Original Article
Proliferation inhibition and apoptosis enhancement of human cervical cancer cells by ultrasound-targeted microbubble destruction delivered double suicide genes

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Abstract: Successful gene therapy requires safe and efficient gene vectors and gene delivery methods. This study is to investigate the effects of double suicide genes on the proliferation and apoptosis of HeLa cells by using the ultrasound-targeted microbubble destruction (UTMD). A lentiviral vector with the KDR promoter was constructed, packaged, and delivered into HeLa cells by UTMD. The results showed that the encapsulation efficiency was 90.6 ± 3.1% and the drug-loading efficiency was 29.2 ± 0.9% assessed by reversed-phase high performance liquid chromatography (RP-HPLC). Cell proliferation was determined by MTT assay and apoptosis was detected by flow cytometry. The proliferation rates of HeLa cells were significantly inhibited when treated with dual-gene lentiviral vectors or lentiviral vector-loaded microbubbles plus UTMD (P < 0.05). Moreover, the inhibiting effects were enhanced along with the increased ultrasonic intensities and declined at 24 h post-irradiation. Additionally, in comparison with the control group, the apoptotic rates of HeLa cells were significantly elevated in the lentiviral vector group and the lentiviral vector microbubble groups (P < 0.05). The apoptotic rates were also elevated as the ultrasonic irradiation intensities were increased (P < 0.05). The results suggest that dual-gene lentiviral vector-loaded microbubbles inhibit proliferation and enhance apoptosis of cervical cancer cells.

Keywords: Cervical cancer, ultrasound targeted microbubble destruction, suicide gene, lentiviral vector

Introduction

Cervical cancer is one of the most common gynecological malignancies, which is also a leading cause of morbidity and mortality in women worldwide. The prevalence of cervical cancer has been increasing during the last decades, especially in Asia countries [1-3]. Due to inadequate or even non-existent screening, the disease is normally detected in the late stage. Improved therapeutic options for this type of malignancy are highly needed [4]. Nowadays, gene therapy has been recognized as a promising method for the treatment of cancer diseases, which means transfecing exogenous genes into the patient to kill tumor cells.

In recent years, suicide gene therapy has become a novel anti-cancer treatment after traditional surgery, radiation therapy, and chemotherapy [5]. Suicide genes usually refer to pro-drug convert genes or cytotoxic gene receptor genes from viruses and bacteria [6], such as E. coli cytosine deaminase (CD) and herpes simplex virus-thymidine kinase (HSV-TK) genes, which have been shown to confer significant effects in treatment for various cancers [7, 8]. In fact, successful suicide gene therapy depends on the safe and efficient delivery systems [9]. Ultrasound-targeted microbubble destruction (UTMD) offers advantages in stimulating cell membrane permeabilisation to help transfer plasmid DNA or drug into cells [10-12]. However, most studies have mainly used UTMD with reporter genes to show high transfection efficiency, while the potential application of this technology in cancer gene therapy has been limitedly investigated.

In this study, the effects of double suicide gene lentiviral vector (pLenti6-KDRP-CD/TK-EGFP)-loaded microbubbles delivered by UTMD on the
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proliferation and apoptosis of cervical cancer cells were investigated. Our results showed that dual-gene lentiviral vector-loaded microbubbles could significantly inhibit the proliferation and enhance the apoptosis of HeLa cells. These findings provide experimental evidence for the clinically visual examination of ultrasound-controlled release of plasmid-loaded microbubbles, and support the promising role of this therapeutic strategy for cancers.

Materials and methods

Construction and packaging of double-gene lentiviral vector

Double suicide gene lentiviral vector pLenti6-KDRP-CD/TK-EGFP was constructed and packaged. Briefly, human gDNA was extracted from peripheral blood and amplified to generate KDRP, CD, and TK genes. After purification via gel extraction, these fragments were inserted into pMD18-T vectors, respectively, to obtain recombinant T plasmids. The target genes of KDRP, CD, and TK were constructed using TA cloning kits. After sequencing, these genes were in turn connected to lentiviral vector pLenti6-EGFP, and then transfected into 293T cells with liposome. 24 h later, the fluorescence of EGFP was observed by a fluorescence microscope. After another 72 h, the supernatant was collected, centrifuged, and then stored at -80°C. Serial dilutions of virus suspension were added into 293T cells. 72 h later, the fluorescence of EGFP was counted, and the virus titer was calculated according to the following formula:

\[
\text{Virus titter (pfu/ml) = number of EGFP positive cells} \times \text{dilution factor of viral supernatant/0.4 ml.}
\]

Preparation of plasmid-loaded microbubbles

Five milliliter saline was injected into Sonovue (Sulphur Hexafluoride Microbubbles for Injection; Bracco International BV, Amsterdam, Netherlands), the phospholipid-encapsulated sulfur hexafluoride (SF6). Then the mixture was shaken to obtain microbubble suspension. 50 μl suspension was mixed with 100 MOI viral supernatant, and then gently shaken and incubated at room temperature for 20 min. The particle sizes of drug-loaded microbubbles were detected by a Malvern laser measurer (Zetasizer 300-0HS; Malvern Instruments Inc., Southborough, MA, USA). The encapsulation and drug-loading efficiencies of microbubbles were then assessed by reversed-phase high performance liquid chromatography (RP-HPLC).

Ultrasound irradiation treatment and grouping

Cervical cancer HeLa cells were divided into the following categories: (1) the control group that was free from intervention; (2) the lentiviral vector group that was treated with only lentiviral vector supernatant (100 MOI); (3) the lentiviral vector microbubble group that was treated with lentiviral vector microbubbles delivered by UTMD with the intensities of 0, 0.25, 0.5, 1.0, and 2.0 W/cm² at 300 kHz for 30 s. The ultrasonic irradiation instrument was developed by Ultrasonic Imaging Institute of Chongqing Medical University, Chongqing, China.

MTT assay

Cell suspension was planted into a 24-well plate at the density of 1 × 10⁶/ml, and incubated in a CO₂ incubator at 37°C for 24 h. After treatments, the cell suspension was planted into to a 96-well microplate (with 0.5 × 10⁴ cells in 200 μl culture medium), and incubated at 37°C for 1-4 d. To assess cell proliferation, 20 μl MTT solution was added into each well for a further incubation at 37°C for 4 h. After the supernatant was withdrawn, 200 μl DMSO was added into each well, and the plate was fully shaken for 10 min. The absorbance at 490 nm was read on an automatic microplate reader (BioRad, Hercules, CA, USA), and the inhibition rate was calculated as follows:

\[
\text{Inhibition rate} = \left(1 - \frac{OD_{\text{treatment}}}{OD_{\text{control}}} \right) \times 100\%.
\]

Annexin V-FITC/PI staining and flow cytometry

HeLa cells in logarithmic growth phase were collected and planted into a 6-well plate at the density of 1 × 10⁶ cells/well. When the 70-80% confluence was reached, these cells were subjected to drug treatments. The cellular apoptosis was assessed with the annexin V-FITC/PI kit by flow cytometry, according to the manufacturer’s instructions. The excitation wavelength was 488 nm, and the emission wavelength was 530 nm. The experiment was independently repeated three times, and the mean values were calculated.

Statistical analysis

Data are expressed as mean ± SD. By SPSS17.0 software for statistical analysis. The t-test and
Results

Dual-gene lentiviral vector construction, packaging, and titration

Recombinant pLenti6-KDRP-CD/TK-EGFP vector (containing tumor-specific promoter KDRP, CD, and TK genes, as well as enhanced green fluorescent protein gene, EGFP) was encapsulated in microbubbles. The plasmid-loaded microbubbles appear in form of milky white suspension, with the average particle size of 2.90 μm (ranging 2-5 μm). The encapsulation and drug-loading efficiencies of these microbubbles were detected by RP-HPLC. The results indicated that the encapsulation efficiency was 90.6 ± 3.1% and the drug-loading efficiency was 29.2 ± 0.9%. These plasmid-loaded microbubbles were transfected into 293T cells with liposome for 72 h. The fluorescence of EGFP was examined by fluorescence microscopy (Figure 1), and the virus titer was calculated accordingly. Our results indicated that the titer of the dual-gene lentiviral vector-loaded microbubbles was 3.5 × 10^9 pfu/ml. In the following experiments, these microbubbles loaded with dual-gene lentiviral vectors were transfected into cervical cancer HeLa cells, and the effects of the dual-gene lentiviral vector microbubbles on cell proliferation and apoptosis were investigated. In addition, UTMD was utilized to enhance the cytoplasmic import of these plasmids.

Effects of dual-gene lentiviral vector microbubbles on proliferation of HeLa cells

To investigate the effects of dual-gene lentiviral vector microbubbles on the proliferation of HeLa cells, the MTT assay was performed. These cells were transfected with either dual-gene lentiviral vectors or lentiviral vector-loaded mi-
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Figure 3. Dual-gene lentiviral vector microbubbles delivered by UTMD enhance the apoptosis of HeLa cells. HeLa cells were treated with lentiviral vectors (LV) or lentiviral vector-loaded microbubbles delivered by UTMD (0, 0.25, 0.5, 1.0, and 2.0 W/cm²) at 300 kHz for 30 s. The cellular apoptosis was detected with annexin V-FITC/PI staining by flow cytometry. Compared with the control group, *P < 0.05, **P < 0.01.

that, compared with the control group, the apoptotic rates of HeLa cells were significantly elevated in the lentiviral vector group and the lentiviral vector microbubble groups (all P < 0.05) (Figure 3). Moreover, within the lentiviral vector microbubble groups, the apoptotic rates of HeLa cells were elevated as the ultrasonic irradiation intensities were increased (Figure 3). These results indicate that the dual-gene lentiviral vector microbubbles delivered by UTMD could significantly enhance the apoptotic process in HeLa cells.

Discussion

Cervical cancer is one of the leading causes of cancer death in women throughout the world [13]. In China, especially in south Xinjiang area, the prevalence of cervical cancer has been widely reported [14]. The mortality rate of cervical cancer in south Xinjiang area ranks first among the national minorities [15]. Clinical statistics show that cervical cancer patients account for 20% of all the patients with malignant tumors in Xinjiang. Moreover, among the patients with cervical cancer, over 75% are Uighur women. Therefore, cervical cancer has been listed as one of the malignant tumors with extremely high incidence in this area. Because cervical cancer patients are often diagnosed in the moderate and advanced stages, and the efficiencies of traditional treatments are always poor [16], it is of clinical significance to establish a safe and effective treatment for the disease.

Suicide genes, also known as pro-drug converting enzyme genes, encode cell-specific enzymes capable of converting non-toxic or low toxic pro-drug into cytotoxic substance [17]. Suicide gene therapy applies genetic engineering technique to transfer suicide genes into tumor cells. In this way, the non-toxic or low toxic pro-drug injected into the body could be converted into cytotoxic substance, which can be incorporated into and interfere with the synthesis of cellular DNA, causing tumor cell death. CD and HSV-TK genes are well studied suicide genes that have been applied in therapeutic and experimental settings. CD gene encodes cytosine deaminase, which can be metabolized into uracil, inhibiting the syntheses of RNA and DNA.

crobubbles. Moreover, HeLa cells transfected with lentiviral vector-loaded microbubbles were subjected to ultrasonic irradiation with the intensities of 0, 0.25, 0.5, 1.0, and 2.0 W/cm², respectively, at 300 kHz for 30 s, to facilitate the plasmid delivery. HeLa cells free from intervention were used as control. Our results from the MTT assay showed that, compared with the control group, the proliferation of HeLa cells was inhibited in the lentiviral vector group and the lentiviral vector microbubble groups subjected to ultrasonic irradiation (P < 0.05). No significant differences in the cell proliferation inhibition rates were observed between the lentiviral vector group and the lentiviral vector microbubble groups subjected to 0 or 0.25 W/cm² ultrasonic irradiation (P > 0.05). When ultrasonic irradiation intensities were increased to more than 0.5 W/cm², the cell proliferation inhibition rates were significantly increased (P < 0.01) (Figure 2). Furthermore, according to the dynamic observation, the inhibiting effects started to decline at 24 h post-irradiation (Figure 2). These results suggest that the dual-gene lentiviral vector microbubbles delivered by UTMD could efficiently inhibit the proliferation of HeLa cells.

Effects of dual-gene lentiviral vector microbubbles on apoptosis of HeLa cells

Next, the effects of dual-gene lentiviral vector microbubbles on the apoptosis of HeLa cells were investigated. Our results from annexin V-FITC/PI staining and flow cytometry showed
HSV-TK gene encodes thymidine kinase, which converts nucleoside analogs into diphosphate and triphosphate, inhibiting DNA polymerases. Furthermore, due to the shortcomings of single suicide gene therapy, gene combination therapy has always been used [18].

In this study, two suicide gene systems, CD/5-Fc and HSV-TK/GCV, were used in the combination therapy to improve the efficacy of the cancer treatment. Our results showed that the combination therapy had complementary and synergic effects, which significantly enhance the tumor cell killing effects. Meanwhile, the tumor-specific promoter KDRP was also applied in this study. As one of the receptors of vascular endothelial growth factor (VEGF), KDRP is closely relevant to vascular endothelial cell proliferation and angiogenesis, and plays an important role in cell growth and differentiation. KDRP is highly expressed in actively proliferating vascular endothelial cells, and various tumor cells, while in normal tissues, the expression of KDRP is rather low, or even does not express at all [19]. Application of KDRP could enhance the targeting of cancer gene therapy and the treatment safety.

Considering the successful rate in incorporating PCR products into expression vectors, an alternative cloning method had been used in this study, i.e., constructing the recombinant T plasmids with the TA cloning kits, followed by the digestion and ligation of the expression vector and the target gene. Lentiviral vector can infect dividing and non-dividing cells, deliver large gene fragments, and sustain a long-term expression. Moreover, lentiviral vector does not induce serious host immune responses, so as to achieve high titer viral production in various host cells. Furthermore, the introduction of EGFP enabled direct observation on target gene expression and viral titer detection. Our results showed that the double suicide gene lentiviral vector microbubbles could greatly affect the proliferation and apoptosis of cervical cancer cells. In addition, ultrasonic irradiation was applied to facilitate the delivery of the drug-loaded microbubbles. Results from the MTT assay indicated that, when the ultrasonic intensity was 0.25 W/cm², cell proliferation was obviously inhibited, although without statistical significance. When the ultrasonic intensity was increased to 0.5 W/cm² or above, the inhibiting effect was significantly superior to the control group. The inhibiting effects of ultrasonic irradiation on cancer cells exhibited an obvious increasing trend along with the irradiation intensities, from 0.25, 0.5, 1.0, to 2.0 W/cm². On the other hand, the cellular apoptosis of the cancer cells was enhanced in the ultrasound-radiated groups, even though no statistical significance was achieved. However, in a certain range, the enhancing effects were gradually elevated along with the increasing irradiation intensities.

In conclusion, our results showed that dual-genenterviral vector-loaded microbubbles could significantly inhibit the proliferation and enhance the apoptosis of cervical cancer HeLa cells. These findings provide experimental evidence for the clinically visual examination of ultrasound-controlled release of plasmid-loaded microbubbles, and support the promising role of this therapeutic strategy for cancers.

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Disclosure of conflict of interest

None.

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