A U87-EGFRvIII cell-specific aptamer mediates small interfering RNA delivery

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Abstract. U87-EGFRvIII is a U87 glioma cell line that overexpresses epidermal growth factor receptor variant III (EGFRvIII). In the present study, we investigated whether a DNA aptamer selected against U87-EGFRvIII using cell-based systematic evolution of ligands by exponential enrichment (cell-SELEX) could deliver c-Met small interfering RNA (siRNA) into U87-EGFRvIII cells and silence the targeted gene expression. The selected biotinylated aptamer (BA) was coupled to biotinylated c-Met siRNA by streptavidin to deliver siRNA into U87-EGFRvIII cells. c-Met siRNA, transfected with lipofectamine 2000, served as a positive control, while control siRNA, transferred with BA, served as a negative control. Western blotting was performed to detect changes in the c-Met protein expression, and MTT and Annexin V-propidium iodine assays were used to determine changes in the proliferation and apoptosis of U87-EGFRvIII cells, respectively. Similar to the liposome-mediated group, U87-EGFRvIII cells that were transfected with BA-c-Met siRNA experienced a significant decrease in the c-Met protein expression (P<0.05). There were also significant increases in the apoptotic rate (P<0.05) and inhibition rate of cell growth (P<0.01) compared with the negative control group, indicating that BA could deliver c-Met siRNA into U87-EGFRvIII and result in target gene silencing. In conclusion, the results demonstrated that this DNA aptamer, obtained through cell-SELEX, can be used as an efficient and targeted carrier for siRNA delivery, providing a novel approach and strategy for the targeted combination therapy of glioblastoma.

Introduction

Small interfering RNAs (siRNAs) are intermediate products in RNA interference (RNAi) pathways that can cause target gene silencing via RNAi pathways (1). Traditional siRNA delivery vehicles fail to address the instability and poor bioavailability of siRNAs and are associated with low delivery efficiency, poor uptake by target cells and immunotoxicity for protein carriers. Effective siRNA delivery to target systems without causing damage to normal cells or tissue remains the biggest challenge for in vivo studies (2). Aptamers are oligonucleotides (single-stranded DNA or RNA) that bind to target molecules with a high affinity and good specificity and can be obtained using the systematic evolution of ligands by exponential enrichment (SELEX) system (3). This technique adopts random oligonucleotide libraries of 10¹⁵-10¹⁶ molecules and exponentially enriches oligonucleotides that bind to the target molecule specifically via polymerase chain reaction amplification. This technique, when used to select aptamers by targeting living cells, is known as cell-SELEX (4), which offers more advantages over protein-based SELEX. Cell-SELEX obviates the requirement of protein purification and immobilization, allows target molecules to maintain their natural spatial structure and keeps protein glycosylation sites on the cell surface in a natural state. Aptamers obtained in this way can bind target molecules in their natural state and can be directly used for cell identification and detection.

Glioblastoma multiforme (GBM) is the most common and aggressive human malignant brain tumor, accounting for 50% of solid brain tumors. A common feature of patients with GBM is the presence of an increased expression and mutation of human epidermal growth factor receptor (EGFR), with the most common mutant being EGFR variant III (EGFRvIII). EGFRvIII is produced from the deletion of 801 base pairs in exons 2-7 of the extracellular domain of EGFR and is closely associated with the progression of GBM and its resistance to radiotherapy and chemotherapy. Varying degrees of a high EGFRvIII expression are found in other malignancies, including non-small cell lung cancer and colorectal cancer. However, EGFRvIII is not expressed in normal tissue (5). Therefore, EGFRvIII can be harnessed as a specific target for tumor therapy. c-Met is encoded by proto-oncogene c-Met and is a tyrosine kinase receptor of hepatocyte growth factor (HGF). In GBM patients, overexpression of c-Met often leads...
to poor prognosis, and the expression of c-Met is significantly higher in recurrent GBM compared with primary GBM (6).

Thus far, aptamers that have been designed for siRNA delivery are mainly aimed at prostate cancer (7-9), AIDS (10-12) and breast cancer (13). We have previously studied the selection of high-affinity and high-specificity aptamers targeting a U87MG glioma cell line with EGFRvIII overexpression (U87-EGFRvIII). One of these aptamers, designated as 32, was internalized and located in the nucleus of U87-EGFRvIII cells (14). In the present study, aptamer 32 was biotinylated and coupled to biotin-labeled c-Met siRNA via streptavidin, and this delivered c-Met siRNA into U87-EGFRvIII cells in a targeted manner. Subsequently, significant changes in the c-Met protein expression, apoptosis and proliferation of U87-EGFRvIII cells were observed.

Materials and methods

Aptamer and siRNA. The 5'-biotin-labeled aptamer 32, termed BA, was synthesized by Invitrogen Life Technologies (Carlsbad, CA, USA). The sequence was as follows: biotin-5'GCAATG GTACGGTACCTCTGATTTTGTCTTT TCTCTATAGTACAAAGTGCAAGCCTATTGGCTAA-3'. The c-Met siRNA sequence (synthesized by Ribo Biotech Co. Ltd., Guangzhou, Guangdong, China) was 5'-AGCCAUUUUAACGAGGUTT-3' (sense); and 5'-ACCUCGUUGAUAAUUGCGUTT-3' (antisense). The negative control siRNA was Cy3-labeled siR-Ribo™ Transfection Control (siN05815122144).

Cell culture. U87MG cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (P/S). The U87-EGFRvIII cell line (15) (obtained from Dr. Webster Cavenee, Ludwig Cancer Institute, San Diego, CA, USA) was grown in DMEM supplemented with 10% FBS, 1% P/S and 100 µg/ml geneticin. The cells were cultured at 37°C in a 5% CO₂/95% air environment.

Lipofectamine 2000 transfection and BA-mediated delivery. The U87-EGFRvIII cells were seeded in 12-well plates at a density of 1x10⁴ cells/well. The following day, the cells were harvested for transfection at 30-50% confluence. The media were switched to 1 ml of serum-free, antibiotic-free medium prior to transfection. A total of 5 µl/well of 20 µM BA and 5 µl/well of 20 µM biotin-labeled c-Met siRNA were added into 100 µl of Opti-MEM (Gibco-BRL, Carlsbad, CA, USA) respectively, gently pipetted 3-5 times and allowed to stand for 5 min. Subsequently, the two solutions were mixed with 2.5 µl/well of 20 µM streptavidin (Sigma-Aldrich, St. Louis, MO, USA) and left to stand for 20 min prior to being added to the culture media. The transfection system was 200 µl, and the final concentration of BA and siRNA was 100 nM. A total of 5 µl/well of liposome (Invitrogen Life Technologies) and 5 µl/well of c-Met siRNA plus 2.5 µl/well of streptavidin served as a positive control and underwent the same aforementioned procedures. BA + control siRNA + streptavidin and liposome + control siRNA + streptavidin were used as negative controls for BA and liposome, respectively. Separate groups exposed to BA or streptavidin alone were provided to exclude the interference from BA or streptavidin, respectively. Following transfection, the cells were cultured in an incubator (37°C, 5% CO₂) for 6 h, following which the cells were cultured for another 72 h in DMEM containing 10% FBS.

Western blotting. Cells were washed with phosphate-buffered saline (PBS) 72 h after transfection and lysed in cell lysis buffer containing 1% protease inhibitor. The protein content was measured using the bicinchoninic acid method. Following denaturation, the samples (10 µg/well) were subjected to 8% SDS-PAGE, transferred to a polyvinylidene fluoride membrane, and incubated with rabbit anti-human c-Met (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and rabbit anti-human GAPDH antibodies (Santa Cruz Biotechnology, Inc.) at 4°C overnight prior to exposure to horseradish peroxidase-labeled secondary antibody at room temperature for 1 h. Target proteins were visualized using enhanced chemiluminescence detection and analyzed with Fluorchem SP imaging system (Alpha Innotech Corporation, San Leandro, CA, USA).

Annexin V-fluorescein isothiocyanate/propiodl iodine (PI) assay. The cells were washed with PBS and digested with 0.25% EDTA-free trypsin 72 h after transfection. Following termination of the digestion, the cell suspension was transferred to a 1.5 ml Eppendorf tube and centrifuged at 100 x g for 5 min. The supernatant was discarded, while the cell pellets were resuspended in PBS (500 µl/tube) and centrifuged as aforementioned. Following the addition of 200 µl detection buffer to each tube, 2 µl of Annexin V and 2 µl of PI (Nanjing KeyGen Biotech Co., Ltd., Nanjing, Jiangsu, China) were added in sequence. The resultant mixture was incubated on ice in the dark for 10 min before performing flow cytometry to detect the percentage of apoptotic cells. To prove the targeting specificity of BA for U87-EGFRvIII cells, U87MG cells were transfected as control cells in this experiment and for the MTT assays.

MTT colorimetric assay. The cells were seeded in 24-well plates at a density of 5x10⁴ cells/well, with each well containing 500 µl of cell suspension. The cells were switched to serum-free medium, 72 h after transfection. Subsequently, 50 µl of MTT (5 mg/ml) was added to each well and was incubated at 37°C for 4 h, the supernatant was discarded, while the cell pellets were exposed to 500 µl of dimethyl sulfoxide and incubated at 37°C for 15 min. Optical absorbance (OD) at 570 nm was measured on a microplate reader.

Statistical analysis. Data were presented as the means ± SD. One-way analysis of variance was performed using SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

BA-mediated delivery of c-Met siRNA into U87-EGFRvIII cells downregulates c-Met protein expression. An agarose gel electrophoresis analysis was performed to identify the amount of BA, c-Met siRNA and streptavidin used for conjugation. When the final ratio for BA, streptavidin and siRNA was 2:1:2, a single and distinct band was observed (Fig. 1A), suggesting
that it was the optimal ratio for BA, streptavidin and siRNA conjugation. Western blotting results showed that 72 h after transfection, with a c-Met siRNA final concentration of 100 nM, the c-Met protein expression in the liposome- and BA-mediated groups was significantly reduced compared with cells transfected with control siRNA (0.418±0.18, P=0.026; 0.57±0.09, P=0.039) (Fig. 1B-C). The presence of streptavidin or BA alone had no effect on the protein levels of c-Met. The liposome-mediated siRNA delivery downregulated the target gene expression by >50%. Similarly, BA-mediated siRNA delivery reduced the target gene expression by ~50%. The results showed that BA mediated the delivery of siRNA into U87-EGFRvIII cells, leading to target gene silencing.

**c-Met gene silencing as a result of BA-mediated c-Met siRNA delivery decreases the proliferation of glioma cells.** The OD values of each group were determined with the MTT assays. Similar to the flow cytometry results, in the U87-EGFRvIII cells the inhibition rate of the liposome- and BA-mediated groups was 61.77±6.73 and 68.43±5.87%, respectively, showing a significant increase compared with that of the respective control groups (P<0.01 and P<0.01). The inhibition rate did not differ between the two groups (P=0.762). In the U87MG cells, the inhibition rate of the liposome-mediated group (45.78±10.53%) increased significantly compared with its control group and the BA-mediated group (6.46±1.78%) (P<0.01 and P<0.01, respectively). The inhibition rate of the BA-mediated group showed no difference from the control group (P=0.971) (Fig. 3). These findings demonstrated that the BA-mediated c-Met siRNA delivery into U87-EGFRvIII cells suppressed U87-EGFRvIII cell proliferation while producing no impact on that of U87MG cells.

**Discussion**

GBM patients have extremely poor prognosis and short median survival rates (16). EGFR-targeting protein tyrosine kinase inhibitors are highly immunogenic and are associated with various degrees of resistance, leading to the failure of cancer chemotherapy. The type and level of biomarkers varies in different types of tumors with different degrees of differentiation and location. Aptamers selected against living cells by cell-SELEX can recognize specific cell surface receptors and can therefore be used to identify tumor cell surface biomarkers (4,17). Following binding to surface proteins on target cells, certain aptamers are internalized within cells (18-20). The advantages of aptamers over conventional antibodies include low molecular weight, rapid synthesis
ZHANG et al: APTAMER MEDIATES siRNA DELIVERY

in vitro, low toxicity, low immunogenicity, good tissue permeability and ease of chemical modification, which minimizes degradation and enhances pharmacokinetic parameters in vivo. There are two key issues in the application of RNAi in the clinical trials of human diseases. The first issue is an effective means for siRNA delivery, and the second issue is the specificity of siRNA-targeted delivery and the safety of the delivery vehicle in humans. The aforementioned characteristics of aptamers make them promising siRNA delivery vehicles (21). An aptamer for human epidermal growth factor receptor 2 (HER2) can carry siRNA into HER2-expressing breast cancer cells to mediate target gene silencing (13). Aptamers targeting human immunodeficiency virus (HIV) glycoprotein 120 and cluster of differentiation 4 can also mediate the delivery of their corresponding siRNAs into target cells in the form of aptamer-siRNA chimeras, thereby inhibiting HIV-1 viral replication (10,11). In the present study, an aptamer obtained through a cell-SELEX procedure mediated the delivery of siRNA into glioma cells with EGFRvIII overexpression, specifically silenced target genes and modulated the apoptosis and proliferation of U87-EGFRvIII cells. This aptamer did not affect the survival rate of U87MG cells as it did not bind and enter the U87MG cells. In a previous report, we proved that EGFRvIII is the target receptor of BA (14), and we therefore hypothesized that receptor-mediated endocytosis is a possible mechanism for siRNA delivery. The present study provides evidence for the feasibility of aptamers as siRNA delivery vehicles.

Abnormal HGF/c-Met signals can lead to the stimulation of tumor cell functions, including cell proliferation, survival, cell dispersion and movement, epithelial to mesenchymal transition, angiogenesis, invasion and metastasis (22,23). HGF/c-Met overexpression and EGFRvIII high expression in gliomas are closely associated with the development and progression of
gliomas, complicating the diagnosis and treatment of gliomas. The synergy among these molecules attenuates the response of gliomas to any single chemotherapeutic agent and tends to induce drug resistance as a result. It has been reported that the overexpression of c-Met can activate HER3 and cause resistance to the chemotherapeutic agent, fibrate gemfibrozil, which is an EGFR inhibitor used in the treatment of patients with lung cancer (24). The combination of c-Met and EGFR kinase inhibitors can enhance the therapeutic effect on EGFRvIII-positive GBM and overcome its chemoresistance (25). In glioma cells overexpressing EGFRvIII, c-Met activation is not dependent on its ligand HGF, but is dependent on the activation of the EGFRvIII-mediated tyrosine kinase pathway. This indicates that the c-Met receptor may be another significant target for the treatment of EGFRvIII-positive GBM. In the case of a high EGFRvIII expression, c-Met is also highly activated in tumors and is detrimental to chemotherapy in cancer patients (26). In the present study, c-Met gene silencing inhibited EGFRvIII overexpressed glioma cell proliferation and induced apoptosis, indicating that the co-suppression of HGF/c-Met and EGFRvIII signaling pathways can be more effective in glioma treatment. Notably, aptamer-mediated c-Met siRNA delivery into U87-EGFRvIII cells offers a combined treatment regimen for patients with a high expression of c-Met and EGFRvIII.

Chu et al (7) created a biotin-labeled anti-prostate-specific membrane antigen (PSMA) aptamer and a biotin-labeled siRNA, which were incubated with streptavidin containing four binding sites. The siRNA, streptavidin and aptamer formed stable conjugates via the association between streptavidin and biotin, resulting in target gene silencing in PSMA-positive cells. Biotinylated aptamers link to biotinylated siRNA via a streptavidin bridge, offering a simple yet effective approach for siRNA delivery. In the present study, c-Met expression did not differ in the presence or absence of streptavidin during liposomal transfection of c-Met siRNA, and the addition of streptavidin alone had no effect on the expression of c-Met, indicating that streptavidin has no cytotoxicity. Future studies should examine the role of aptamer-mediated c-Met siRNA delivery in tumor suppression in an animal model of glioma.

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