Generation of an AEG-1 Knockout U251 Cell Line Using CRISPR/Cas9 Technology and Characterization of Its Metastatic Behavior: Postprint

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Abstract

Astrocyte elevated gene-1 (AEG-1) is overexpressed in multiple tumor types and participates in tumorigenesis, metastasis, and other processes. The present study employed CRISPR/Cas9 technology to knockout the AEG-1 gene and investigate its role in glioma cell metastasis. Initially, a sgRNA/Cas9 all-in-one expression vector was designed and constructed, then transfected into human glioma U251 cells, with sgRNA activity validated through TA cloning and sequencing; subsequently, a stable AEG-1 knockout U251 cell line was screened and established, and knockout efficiency was assessed via Western blot analysis; finally, the impact of AEG-1 knockout on tumor cell migratory capacity was evaluated using Transwell chamber and wound healing assays. The results demonstrated successful construction of a sgRNA/Cas9 all-in-one expression vector targeting the AEG-1 gene that was consistent with the experimental design, with sgRNA activity confirmed by TA cloning and sequencing; a stable AEG-1 knockout U251 cell line was successfully established, exhibiting a knockout efficiency of up to 98% as determined by Western blot; Transwell chamber and wound healing assay results revealed that the metastatic capacity of the AEG-1 knockout U251 cell line was significantly diminished.

Full Text

The Construction of AEG-1-Knockout U251 Cell Line by CRISPR/Cas9 Technology and Investigation of Its Metastatic Characteristics

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Abstract

Astrocyte elevated gene-1 (AEG-1) is overexpressed in multiple tumor types and participates in tumor formation and metastasis. This study utilized CRISPR/Cas9 technology to knock out the AEG-1 gene and investigate its role in glioma cell metastasis. We designed and constructed a sgRNA/Cas9 dual-expression vector, which was transfected into human glioma U251 cells. TA cloning and sequencing were performed to validate sgRNA activity. Stable AEG-1-knockout U251 cell lines were then screened and established, with knockout efficiency detected via Western blot. Finally, Transwell chamber and scratch assays were employed to evaluate the effect of AEG-1 knockout on tumor cell migration. The results demonstrated successful construction of the AEG-1-targeting sgRNA/Cas9 expression vector, confirmed by TA cloning sequencing showing active sgRNA. We established stable AEG-1-knockout U251 cell lines with knockout efficiency reaching 98% as determined by Western blot. Both Transwell and scratch assays revealed significantly reduced metastatic capacity in AEG-1-knockout cells.

Keywords: Astrocyte elevated gene-1 (AEG-1); CRISPR/Cas9; gene knockout; U251 cells

Introduction

Glioma is a common intracranial tumor originating from neuroectodermal tissue, accounting for over 50% of intracranial tumors and characterized by high incidence, recurrence, mortality, and low cure rates. Current treatment modalities include surgery, radiotherapy, chemotherapy, X-knife, and gamma knife, yet no effective cure exists. Astrocyte elevated gene-1 (AEG-1) was first identified by Su et al. in 2002 using modified rapid subtraction hybridization (RaSH) as a novel gene upregulated in astrocytes following type 1 human immunodeficiency virus (HIV-1) infection. This gene is located in a region highly associated with human glioma development. The AEG-1 protein product is a single-pass transmembrane protein composed of 582 amino acids. AEG-1 has been confirmed as a downstream target molecule of the Ras/PI3K/Akt/c-Myc signaling pathway and can regulate multiple signaling pathways including PI3K/Akt, NF- B, Wnt/β-catenin, and MAPK, thereby participating in various stages of tumorigenesis such as normal cell transformation, tumor cell proliferation, invasion,

metastasis, angiogenesis, autophagy, and chemoresistance. Recent research has focused on AEG-1' s role in promoting tumor progression and metastasis, with the key mechanism being increased tumor cell invasiveness and adhesiveness. Multiple studies have demonstrated AEG-1 overexpression in glioma, where it functions as an oncogene closely associated with glioma development and progression, with expression levels increasing with malignancy grade. Although the specific mechanisms by which AEG-1 promotes glioma growth, invasion, and metastasis remain unclear, it represents both an important biomarker for glioma evaluation and an effective target for gene therapy.

CRISPR/Cas9 (Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) is a novel genome editing technology discovered in recent years. The CRISPR system is an adaptive immune defense mechanism evolved by bacteria during long-term conflict with bacteriophages, widely present in bacteria and archaea. The system consists of repeat sequences (CRISPR array) and upstream Cas protein family operons. Foreign sequences are recognized and inserted between repeat sequences as spacers (protospacers), endowing the CRISPR system with the ability to recognize invasive DNA. After artificial modification, this system enables highly flexible and specific genome editing in eukaryotic cells. The CRISPR system primarily relies on crRNA (CRISPR RNA) and tracrRNA (trans-activating chimeric RNA) binding to guide Cas proteins for sequence-specific degradation of foreign DNA. Three types of CRISPR/Cas systems have been identified: Type I, II, and III. The Type II system is relatively simple, mainly depending on the Cas9 core protein, which can recognize and cleave target sequences under RNA guidance, causing DNA double-strand breaks (DSBs). This enables various genetic manipulations including gene targeting, insertion, and repair at specific genomic loci. This novel genome editing technology offers simpler design and easier operation compared to transcription activator-like effector nuclease (TALEN) and zinc-finger nuclease (ZFN) technologies, promising broader applications.

In this study, we designed AEG-1 gene-specific sgRNA sequences and synthesized corresponding sense and antisense oligonucleotides. The CRISPR/Cas9 plasmid pX459 from Addgene is a dual-function plasmid containing a sgRNA transcription structure (sgRNA scaffold) under the U6 promoter with two tandem BbsI restriction sites. Downstream elements include a Cas9 gene driven by the CAG promoter (CMV early enhancer/chicken β-actin promoter) and other transcription-related components such as enhancers and nuclear localization sequences. After annealing the two oligonucleotides, they were inserted into the BbsI site of pX459 to construct an AEG-1-specific CRISPR/Cas9 gene editing vector. Utilizing the plasmid' s puromycin resistance feature, we established stable AEG-1-knockout glioma U251 cell lines to investigate AEG-1' s role in tumor metastasis.

Materials and Methods

1.1 Materials and Reagents

The pSpCas9(BB)-2A-Puro (pX459) V2.0 plasmid was purchased from Addgene (a non-profit plasmid repository) Figure 1: see original paper. sgRNA sequences for AEG-1 knockout were designed in-house, with corresponding oligonucleotides synthesized by Genewiz Biotechnology. U251 (human glioma cell line) cells were maintained in our laboratory. Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum. DNA extraction kits were purchased from Omega, agarose gel recovery kits from Tiangen Biotech, and primers for sgRNA activity detection from Genewiz Biotechnology.

1.2 sgRNA Sequence Design and Oligonucleotide Synthesis

The human AEG-1 gene has six transcripts (ENST00000336273.7), 12 exons, and is located on chromosome 8 (97,644,179-97,728,770). Exons 2, 3, and 4 were selected for target screening. After analyzing specific sgRNA scores using the CRISPR design tool (http://crispr.mit.edu/), one high-scoring sequence was selected from each exon as candidate sequences: AEG-1-sgRNA-1, AEG-1-sgRNA-2, and AEG-1-sgRNA-3. Based on the cohesive ends generated by BbsI digestion of pX459 Figure 1: see original paper, we added CACCG to the 5' end of the designed sgRNA, C to the 3' end of the reverse strand, and AAAC to the 5' end to match the cohesive ends of digested pX459. Six oligonucleotide sequences were designed .

1.3 Construction of pX459-AEG-1 Plasmid

1.3.1 pX459 Plasmid Digestion The pX459 plasmid was digested with restriction endonuclease BbsI, followed by agarose gel electrophoresis for identification. The linearized plasmid was recovered from the gel, and the recovered product was re-analyzed by agarose gel electrophoresis to confirm successful recovery. The digested product was stored at -20°C for subsequent ligation.

1.3.2 Annealing of AEG-1 sgRNA Oligonucleotides Synthetic sgRNA oligonucleotides were diluted to 10 mol/L. Twenty microliters each of sense and antisense sgRNA oligo solutions were combined with 20 L annealing buffer and double-distilled water to a final volume of 200 L. After mixing, the solution was boiled for 5 minutes and then allowed to cool naturally to room temperature to form double-stranded DNA for subsequent ligation.

1.3.3 Cloning and Identification of pX459-AEG-1 Vector One microliter of digested linearized pX459 vector and 5 L of annealed double-stranded DNA were ligated with 1 L T4 ligase and corresponding buffer overnight at 16°C. The ligation product was transformed into DH5α competent cells, plated, and incubated at 37°C overnight. Single colonies were selected and cultured in LB medium with ampicillin resistance for 12 hours at 37°C with shaking.

Plasmids were extracted and identified by EcoRI and BbsI digestion to confirm donor DNA insertion, followed by DNA sequencing to verify sequence accuracy.

1.3.4 Validation of sgRNA Activity in pX459-AEG-1 Vector Cloned plasmids pX459-AEG-1-1, pX459-AEG-1-2, and pX459-AEG-1-3 were transfected into U251 cells. After 48 hours, genomic DNA was extracted. Primers flanking the sgRNA target sites were designed for PCR amplification. Short PCR products were purified and cloned into the TA cloning vector PMD-19. Blue-white screening was performed, positive clones were selected, plasmids were extracted, and sequencing analysis was conducted to detect sgRNA-mediated gene editing.

1.4 Transfection and Screening of U251 Cells

1.4.1 Transfection of Active pX459-AEG-1 Plasmid into U251 Cells U251 cells were seeded in 6-well plates at 8$×10^{5}$ cells per well. When cells reached 90% confluence the following day, transfection was performed using Lipofectamine 3000 according to the manufacturer' s protocol without medium change. After 2-4 days, medium was replaced based on cell condition. On day 5, puromycin was added to the medium at a final concentration of 3 g/mL. Under this concentration, untransfected cells were eliminated during a 7-day selection period. Prior gradient experiments confirmed that untransfected cells were completely killed within 5-7 days. Surviving cells had successfully integrated the pX459-AEG-1 plasmid.

1.4.2 Construction of Monoclonal AEG-1 Knockout Cell Lines Cells were digested and subjected to infinite dilution, then seeded in 96-well plates. Wells containing only a single cell were selected for culture, with puromycin concentration reduced to 1 g/mL. Once monoclonal cell colonies formed, they were digested and transferred to 48-well plates for expansion, then eventually to culture flasks for stable growth.

1.5 Western Blot Detection of AEG-1 Protein Expression

Total protein was extracted from U251 cells and AEG-1 knockout cell lines using lysis buffer, vortexed every 5 minutes for 5 cycles, then centrifuged at 15,000 r/min for 15 minutes. Supernatants were collected and protein concentrations were determined (with correlation coefficients above 0.99). Protein samples were mixed with loading buffer and water, denatured at 100°C for 5 minutes, and subjected to Western blot analysis with 50 g total protein loaded per well. After SDS-PAGE, proteins were transferred to membranes, blocked with 5% skim milk, incubated with primary antibody overnight at 4°C, and with fluorescent secondary antibody for 2 hours at room temperature. Membranes were scanned using an Odyssey CLX scanner, and data were processed using Image J software with statistical analysis performed in GraphPad Prism.

1.6 Functional Assays

1.6.1 Transwell Chamber Assay Matrigel matrix was diluted with pre-chilled serum-free DMEM medium (medium:Matrigel = 5:1), mixed thoroughly, and 100 L was immediately added to the upper chamber of each insert. After drying at 37°C for 2 hours, cells were prepared as suspensions and 100 L containing 1$×10^{5}$ cells was added to each upper chamber. The lower chamber received 500 L medium with 10% FBS. Cells were incubated at 37°C with 5% CO_2 for 24 hours. Inserts were then removed, stained with crystal violet for 30 minutes, washed 5 times with PBS, and observed/photographed under a microscope.

1.6.2 Scratch Assay Healthy cells were washed 3 times with PBS, digested with trypsin, and dispersed by repeated pipetting. Using a marker pen and 6-well plate lid as a guide, horizontal lines were drawn uniformly on the back of the plate approximately 1 cm apart (minimum 5 lines per well). Cells were seeded into 6-well plates, 3 wells per cell type. Once confluent, scratches were made perpendicular to the back lines using a pipette tip. Cells were washed 3 times with PBS to remove detached cells, then cultured in 1% serum medium at 37°C with 5% CO_2. Photographs were taken at 0 and 48 hours. Relative migration distance was calculated as: (scratch width at 0h) - (scratch width at 48h).

Results and Analysis

2.1 Identification of pX459-AEG-1 Plasmid

After ligating pX459 plasmid with the three oligo fragments, extracted plasmids were identified by EcoRI and BbsI digestion Figure 2: see original paper. Plasmids were also sent to Genewiz Biotechnology for DNA sequencing Figure 2: see original paper. Both digestion and sequencing results confirmed successful insertion of donor DNA with sequences matching the experimental design.

2.2 Analysis of sgRNA Activity by TA Cloning

Short PCR products were cloned into the PMD-19 TA vector, and positive clones were selected for sequence analysis to detect indel mutations. Results showed no mutations in pX459-AEG-1-1, while multiple mutation forms were detected in pX459-AEG-1-2 Figure 3: see original paper and pX459-AEG-1-3 Figure 3: see original paper.

2.4 Western Blot Analysis of AEG-1 Expression and Knockout Efficiency

Protein was extracted from established stable AEG-1 knockout cell lines, with protein from pX459-empty vector-transfected U251 cells and normal U251 cells

as controls. Western blot analysis Figure 4: see original paper and Image J quantification with GraphPad Prism statistical analysis Figure 4: see original paper revealed that AEG-1 protein expression was reduced in pX459-empty vector-transfected cells. Among the stable knockout cell lines, pX459-AEG-1-3 showed the highest efficiency, achieving 98% knockout.

2.5 Transwell Chamber Assay of AEG-1 Knockout Cell Invasiveness

After 24-hour incubation, Transwell inserts were removed, stained with crystal violet for 30 minutes, washed 5 times with PBS, and observed/photographed under a microscope. Results [Figure 5: see original paper] showed that Cas9 control cells had slightly reduced metastatic ability compared to normal U251 cells, but were significantly more invasive than all three AEG-1 knockout cell lines.

2.6 Scratch Assay of AEG-1 Knockout Cell Migration

Photographs were taken at 0 and 48 hours after scratching, with plates maintained in the incubator between time points. Representative images are shown Figure 6: see original paper. Relative migration distances for each group were normalized to the normal control group and statistically analyzed using GraphPad Prism Figure 6: see original paper.

Discussion

Glioma is the most common malignant tumor of the central nervous system, with development and progression involving multiple interacting genes. Numerous studies have identified AEG-1 as a key oncogene in glioma pathogenesis. Glioma invasive growth involves complex molecular mechanisms including cell adhesion, motility, and extracellular matrix (ECM) degradation. AEG-1 enhances glioma cell adhesiveness and invasiveness through the signaling pathway where AEG-1 acts as a downstream target of Ha-ras to activate NF- B. RNA interference (RNAi) studies have demonstrated that silencing AEG-1 reduces glioma cell invasion and metastasis. However, despite its high specificity, RNAi technology suffers from high production costs, long customization cycles, requirement for multiple siRNA pairs, and inability to meet special gene editing needs. The common practice of screening for the most effective sequences using other methods before chemical synthesis is cumbersome and limits widespread application.

CRISPR/Cas9 gene editing technology has attracted extensive attention as an emerging genome editing tool. This efficient and simple technology provides a powerful weapon for studying gene function across experimental platforms. Following the discovery that CRISPR/Cas9 could edit mammalian genomes, the technology has been rapidly applied to animal model construction, gene function

studies, and tumor gene knockout since the early 21st century, as well as recent applications in tumor immunotherapy including immune checkpoint blockade, adoptive cell therapy, and antibody-targeted therapy. Tumor-targeted therapy, a long-standing research focus in biology, has also benefited tremendously from CRISPR/Cas9 development, ranging from single tumor-related gene function studies to mouse tumor model construction, Cas9-derived technologies for tumor-related phenotype screening, and exploration in tumor gene therapy. In future medical fields, CRISPR/Cas9 technology will undoubtedly revolutionize traditional tumor treatment approaches.

This study utilized CRISPR/Cas9 to knock out AEG-1 in human glioma U251 cells, confirming AEG-1' s effect on glioma metastatic capacity. Results demonstrate that CRISPR/Cas9 can effectively and rapidly knock out AEG-1 in human glioma cell lines, and that AEG-1 knockout significantly reduces glioma cell metastatic ability. This confirms that AEG-1 effectively promotes glioma metastasis and validates CRISPR/Cas9 as an efficient and simple gene editing technology. Although the signaling pathways through which AEG-1 regulates glioma metastasis are not yet fully understood and require further validation in additional model systems, and while CRISPR/Cas9 still has technical limitations such as off-target effects and inability to ensure cell targeting specificity, inhibiting AEG-1 expression in glioma using CRISPR/Cas9 likely represents a promising new therapeutic strategy. This approach is simple to operate, can effectively and rapidly inhibit glioma proliferation, reduce systemic dissemination, and may even enable complete cure or prevention of glioma. We anticipate that in the near future, CRISPR/Cas9-mediated AEG-1 editing for glioma treatment will be realized, bringing hope to glioma patients.

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Note: Figure translations are in progress. See original paper for figures.