the ZZ control. Western blotting confirmed that ZZ-GPcR induced apoptosis via the regulation of the Bcl-2-Bax ratio. Expression levels of cell cycle-related factors were also altered, suggesting that ZZ-GPcR may also induce cell cycle arrest. In conclusion, our result suggests that the fusion protein ZZ-GPcR could be effectively delivered into glioma cells where the P53 C terminus could exert its function. It provides the potential to utilize this domain in combination with other functional domains for protein therapy.

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Compliance with ethical standards

Conflict of interest All authors declare no conflict of interests.

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