



Interference on cytoplasmic polyadenylation element-binding proteins affects the invasion ability of glioma stem cells

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ABSTRACT. Glioma stem cells derived from primary cultures were divided into an experiment group, a control group, and a blank group and subjected to cytoplasmic polyadenylation element-binding protein (CPEBs) interference, transfection with empty vector, and normal culture, respectively, to compare their invasion abilities. Western blotting showed that siRNA-3 had the strongest interfering effect on CPEBs. CPEBs were expressed in the experiment group with green fluorescence at an expression rate of over 70%. Significantly lower CPEB expression was observed in the experiment group compared to in the control and blank groups ($P < 0.05$). After 48-h treatment, the apoptotic rate in the experiment group was 21.43%, which was significantly higher than that in the blank (0.51%) and control (1.43%) groups ($P < 0.05$). After 3 days of treatment, the experiment group grew significantly more slowly than did the control and blank groups ($P < 0.05$). The transwell invasion assay showed that significantly fewer cells in the experiment group penetrated the membrane than did cells in the control and blank groups ($P < 0.05$). After CPEB interference, the growth, proliferation,

and invasion of glioma stem cells were substantially inhibited, providing support for targeted therapy of glioma and for improving prognosis.

Key words: Cytoplasmic polyadenylation element-binding protein; Glioma stem cell; Invasive capability

INTRODUCTION

Cytoplasmic polyadenylation element-binding proteins (CPEBs) play important roles in the development, differentiation, aging, and apoptosis of stem cells by affecting cytoplasmic polyadenylation through mediating mRNA translation (D'Ambrogio et al., 2013). Although the expression of CPEBs in various tumor tissues has been studied, few studies have examined targeted therapy (Bishnoi et al., 2008). In this study, we analyzed the effects of CPEB interference on the invasion ability of glioma stem cells (GSCs).

MATERIAL AND METHODS

Main materials

The U87 cell line was provided by the American Type Culture Collection (Manassas, VA, USA) and used for primary culture at room temperature in DMEM containing 10% fetal bovine serum to obtain GSCs. The pSRL-SIH1-H1-GFP plasmid (Tianjin Ssier Biotechnology Co., Ltd., Tianjin, China) was used as the lentiviral vector (Lee et al., 2013). *Escherichia coli* strain DH5 α [TaKaRa Biotechnology (Dalian) Co., Ltd., Shiga, Japan] was used as competent cells (Xiao et al., 2008).

Methods

Preparation of lentiviral vector

siRNAs were synthesized by designing 3 target sequences for RNA interference based on the gene sequences of CPEBs to transfect U87 cells. The siRNA sequence with the most evident interfering effect on CPEBs was identified by western blotting according to which DNA oligo was prepared, with the sticky ends identical to the restriction sites of the pSRL-SIH1-H1-GFP plasmid (Lee et al., 2013). The products were then transfected into *E. coli* competent cells, and preparation of lentiviral vector was confirmed through cloning, extraction, digestion, identification, and sequencing of the cultured plasmid as well as comparison with positive clones (Ji et al., 2007; Kang et al., 2007) (Figure 1).

Transfection with lentivirus

GSCs were divided into an experiment group, a control group, and a blank group and subjected to CPEB interference, transfection with empty vector, and normal culture, respectively. All cells were inoculated on 6-well plates 1 day before transfection and cultured at room temperature. When cell confluence exceeded 30%, serum-free culture medium was used. When the multiplicity of infection reached 15, lentiviral vector and 5 μ g/mL transfection enhancer (Pomerantz et al., 1990; Equils et al., 2006) were added to each well of the experiment group. Empty vector was added to each well of the control group.

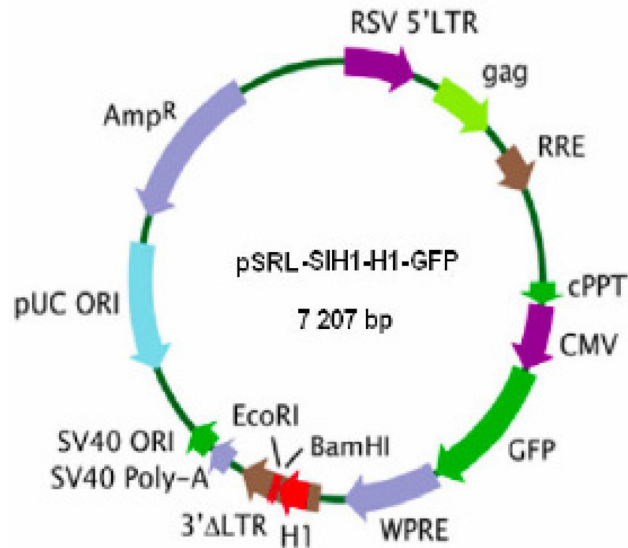


Figure 1. Constitution of lentiviral vector (including pSRL-SIH1-H1-GFP, pHelper 1.0, and pHelper 3.0 plasmids).

Observation indices

Detection of CPEB expressions

Total DNA and total proteins of each group were extracted 24, 48, 72, 96, and 120 h after treatment and reverse-transcribed. Expression changes in mRNA and proteins were detected by western blotting (Hansen et al., 2009). Cell cycle was detected by flow cytometry to observe proliferation. Cell viability was detected using an MTT assay.

Detection of invasion ability

Cell invasion ability was determined using a transwell assay (Li et al., 2013) in triplicate. Invading cells were counted under 8 randomly selected visual fields (200X).

Statistical analysis

All data were analyzed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). The numerical data were compared by χ^2 test, and the categorical data were compared by the *t*-test. $P < 0.05$ was considered to be statistically significant.

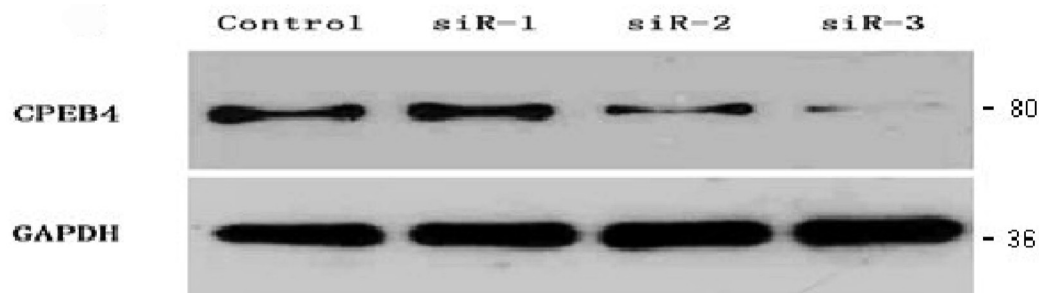
RESULTS

Effects of siRNA interference

The primer sequences and interference outcomes are summarized in Table 1. Western blotting showed that siRNA-3 had the best interfering effect on CPEBs (Figure 2).

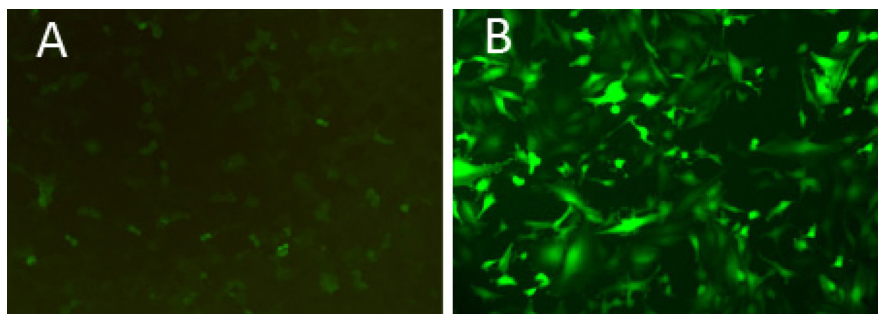
Table 1. siRNA primer sequences and interference outcomes.

| No. | Starting point | DNA sequence | Relative expression (means \pm SD) |
|---------|----------------|----------------------------|--------------------------------------|
| siRNA-1 | 1436 | GCAAGCAACTGGAAT | 0.581 \pm 0.039 |
| siRNA-2 | 1716 | GCTGGAATACTGCCTGAAA | 0.312 \pm 0.041 |
| siRNA-3 | 1807 | GGATCGAATCTCCAGTGTT | 0.070 \pm 0.009 |
| Control | - | 5'-AATTCTCCGAACGTGCACGT-3' | - |

**Figure 2.** siRNA interference outcomes detected by western blotting.

Transfection outcomes

CPEBs were expressed in the experiment group with green fluorescence at an expression rate of over 70% (Figure 3).

**Figure 3.** Green fluorescence of blank group (A) and experiment group (B) (200X).

Expressions of CPEBs

Significantly lower CPEB levels were observed in the experiment group compared to in the control and blank groups ($P < 0.05$) (Table 2).

Table 2. CPEBs expression (means \pm SD).

| Group | 24 h | 48 h | 72 h |
|------------|--------------------|--------------------|--------------------|
| Experiment | 0.589 \pm 0.102 | 0.196 \pm 0.084 | 0.085 \pm 0.014 |
| Control | 0.823 \pm 0.114* | 0.819 \pm 0.118* | 0.820 \pm 0.121* |
| Blank | 0.858 \pm 0.129* | 0.843 \pm 0.104* | 0.844 \pm 0.117* |

*Compared with experiment group, $P < 0.05$.

Cell apoptotic rates

After 48-h treatment, the apoptotic rate of the experiment group was 21.43%, which was significantly higher than in the blank (0.51%) and control (1.43%) groups ($P < 0.05$) (Figure 4).

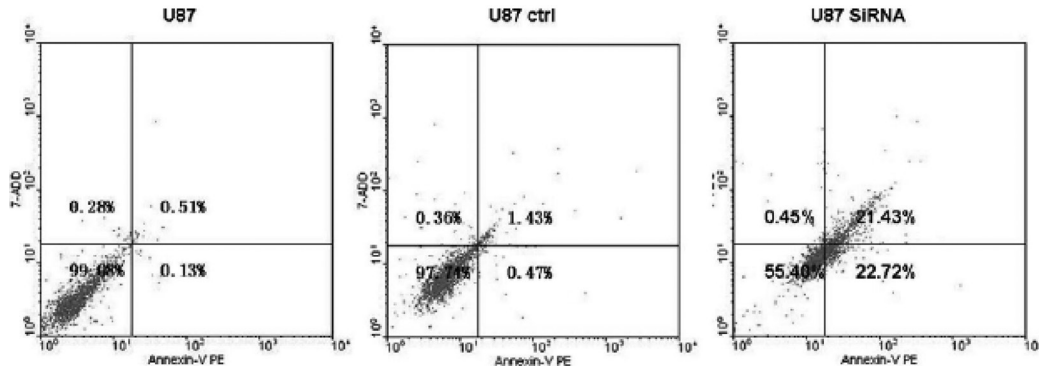


Figure 4. Cell apoptosis rates.

Cell viability

After 3 days of treatment, the experiment group grew significantly more slowly than control and blank groups ($P < 0.05$) (Table 3).

Table 3. Cell viability (means \pm SD).

| Group | 1 day | 2 days | 3 days | 4 days |
|------------|-------------------|-------------------|--------------------|--------------------|
| Experiment | 0.397 \pm 0.025 | 0.391 \pm 0.038 | 0.415 \pm 0.049 | 0.426 \pm 0.038 |
| Control | 0.385 \pm 0.026 | 0.402 \pm 0.065 | 0.481 \pm 0.052* | 0.591 \pm 0.042* |
| Blank | 0.390 \pm 0.031 | 0.410 \pm 0.046 | 0.490 \pm 0.048* | 0.609 \pm 0.035* |

*Compared with experiment group, $P < 0.05$.

Cell invasion ability

The transwell invasion assay showed that significantly fewer cells in the experiment group penetrated the membrane than those in control and blank groups did ($P < 0.05$) (Table 4).

Table 4. Cell invasion ability (means \pm SD).

| Group | Number of membrane-penetrating cells (per visual field) |
|------------|---|
| Experiment | 49.32 \pm 6.97 |
| Control | 153.61 \pm 11.84* |
| Blank | 201.43 \pm 19.33* |

*Compared with experiment group, $P < 0.05$.

DISCUSSION

As one of the most common tumors in the human body, glioma has been treated clinically using many methods, but there are no effective protocols for improving the prognosis and quality

of life (Dong and Huang, 2011). Therefore, studies have focused on the pathogenesis and targeted therapy of glioma (Ahmed et al., 2013). CPEBs are highly expressed in many types of malignant tumors and associated with study and memory, indicating that they may be involved in inherited neurological disorders in addition to the mediation of stem cell development, cell differentiation, aging, and synaptic plasticity (Bishnoi et al., 2008; Fernández-Miranda and Méndez, 2012).

Glioma affects the prognosis of patients mainly because of strong invasion and infiltration, and thus inhibiting the migration of GSCs using non-surgical methods is of great significance (Das et al., 2008). CPEBs are highly conservative RNA binding proteins that predominantly induce the translation of polyadenylation. Because overexpressed CPEBs remarkably promote the growth, development, and angiogenesis of tumor cells (Hansen et al., 2009; D'Ambrogio et al., 2013), we inhibited CPEBs using siRNA-3 for transfection. Characterized by a wide range of host and regular expressions of exogenous genes, lentivirus has been extensively used in gene therapy (Lee et al., 2013). In this study, transfection with a CPEB RNA-interfering lentiviral vector generated stable siRNA, and more than 70% of cells in the experiment group emitted green fluorescence, suggesting that the lentivirus carrying the specific target interference sequence worked well after transfection. With an extended transfection time, the experiment group showed dramatically decreased in CPEB expression, cell viability and invasion ability as well as an increased cell apoptotic rate. Thus, CPEB interference effectively suppressed the growth and proliferation of GSCs, showing promise for the clinical treatment of glioma. Biological functions were altered with decreasing CPEB expressions. Inhibition of mRNA translation was weakened. By forming cytoplasmic polyadenylation ribonucleoprotein complexes or by binding the Maskin protein, highly-expressed CPEBs maintain the poly A tail of mRNA to be short and make recognition of the start codon by 40S ribosomal subunit difficult. As a result, translational output from mRNA cannot be continued (D'Ambrogio et al., 2013). Activation of mRNA translation is attenuated. In contrast, CPEBs extend the poly A tail and the length of mRNA, facilitating the activation and translation of dormant mRNA, generating the cell signaling cascade, and inducing abnormal mRNA translation. Furthermore, CPEBs significantly control aggravating cancerization by playing crucial roles in cell aging and synaptic plasticity (Richter, 2007; Burns and Richter, 2008), and thus are important in the onset, proliferation, invasion, and metastasis of glioma. Hence, it is feasible that controlling CPEBs is of great significance for glioma treatment.

After CPEB interference, the growth, proliferation, and invasion of GSCs were considerably suppressed, providing valuable support for targeted therapy of glioma and for improving prognosis.

Conflicts of interest

The authors declare no conflict of interest.

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