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Novel cell-penetrating peptide-loaded nanobubbles synergized with ultrasound irradiation enhance EGFR siRNA delivery for triple negative Breast cancer therapy

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ABSTRACT

The lack of safe and effective gene delivery strategies remains a bottleneck for cancer gene therapy. Here, we describe the synthesis, characterization, and application of cell-penetrating peptide (CPP)-loaded nanobubbles (NBs), which are characterized by their safety, strong penetrating power and high gene loading capability for gene delivery. An epidermal growth factor receptor (EGFR)-targeted small interfering RNA (siEGFR) was transfected into triple negative breast cancer (TNBC) cells via prepared CPP-NBs synergized with ultrasound-targeted microbubble destruction (UTMD) technology. Fluorescence microscopy showed that siEGFR and CPP were loaded on the shells of the NBs. The transfection efficiency and cell proliferation levels were evaluated by FACS and MTT assays, respectively. In addition, in vivo experiments showed that the expression of EGFR mRNA and protein could be efficiently downregulated and that the growth of a xenograft tumor derived from TNBC cells could be inhibited. Our results indicate that CPP-NBs carrying siEGFR could potentially be used as a promising non-viral gene vector that can be synergized with UTMD technology for efficient TNBC therapy.

1. Introduction

Triple negative breast cancer (TNBC) is an independent clinical-pathological subtype of invasive breast cancer and is characterized by a lack of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2). TNBC is aggressive, has a high risk of metastasis and presents a poor prognosis [1]. Because of a lack of specific molecular marker expression, TNBC primarily presents as an advanced-stage disease, and its treatment is challenging and usually characterized by the systemic administration of conventional chemotherapy [2]. Microarray analysis showed that a majority of TNBC cases are generally associated with overexpression of epidermal growth factor receptor (EGFR), which is closely correlated with disease progression, chemoresistance, and metastasis [3]; therefore, EGFR is a potential therapeutic target for the successful treatment of TNBC. Increasingly, anti-EGFR therapies, such as anti-EGFR monoclonal antibodies (mAbs) and EGFR tyrosine kinase inhibitors (TKI), have been recognized as significant treatments for TNBC [4]. However, all of the conventional small-molecule drugs are quickly metabolized and cleared through the kidneys; thus, high therapeutic concentrations are required that result in cardiotoxicity or other toxicities as side effects.

With the development of biotechnology, the potential uses of gene therapy in cancer treatment have increased, especially after the discovery of RNA interference (RNAi), which can block target gene expression in a highly efficient and specific manner [5,6]. However, because of a lack of safe, reliable, and highly efficient gene delivery systems, the development and clinical application of gene therapy have been hindered. Viral vectors provide a highly efficient transfection concentration; however, the subsequent immunotoxicity and lack of site specificity increase the difficulty of achieving stable and lasting gene expression changes. Non-viral vectors are much safer, although they are limited by their lower transfection

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efficiency and significant technical challenges [7]. Recently, the use of non-viral vector-based therapies in gene therapy has received much attention [8,9].

Cell-penetrating peptides (CPPs) are usually short (up to 30 amino acids) and positively charged. These peptides can translocate through the cell membrane independent of receptors and can transport cargo, including proteins, small organic molecules, nanoparticles, and oligonucleotides [10]. CPPs represent a promising class of non-viral vectors and have garnered widespread attention because of their ability to autonomously translocate into a cell with comparably low cytotoxicity and immune response risk [11]. The HIV-1 transcription-transactivating (Tat) protein, which is one of the most popular CPPs, can enhance the ability of other vector systems. Torchilin et al. reported that liposomes were more efficiently transfected when their surfaces were coated with Tat molecules [12]. However, because of the lack of specificity for a single cell type, CPPs can directly or actively enter and deliver therapeutic genes into any neighboring cell. This property represents a weakness, as it may threaten the safety of the surrounding normal cells; thus, modifications are required to improve the specificity of CPPs in their application.

As an emerging biotechnology in gene therapy, ultrasound-targeted microbubble destruction (UTMD) has evolved as a new, promising tool for targeted drug and gene delivery in vitro and in vivo [13,14] and presents a number of advantages, including low toxicity, low invasiveness and tissue specificity. UTMD generates a cavitation effect that renders the cell membrane temporarily permeable. This phenomenon is called "sonoporation" and can safely promote exogenous gene transfer into the targeted cells [15,16]. With the rapid development of nanotechnology, the size of microbubbles (MBs) continues to be reduced to the nanoscale. Nanobubbles (NBs) have stronger penetrating power and stability and a longer half-life in blood circulation than do larger MBs [17]. Although many in-depth studies on NBs have achieved good results, the gene loading capability and gene transfection efficiency of NBs remain low.

In the present study, we attempted to load CPPs onto NBs and then utilize UTMD to deliver EGFR-targeted siRNA (siEGFR) into TNBC cells in vitro and in vivo. To the best of our knowledge, limited information is available on the use of CPP-loaded NBs synergized with UTMD technology to downregulate EGFR expression in TNBC cells.

2. Materials and methods

2.1. Cell culture

MDA-MB-231 cells from a TNBC cell line were kindly donated by the Institute of Cancer Research (affiliated with Harbin Medical University) and maintained in L15 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were cultured at 37 °C in a humidified incubator of ambient air, and exponentially growing cells were used for all experiments. The local ethics committee of Harbin Medical University approved the protocol for using the donated human TNBC lines in this study.

2.2. Design of the EGFR siRNA

Three EGFR-targeted siRNA oligonucleotides were designed and synthesized by Shanghai GenePharma Co. Ltd. (Shanghai, China; Table 1). The siRNAs were designed according to an EGFR complementary DNA (cDNA) sequence (GenBank accession no. NM_005228) and checked on the human genome database (National Center for Biotechnology Information, NCBI). All of the siRNAs were labeled with FITC at the end of the 5′ sense strand and dissolved in RNase-free water at a final concentration of 20 μM according to the manufacturer's instructions.

Table 1
Short interfering RNA sequences targeting EGFR.

Genes	Short interfering RNA duplex
siEGFR-1	Sense: 5′- CCU UAG CAG UCU UAU CUA A dTdT-3′ Antisense: 5′-UUA GAU AAG ACU GCU AAG G dTdT-3′
siEGFR-2	Sense: 5′-GAU CCA CAG GAA CUG GAU A dTdT-3′ Antisense: 5′-UAU CCA GUU CCU GUG GAU C dTdT-3′
siEGFR-3	Sense: 5′-GUA AUU AUG UGG UGA CAG A dTdT-3′ Antisense: 5′-UCU GUC ACC ACA UAA UUA C dTdT-3′
Scrambled siEGFR	Sense: 5′-UUC UCC GAA CGU GUC ACG U dTdT-3′ Antisense: 5′-ACG UGA CAC GUU CGG AGA A dTdT-3′

2.3. Screening of effective siEGFR by RT-PCR

To screen for the most efficient siRNA, we transfected the three siRNAs into MDA-MB-231 cells using Lipofectamine 2000 (Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions and determined the inhibition ratio of each siRNA using reverse transcription-polymerase chain reaction (RT-PCR) analysis. Briefly, the MDA-MB 231 cells were seeded without antibiotics at 1×10^6 cells in 25-cm^2 culture bottles and cultured for 24 h. Prior to transfection, the cells were washed with phosphate-buffered saline (PBS). The cells were divided into 5 groups (n = 3): three test groups transfected with the different siEGFR sequences; a scrambled siEGFR group; and an untreated control group.

At 48 h after transfection, the cells were collected, and total RNA was extracted using TRIzol (Invitrogen Life Technologies, Carlsbad, CA). RNA purity was analyzed using a BioSpec-nano spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at an OD260/OD280 ratio of 1.8–2.0, and 3 μg of total RNA was reverse transcribed into cDNA using a PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). The resultant cDNA was amplified using specific primers for EGFR: sense, 5′-CTAAGATCCCGTCCATCGCC-3′(2168-2187); and antisense, 5′-GGAGCCCAGCACTTTGATCT-3′ (2406-2387). This process yielded a predicted amplicon of 239 bp. β-Actin was used as an internal standard and was amplified using the following primer sequences: 5′-CTCCATCCTGGCCTCGCTGT-3′ (1113-1132) and reverse, 5′-AACTTTGGGGGATGCTCGCT-3′ (1410-1391). This process yielded a predicted amplicon of 298 bp. The cycling program was performed as follows: denaturing at 94 °C for 2 min; 35 cycles of denaturation at 94 °C for 30 s, renaturation at 57 °C for 30 s, and extension at 72 °C for 30 s; and a final extension at 72 °C for 10 min. The PCR products were electrophoresed through a 2% agarose gel, and the bands were visualized using an electrophoresis imaging system (Bio-Rad, CA, USA) and analyzed using semiquantitative software. The grey ratios of EGFR/β-actin were calculated. After validation, the most effective siEGFR was used for subsequent experiments.

2.4. Preparation of NBs

Blank NBs were prepared using thin-film hydration-sonication and low-speed centrifugation methods. Briefly, 10 mg 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 4 mg 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG2000-MAL), 1 mg diphenylphosphoryl azide (DPPA) (all phospholipids were purchased from Avanti Polar Lipids, Alabaster, AL, USA), and 5 mg polyethylene glycol (PEG)-40 stearate (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in 5 ml of chloroform and transferred to a culture dish. The suspension formed a thin phospholipid film by natural evaporation in a fume cupboard overnight. The film was