Targeted Protein Liposomes-Mediated AChE Gene Therapy for Effective Liver Cancer Treatment

Kai Wang  
Fudan University School of Basic Medical Sciences

Fusheng Shang  
Shanghai University

Dagui Chen  
Shanghai University

Jianpeng Jiao  
Changzheng Hospital

Tieliu Cao  
Fudan University Shanghai Cancer Center

Xiaowei Wang  
Changzheng Hospital

Shengli He  
Fudan University Shanghai Cancer Center

Xiaofei Liang  
Shanghai Jiao Tong University School of Medicine  
https://orcid.org/0000-0003-3363-9380

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Abstract

The development of highly efficient non-viral gene vector systems has very important application value in the field of cancer therapy. The high protein content of proteolipids allows for high biocompatibility, low immunogenicity, and surface modification of proteins to confer more targeted drug/gene function. For the first time, this study selected transferrin, which has hepatocellular carcinoma cell targeting function, with a liposome backbone material to construct transferrin liposome (Tf-PL), and load acetylcholinesterase (AChE) therapeutic gene for in vitro and in vivo functions evaluation. The results showed that the Tf-PL transfection efficiency was higher than that of commercial Lipo 2000, low cytotoxicity and targeted ability to liver cancer SMMC-7721 cells. After tail vein injection, Tf-PL/AChE can effectively target to liver cancer, significantly inhibiting the growth of liver cancer xenografts in nude mice, prolonging the survival time of tumor-bearing nude mice, and also does not cause significant systemic toxicities. Our study provides a strategy for proteolipids targeting the transferrin receptor to carry therapeutic gene therapy for tumors. This method has strong tumor affinity and can provide an effective vector selection for precise tumor therapy.

1. Introduction

Liver cancer is a serious disease that can progress and spread to other parts of the body and difficult to treat, ranking third in cancer-related deaths[1–3]. Gene therapy provides a powerful and promising approach to the treatment of deep tumors such as liver cancer[4–7]. To date, more than 1,500 cancer gene therapy protocols have been demonstrated for use in global clinical trials[8–10]. However, developing a better quality and less toxic gene delivery system remains the biggest challenge for gene therapy. The core of targeted gene therapy delivery systems is to ensure the safety and high degree of targeting of the delivery system[11, 12]. In the currently widely used biodegradable positive non-viral vector system, the biggest defect is the rapidly cleansed by reticulo-endothelial system and the high cytotoxicity caused by the positive surface of the microsphere[13, 14]. In order to solve the above key problems as much as possible, we have effectively designed a proteoliposome system, which uses a targeted protein or antibody to directly construct a "protein liposomes" (PL) by means of lipid assembly. As part of the microspheres, the protein content in the microspheres is directly increased, and the in vivo circulation time and targeting of the gene carrier complex are effectively improved. The system can select different targeting proteins or active substances such as antibodies, such as transferrin and lectin, so it has great application value as a novel gene delivery non-viral vector system.

In order to achieve targeted drug delivery to liver cancer cells, receptor-mediated active targeting has been used in recent years[15–19]. Transferrin (Tf) is a well-known plasma protein responsible for transporting iron into cells via the transferrin receptor (TfR) that binds to the cell membrane[20–22]. TfR is overexpressed in many human tumors, and it is possible to improve the uptake efficiency of liver cancer cells by transferrin[23–26]. Transferrin receptors are present on the membrane of liver parenchyma, mannose receptors are distributed on non-parenchymal membranes, and low-density lipoprotein is regulated[27–29]. The content of transferrin receptor in liver cancer tissues is higher than that in adjacent
cancer tissues and normal liver tissues[30, 31]. Oncomine data showed that the expression level of transferrin receptor in liver cancer tissues was 2.780 times that of normal liver tissue (p < 0.01, Figure S1A, Supporting Information). At the same time, the survival rate of patients with high TfR expression was lower than that of patients with low expression (Figure S1B, Supporting Information). Therefore, transferrin receptors can be a drug delivery target for liver cancer treatment. Acetyl cholinesterase (AChE) has many non-choline functions in addition to the classic function of neurotransmitter by breaking down the neurotransmitter Acetylcholine (ACh)[32, 33]. Oncomine data showed extremely high expression of ACh in various tumor tissues including liver cancer, p = 0.002, (Figure S2A, Supporting Information), while AChE showed significant low expression (p = 0.012, Figure S2B, Supporting Information). Therefore, AChE can be used as a gene therapy target for cancer.

In this study, we first attempted to construct Tf-protein liposome (Tf-PL) using transferrin derivatives (Tf-GHDC) instead of lipids such as lecithin and small mterol, and effectively carry the AChE gene. Targeted treatment of liver cancer. The Tf-PL preparation process, as shown in Fig. 1A, utilizes transferrin to mediate delivery of the AChE gene to the nucleus via transferrin receptor-mediated endocytosis (Fig. 1B) and study the targeting and inhibitory effect of transferrin liposome pairs in liver cancer cells.

2. Experimental Methods

2.1 Reagents and kits

N, N-Dimethyldiethyldimethylamine (≥ 99%) was purchased from Feixiang Corporation. Fluorescein isothiocyanate I (FITC); succinimidyl-3-(2-pyridyldithio) propionate (SPDP, MW 312.37); 3-(4,5-dimethyl Thiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT); cholesterol and other molecular biology reagents were purchased from Sigma, USA. The AChE gene was deposited by the National Laboratory of Oncogenes and Related Genes of the Shanghai Cancer Institute (sequence number: NG007474.1). The micro BCA protein assay kit, the apoptosis and cell cycle assay kit, and the luciferase activity assay kit were purchased from Promega (Madison, USA). Lipofectamine 2000 (Lipo 2000) was purchased from Invitrogen, USA. 1,2-dioleoyl-sn-glyceryl-3-phosphoethanolamine (DOPE), 1,2-distearoyl-sn-glyceryl-3-phosphoethanolamine-N-[(polyethylene glycol)-2000] (DSPE-PEG) was purchased from Avanti, USA. Fetal bovine serum, penicillin/streptomycin, Dulbecco's modified Eagle's medium, DMEM were purchased from Gibco, USA. RIPA lysis buffer, DMSO, and PMSF were purchased from Shanghai Shenggong Biotechnology Co., Ltd. The Transwell chamber was purchased from Corning, USA. Apoptosis Detection Kit (BD, USA), Tunel staining kit was purchased from Biyuntian Bio. Mouse anti-human AChE monoclonal antibody, mouse anti-human TfR monoclonal antibody was purchased from Santa Cruz, USA. Goat anti-mouse IgG (H + L)-HRP, goat anti-rabbit IgG (H + L)-HRP was purchased from Abcam, USA. ECL (Millipore, USA), TRIzol, SYBR MIX were purchased from TaKaRa. Epichlorohydrin (≥ 99%), absolute ethanol, isopropanol (≥ 99%), chloroform (≥ 99%), ethylenediaminetetraacetic acid (EDTA, 99.9%), epichlorohydrin and other biochemical reagents All are analytical grades and are purchased from Sinopharm. Glycidylhexadecyldimethylammonium chloride (GHDC) and other polymers are synthesized and stored by our own laboratory.
2.2 Preparation of AChE plasmid

The therapeutic acetylcholinesterase (AChE) plasmid was obtained from the National Laboratory for Oncogenes and Related Genes, Cancer Institute of Shanghai JiaoTong University (sequence: NG007474.1). Plasmid amplification was achieved by transforming competent E. coli and enlarging the number of E. coli in large quantities, and the plasmid was extracted using the Qiagen EndoFree Plasmid Mega Kit (Qiagen, Hilden, Germany). After passing the test, the plasmid was then dissolved in sterile endotoxin-free water and stored at -20 °C for later use.

2.3 Cell culture and transfection experiments

Human embryonic kidney cell 293T cells, liver cancer cell line SMMC-7721 were preserved by the laboratory. And cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin in a humidified incubator with 5% CO$_2$ at 37 °C. SMMC-7721 cells were seeded at $2 \times 10^5$ cells/well in 6-well plate (Coming Inc., NY, NJ, USA) in 2 mL of complete medium. After 24 hours of incubation, the medium in each well was replaced with 2 mL of fresh serum-free medium. The pVAX-GFP (pGFP) reporter plasmid was maintained at 2 µg per well, while the mass ratios of PL/pGFP, Tf-PL/pGFP and Lipo 2000/pGFP were 25:1. The serum-free medium was then replaced with complete medium after 6 hours. Then, the cells were cultured for an additional 48 hours at 37 °C. Expression of GFP was visualized by an Olympus IX 71 inverted fluorescence microscope (Olympus Corp., Tokyo, Japan). Cell suspensions were harvested and analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA) to determine transfection efficiency.

2.4 Liver cancer cell lines and animal experiments

SMMC-7721 cells in Dulbecco's Modified Eagle's medium containing 10% fetal bovine serum (FBS) (GIBCO BRL, Grand Island, NY, USA) or RPMI 1640 (GIBCO BRL, Grand Island, NY, USA). The concentration of penicillin and streptomycin in the medium was 50 µg/mL, respectively; and the cells were cultured at 37 °C in a humidified environment containing 5% CO$_2$. Female BALB/c-nu nude mice (5–6 weeks old; body weight, 18–20 g) were obtained from Shanghai Experimental Animals Inc. (SLAC; Shanghai, China) and maintained under conditions free of specific pathogens. All animal experiments were conducted in accordance with the guidelines set by the Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine (Shanghai, China).

2.5 Synthesis and Analysis of Tf-GHDC

Tf-GHDC was prepared by modifying Tf using GHDC. The conjugate was formed by gently stirring 20 mg of Tf into 20 mg of GHDC dissolved in 20 mL of double distilled water (ddH$_2$O). The resulting solution was incubated at 37 °C for 24 hours to allow the reaction to proceed. Unreacted GHDC was separated from the conjugate by dialysis against ddH$_2$O for 36 hours. Nuclear magnetic resonance analyzer was performed to obtain spectral absorption peaks of Tf, CHDC and Tf-GHDC to compare changes in Tf before and after conjugation.
2.6 Preparation and characterization of transferrin liposomes

Transferrin liposomes were prepared by reverse evaporation method, dissolving macromolecular quaternary ammonium salt (mass concentration 1–100%) and lipid component (mass concentration 0.1–60%) in chloroform. The GHDC-modified macromolecular quaternary ammonium salt Tf-GHDC (mass concentration 0.1–39%) was dissolved in the aqueous phase, and then PEG-modified transferrin liposome (Tf-PL) was prepared by reverse evaporation. The average particle size, size distribution and zeta potential of the proteoliposome were determined using a Malvern Zetasizer (Nano-ZS 90, Malvern Instruments Limited, UK) based on quasi-elastic light scattering at 25 °C. The morphology and shape of the liposomes were imaged by TEM. Prior to imaging, the liposomes were coated on a carbon coated copper grid, stained with 4% uranyl acetate for 10 min and allowed to dry. TEM was carried out using a 7650 TEM (Hitachi; Kyoto, Japan) at 120 kV. A 10 µL sample was taken for atomic force microscopy (AFM) analysis. Approximately 30 µL of the sample was dropped on freshly cleaved mica for 10 min and subjected to atomic force microscopy (AFM) analysis (Bioscope SPM, DI, USA). Agarose gel electrophoresis experiments were performed with different weight ratios of TfPL and DNA. X-ray photoelectron spectroscopy (XPS) analysis of the samples was performed using a PHI-1600 instrument (PerkinElmer) and a MgKα X-ray source (1253.6 eV). The prepared transferrin liposome solution was placed in an ultrafiltration centrifuge tube, centrifuged at room temperature for 15 min at 8 000 r/min, and the flow-through was removed and then using an anti-human transferrin ELISA kit to qualify the dose according to the manufacturer's instructions. The conjugation efficiency of Tf was calculated. The calculation formula is: CE% = (transferrin addition amount - transferrin outflow amount) / transferrin addition amount × 100%. In the same way, non-targeted liposomes (PL) were prepared only with GHDC and cholesterol (Chol). FITC and Cy5.5 labeled liposomes were prepared by adding the desired amount of FITC to the lipid organic solution prior to the solvent evaporation step and adding Cy5.5 to ddH₂O prior to the hydration step.

2.7 Characterization of the transgenic performance of proteoliposome

The appropriate amount of proteoliposome was taken and demulsified with methanol. Nanodrop 2000 was used as the main absorption peak of nucleic acid with 260 nm ultraviolet absorption peak. The gene load (DL) of the liposome and the encapsulation efficiency (EE) of the liposome to the gene were determined according to the formula. The calculation formula is: DL% = (total amount of gene - unencapsulated free gene) / total amount of system × 100%; EE% = (total amount of gene - unencapsulated free gene) / gene × 100%. The release rate of the in vitro gene of the proteoliposome carrying the gene was determined using a dialysis method. Briefly, 2 mL of plasmid-loaded liposomes were suspended in a dialysis bag with a molecular weight cut-off of 12 kDa and dialyzed against 18 mL PBS (pH 7.4) containing 0.1% Tween-80 for 7 days (v/ v) On a horizontal shaker (100 rpm) at 37 °C. A
2 mL aliquot was taken at predetermined intervals and replaced with an equal volume of fresh medium. The DNA content of the samples collected at each time point was measured using Nanodrop 2000.

2.8 Expression of transferrin receptor and acetylcholinesterase in hepatocellular carcinoma cell lines

Expression of transferrin receptor in hepatoma cells was analyzed by Western Blot and flow cytometry. The results were compared to TfR expression levels in normal liver tissue and liver cancer samples. The normal liver cells and three human hepatoma cell lines were selected, and the expression level of TfR on the cell membrane was analyzed by Western Blot. The expression of acetylcholinesterase in normal liver cells and three human hepatoma cell lines was analyzed. The cells were collected in 1.5 ml tubes, washed twice with PBS, then 0.1 ml RIPA lysis buffer containing 1 mM PMSF was added, and then placed on ice for 30 minutes. The supernatant was obtained by centrifugation at 13,000 rpm for 15 minutes at 4 °C. Subsequently, the protein concentration was determined by BCA protein quantification. A total of 20 µg of protein sample was separated on a 12% SDS-PAGE gel and then transferred to a PVDF membrane which was blocked in 5% skim milk for 1 hour. Membranes were incubated with mouse anti-human AChE monoclonal antibody or mouse anti-human TfR monoclonal antibody (1:500) overnight at 4 °C and washed three times with PBST followed by goat anti-mouse IgG (H+L)-HRP was incubated for 2 hours at room temperature. Finally, ECL luminescence is used for detection.

2.9 Cellular uptake and localization of liposomes in SMMC-7721 cells

SMMC-7721 cells were incubated with proteoliposomes at a series of FITC concentrations (0.33, 1 and 2 nM) for 2 hours at 37 °C and then washed three times with PBS. Cellular uptake of FITC-labeled proteoliposomes was qualitatively and quantitatively analyzed by fluorescence microscopy (TE2000; Nikon; Kyoto, Japan) and flow cytometry (FACS; BD Biosciences; San Jose, CA, USA), respectively. Transfection medium was replaced with normal medium and then replaced with Tf-PL-GFP and PL-GFP for uptake studies. The cells were washed three times with PBS and fixed in 4% paraformaldehyde, then DAPI stained the nuclei, and finally subjected to fluorescence microscopic observation.

2.10 Tumor spheroid uptake analysis

To form tumor cell spheroids, SMMC-7721 cells were plated in a 24-well plate (1 x 10^3 cells/well) in 100 µL Matrigel: DMEM medium (1:1 v/v). Incubate for 12 hours at 37 °C. The cells were then cultured for 7 days in complete growth medium (1 mL/well) and then used in subsequent experiments. The obtained spheroids were incubated with transferrin liposomes containing 4 nM-rhodamine B for 3 hours at 37 °C. They were then rinsed three times with PBS and fixed in 4% paraformaldehyde for 15 minutes. Tumor sphere uptake was analyzed by confocal laser scanning microscopy (C2; Nikon; Kyoto, Japan).

2.11 In vitro cytotoxicity assays
SMMC-7721 cells were seeded in triplicate in 96-well plate (5 × 10^3 cells/well). After 24 hours, the medium was replaced with 100 µL of complete growth medium containing different concentrations of transferrin liposomes and incubated for an additional 24, 48, 72 hours. Cells that were not exposed to the transfected protein liposomes were used as controls. Cell viability was measured by the MTT assay according to the manufacturer's instructions.

2.12 Cell migration assay

SMMC-7721 cell migration was measured using a transwell assay kit (Corning Life Sciences; Tewksbury, MA, USA) with 8 µm pores as previously described. SMMC-7721 cells were suspended in serum-free medium containing the gene preparation and DMEM supplemented with 10% FBS as a chemoattractant. Cells migrated after 16 hours were stained with 0.1% crystal violet and counted from 5 randomly selected regions under an inverted microscope.

2.13 Cell cycle and apoptosis analysis

SMMC-7721 cells were subjected to gene therapy intervention with transfectant liposomes, then harvested after 24, 48 and 72 hours, while untreated cells were used as controls. For cell cycle analysis, cells were fixed in 1 mL of 70% ethanol at 4 ºC for 24 hours, then centrifuged, rinsed with cold PBS and stained with PI (200 µg/mL) for 10 minutes at 4 ºC in the dark. Apoptosis was assessed using the Annexin V-FITC/PI Apoptosis Detection Kit. After treatment and harvest, cells were suspended in 200 µL of binding buffer and incubated with 5 µL of Annexin V-FITC and 5 µL of PI for 15 minutes in the dark. Flow cytometry analysis was performed using a BD flow cytometer (Calibur; USA) to assess cell cycle distribution and apoptosis.

2.14 In vivo imaging of mice inoculated with SMMC-7721

SMMC-7721 cells (2 × 10^6) were injected subcutaneously into the right dorsal skin of 4-5-week-old female BALB/c nude mice. The tumor is allowed to grow for 10 days to about 100 mm^3 after inoculation. Mice were randomly divided into three groups, a blank control group, a non-targeted treatment group, and a targeted treatment group, n = 3 in each group. 200 µL of physiological saline containing Cy5.5, Cy5.5-labeled non-targeted liposome (PL), Cy5.5-labeled transferrin liposome (TfPL) were injected at a dose of 100 µg/kg Cy5, respectively. Images were taken at 24 hours after injection using the MAESTRO in vivo imaging system (Cambridge Research & Instrumentation; Hopkinton, MA, USA). The mice were then harvested and the heart, liver, spleen, lung, kidney and tumor were dissected, washed with saline and imaged using the MAESTRO in vitro imaging system.

2.15 Evaluation of antitumor efficiency and safety in vivo
Mice bearing SMMC-7721 liver cancer were established as described above and randomly divided into 4 groups (n = 6 per group). Mice were injected intravenously with saline (control) or transfected proteoliposome or proteoliposome 50 µg/kg at 10, 12, 14, 16, 18, 20, 22, 24 and 26 days after implantation. The anti-tumor efficiency was determined according to the tumor volume using the following formula: larger diameter × (smaller diameter/2)^2. Systemic toxicities were assessed by monitoring body weight changes and nephrotoxicity.

2.16 HE Staining

For HE staining, tissues were fixed in 4% paraformaldehyde for more than 24 hours. Paraffin-embedded tissue sections (4 µm) were dewaxed and rehydrated. Hydration sections were stained with Mayer's hematoxylin and eosin.

2.17 Statistical Analysis

For multiple comparisons, a one-way ANOVA test was performed. The t test (two-tailed) was used for comparison between the two groups. Data are expressed as mean ± standard deviation (S.D.). Survivors were estimated using a log-rank test. *p < 0.05, **p < 0.01, ***p < 0.001.

3. Results

3.1. Preparation and characterization of the protein liposomes

Different from the conventional protein-modified nanospheres or liposomes, this study prepared the protein liposome of the double-layer skeleton structure by physically assembling the amphiphilic protein derivative modified by GHDC with cholesterol. The flow chart is shown in Fig. 1A. The UV spectrum of Fig. 2A showed that Tf-GHDC and Tf-PL have similar UV absorption curves to Tf. The prepared transferrin liposome had a clear UV absorption peak map of transferrin, and transferrin was present in lipid. The protein electrophoresis results in Fig. 2B also indicated that Tf-PL has the transferrin content. The characteristic peaks of transferrin and GHDC were also observed in the nuclear magnetic H spectrum of Tf-GHDC, and the long-chain methylene stretching vibration absorption peak at 2922 cm\(^{-1}\) and 2852 cm\(^{-1}\) (Figure S3). It can be seen from the above that the proteoliposome prepared in this study had a higher protein component. By studying the effects of different distribution ratios on liposome size, charge, gene encapsulation efficiency (EL), and loading (DL) (Table S1-4, Supporting Information), an optimized distribution ratio was obtained. The loading of Tf-PL to AChE optimized to the distribution ratio was (6.31 ± 0.32) %, the encapsulation efficiency was (94.3 ± 1.01) %, the load corresponding to PL (6.07 ± 0.43) %, and the encapsulation ratio (91.2 ± 0.79) % (Table S5, Supporting Information). At the same time, the quantitative analysis of transferrin on the surface of Tf-PL by enzyme-linked immunosorbent assay
showed that the efficiency of transferrin to the surface of liposome was \((88.7 \pm 2.31)\%\), so most of the transferrin was distributed in proteoliposome's surface. The morphology of PL-AChE and Tf-PL-AChE (Figure S4, Supporting Information) was analyzed by transmission electron microscopy. Figure 2C, D-E showed the basic characteristics of the morphology, particle size and potential of PL-AChE and Tf-PL-AChE. In Fig. 2C, the results of atomic force microscopy showed that the morphology of GL-AChE and Tf-PL-AChE was stable and showed a stable and regular spherical shape, the size of the two liposomes was relatively close, about 80 nm. Figure 2D,E showed the particle size and potential data of proteoliposomes in aqueous solution. The particle size of Tf-PL-AChE was \(113.0 \pm 5.2\) nm, PDI = 0.113, the potential was 28.5 mV, and the particle size of PL-AChE was \(96.0 \pm 7.0\) nm, PDI = 0.187, and potential was 30.0 mV. The particle size and potential data in the aqueous solutions of the two liposomes were relatively close and correspond to the particle size data analyzed by atomic force microscopy. Comprehensive analysis of the above characterization data indicated that the direct preparation of proteoliposome strategy, increasing the content of transferrin in protein liposomes, which was beneficial to increase the targeting affinity of liposomes, cells with high expression of transferrin receptor. It had a higher targeting effect than tissue, and high physicochemical stability can be achieved by adding stabilizer cholesterol (Chol).

### 3.2. In vitro cytotoxicity of the proteoliposomes and gene release study

In vitro cytotoxicity of the prepared proteoliposomes is shown in Figure S5. The results indicated that the relative survival rate of 293T cells and liver cancer SMMC-7721 cells were still high when the concentration of PL and Tf-PL reached 500 µg/mL at 48 h. So the proteolipid prepared in this study is less toxic and has good biocompatibility, which lays a foundation for subsequent applications. As gene carrier, Tf-PL should have high stability during storage as well as a high gene delivery efficiency. We studied the gene release efficiency of PL/AChE and Tf-PL/AChE in vitro under different pH conditions (Figure S6), in which Figure S6A showed the total release at different time points, and in Figure S6B showed the gene release rate within 12 hours. Compared with the rapid release of free AChE, PL/AChE and Tf-PL/AChE showed similar sustained release of AChE at pH 7.4 and pH 5.5; the burst release was not obvious, and the stability of Tf-PL/gene complex was higher.

### 3.3. In vitro cellular uptake and transfection efficiency study

To determine if TfR might be a therapeutic target for liver cancer, we compared TfR expression between liver cancer (N2 = 225) and normal liver tissue (N1 = 220) by downloading the Roessler liver 2 dataset from Oncomine. The data showed that TfR expression was significantly increased in liver cancer specimens, which was 2.780 times that of normal specimens (Figure S1A, Supporting Information). This suggested that TfR may be a potential drug delivery target for liver cancer. Then, we compared the expression of TfR in 293T cells and liver cancer SMMC-7721 cell lines, as shown in Fig. 3A and B. Western blot and flow cytometry results all showed that SMMC-7721 had significant TfR expression compared to 293T cells, \(p < 0.001\), which can be used for further experimentations. In order to study the interaction between liposomes and cell surface receptors, to assess the ability of Tf-PL to specifically
bind to TfR and trigger receptor-mediated internalization of liposome gene delivery in TfR-positive cells, we compared FITC-labeled PL to Tf-PL in SMMC-7721 cells. As shown in Fig. 3C, SMMC-7721 has a significant time-dependent endocytosis both in PL and Tf-PL. However, cellular uptake of Tf-PL was greater than uptake of PL. To evaluate the efficiency of Tf-PL as a gene vector for gene delivery to cells, we compared the transfection efficiency of PL and Tf-PL with commercial transfection reagent Lipo 2000 on SMMC-7721 cells. By comparing the amount of GFP fluorescence in the fluorescence microscope at 36 h and 72 h, it can be found that the gene transfection efficiency of Tf-PL is significantly stronger than that of Lipo 2000 (Figure S7), so Tf-PL is suitable to be used as a vector for SMMC-7721 cells gene therapy.

3.4. In vitro cytotoxicity study

The data in Figure S2 showed that overexpression of Ach and low expression of AChE were expressed in liver cancer. Therefore, we measured the effect of Ach and AChE on the proliferation of liver cancer SMMC-7721 cells by CCK-8 assay. As shown in Figure S8 A, Ach can significantly promote the proliferation of SMMC-7721, with the increase of Ach concentration and the prolongation of action time, the proliferation promotion effect is more obvious. On the contrary, as shown in Figure S8 B, AChE can significantly inhibit the proliferation of SMMC-7721, and the inhibition of proliferation is more obvious with the increase of AChE concentration and the prolongation of action time. Therefore, this study demonstrates that overexpression of AChE is theoretically a strategy to inhibit proliferation of liver cancer cells.

The effect of AChE gene therapy on liver cancer SMMC-7721 cells was studied. It was found that PL/AChE and Tf-PL/AChE inhibited the growth of SMMC-7721 cells in a concentration- and time-dependent manner. Free AChE had little effect on the proliferation of SMMC-7721 cells, and the effect of gene therapy on 293T cells was not large, too (Fig. 4A). Tf-PL/AChE showed the strongest cytotoxicity with IC50 values of 3.45 and 4.25 µg/mL at 48 and 72 hours, respectively (Fig. 4B). This is the result of Tf modification significantly enhancing the uptake of Tf-PL/AChE by liver cancer SMMC-7721 cells. Tf-PL delivers more AChE to cells and subsequently inhibits cell growth.

3.5. In vitro cell migration study

Cell migration plays an important role in tumor growth and metastasis, so the role of Tf-PL/AChE in cell migration of SMMC-7721 cells can be assessed by transwell migration and scratch repair experiments (Fig. 4 and Figure S9). Although SMMC-7721 cells in the untreated control group showed high levels of migration, cells treated with Tf-PL/AChE and PL/AChE showed significantly reduced motility, and Tf-PL/AChE was the most effective, the inhibition rate was 45.1%, p < 0.05. These observations indicate that Tf-PL/AChE treatment can effectively block the migration of SMMC-7721 cells. It is similar in the scratch repair experiment, the cell growth time increased from 24 h to 72 h, and the control group, free AChE and PL/AChE group showed significant cell healing, while Tf-PL/AChE Group cell repair showed a significant lag, so Tf-PL/AChE treatment significantly affected the migration and proliferation of SMMC-7721.

3.6. Cell cycle and apoptosis study
To further investigate the anti-proliferative effect of Tf-PL/AChE in hepatocarcinoma cells, SMMC-7721 cells were treated with flow cytometry for 24, 48 and 72 h, respectively, to evaluate the apoptosis and cycle distribution of SMMC-7721 cells. The results showed that PL/AChE and Tf-PL/AChE induced apoptosis in SMMC-7721 cells in a time-dependent manner (Fig. 5A). Compared with PL/AChE, the apoptosis rate of Tf-PL/AChE treatment was significantly increased. The apoptosis rate after incubation at 24, 48 and 72 h were (3.23 ± 0.06) % Vs (2.64 ± 0.14) %, (8.90 ± 0.10) % Vs (4.22 ± 0.18) % and (25.45% ±0.45) % Vs (7.73 ± 0.21) % respectively, and the statistical results were significantly different (p < 0.05).

At the same time, the cell cycle analysis was also evaluated. As shown in Fig. 6A and 6B, SMMC-7721 cells showed significant G0/G1-S phase accumulation after gene therapy, while the G2/M phase decreased and was time-dependent. This indicated that the transition from the S phase to the G2/M phase can be disrupted in cells treated with the transferrin liposome carrying the AChE gene, with the Tf-PL/AChE group having the best effect. Therefore, Tf-PL/AChE-treated cells can inhibit the growth of liver cancer cells by inducing apoptosis and cell cycle arrest.

3.7. In vivo imaging distribution study

Effective targeting of tumor cells is essential for inhibiting tumor growth. To investigate the tumor targeting efficiency of Tf-PL/AChE in vivo, we established a nude mouse model of SMMC-7721 tumor. Real-time fluorescence imaging of nude mouse organs and tumors was performed by injecting near-infrared dye (Cy5.5)-labeled liposomes into the tumor-bearing nude mice, and the target effect of the materials was studied by the distribution of fluorescent signals (Fig. 7). The fluorescence image of Cy5.5 showed that after 24 hours, the fluorescence signal was mainly found in kidney, lung, liver, tumor and peripheral blood. Moreover, the fluorescence signal of mouse tumors in the Tf-PL group was stronger than the other groups. After 72 hours, the fluorescence signals in kidney, lung, liver, tumor and peripheral blood were significantly decreased. There was still obvious Cy5.5 signal distribution in the tumor of Tf-PL group, but the intratumoral signal of PL group was significantly decreased. At the same time, green fluorescent protein (GFP) expression was studied to indirectly mimic the expression of the acetylcholine ester gene to analyze the targeted delivery of the Tf-PL-carrying gene to the tumor. There was a significant GFP fluorescence signal at the tumor site in mice, 24 h or 72 h. Based on the above experimental results, it can be proved that the Tf-PL group has extremely high intensity and distribution in tumors, so Tf-PL can effectively target liver cancer.

3.8. In vivo antitumor efficacy study

In this study, a nude mouse xenograft model of SMMC-7721 tumor was established, and different therapy treatments were given to analyze the anti-tumor effects of different groups. Mice bearing SMMC-7721 tumors were injected with normal saline, free AChE, PL/AChE and Tf-PL/AChE every 7 days for 4 consecutive injections. As shown in Fig. 8A, Free AChE had some efficiency in inhibiting the tumor growth. But tumor growth in mice treated with GL/AChE and Tf-PL/AChE was significantly inhibited. Tumor growth in mice treated with GL/AChE and Tf-PL/AChE was significantly inhibited. Mice treated with Tf-PL/AChE had the most pronounced inhibitory effect on tumor growth compared to the other
groups (Fig. 8A and Figure S10). The final weight and volume of the tumor in the Tf-PL/AChE group were $(0.52 \pm 0.12)$ g and $(517.14 \pm 112.63) \text{mm}^3$, respectively. Statistical analysis showed that these tumors were significantly smaller than those in the other groups (Fig. 8B, C). HE staining analysis was performed on the tumors of the above four groups. The results are shown in Fig. 9, the tumors in the PL/AChE and Tf-PL/AChE groups showed mild staining and a large area of blank, in Tf-PL/AChE group tumor tissue showed a large area of necrosis, so the tumor tissue necrosis was very serious. The tumor tissues of control and the Free AChE groups were darker, showed identifiable tissues. Taken together, it was shown that Tf-PL/AChE induced an effective response against SMMC-7721 cells in vivo.

4. Discussion

Currently, non-viral vectors have been widely used for gene therapy, but carrier toxicity is an important problem in the clinical application of non-viral vectors, usually due to their positively charged surface\[32, 33\]. Previous studies have found that cationic carriers (such as liposomes and PEI, etc.) accumulate in lung tissue immediately after administration, thereby inducing an inflammatory response. Excessive positive charge on the surface of cationic nanocomposites typically results in irreversible binding components to plasma including albumin and fibrinogen. In this work, we first attempted to directly construct protein liposomes using protein derivatives by amphiphilic modification of the tumor targeting protein-transferrin, and by in vitro and in vivo delivery of the gene. The effect evaluation confirmed the significance of the effectiveness and targeted effect of the constructed gene/proteoliposome. Transferrin liposome can effectively reduce the charge on the surface of proteoliposome by increasing the protein content while reducing the content of cation, which helps to reduce the cytotoxicity of liposome. Typically, nanoparticles of size 50–200 nm are suitable for prolonged circulation in the blood and efficiently accumulate into the tumor by enhanced permeability and retention (EPR) effects.

In our study, Tf-PL/AChE has a particle size of $113.0 \pm 5.2$ nm, a PDI of 0.113, and a potential of 28.5 mV, and these features confer its use for intravenous administration. Our study describes a method for easily preparing a neutral targeting gene delivery system by complexing cationic nanoparticles with a therapeutic plasmid by electrostatic interaction. The obtained neutral targeting gene delivery system provides a robust and flexible non-viral platform for mediating cancer gene therapy for intravenous administration.

Transferrin can specifically bind to transferrin receptor, transferrin receptor is expressed in a large amount on the surface of tumor cells, and transferrin receptor has become a hot spot in tumor targeting research. In this study, transferrin was directly constructed as transferrin liposome, which increased the water solubility of liposomes and increased the amount of protein on the surface of liposomes, using transferrin receptor-mediated lipids expressed on the surface of hepatoma cells. The plastid enters the cell and achieves targeted administration of liver cancer cells. Qualitative confocal experiments confirmed that proteolipids directly formed by transferrin can have specific affinity with liver cancer SMMC-7721 cells, but have no affinity for normal hepatocytes. This indicates that the proteolipid formed by transferrin can target liver cancer cells and introduce into the cells through the transferrin receptor
without affecting normal cells. Secondly, this study confirmed the inhibitory activity of Tf-PL/AChE on hepatocarcinoma cells in a concentration-dependent manner by cell proliferation inhibition assay, and the proliferation inhibition effect of Tf liposome on hepatoma cells was significantly stronger than that of free AChE group. The common bovine serum albumin liposome group indicates that the anti-tumor effect of Tf-PL/AChE depends on the uptake of proteoliposome by tumor cells. The amount of proteoliposome uptake after Tf modification is enhanced and the toxicity is enhanced. Transwell experiments showed that the cell migration ability of tumor cells after Tf-PL/AChE treatment was reduced, and the apoptosis and cycle experiments were also the same as the cytotoxicity experiments. In vivo imaging experiments confirmed that the proteosome has a tumor-specific targeted delivery of the therapeutic gene and prolonged the in vivo circulation time of the proteoliposome to increase the bioavailability of the therapeutic gene. The ectopic tumor model of liver cancer confirmed the significant inhibitory effect of Tf-PL/AChE on tumor growth. Our data suggest that the AChE gene is safe and effective for the treatment of liver cancer and shows promising clinical applications.

5. Conclusions

In this work, we show a strategy for the delivery of AChE gene by transferrin liposome targeting TfR on the surface of liver cancer cells to treat liver cancer. This strategy provides an alternative to live virus as a gene or drug carrier for the treatment of tumors, which can effectively avoid the biosafety problems that live viruses may cause. The gene delivery system has good blood compatibility and degradability, low toxicity, good tumor targeting ability and high transfection efficiency for liver cancer cells. Overexpression of the AChE gene can significantly inhibit the growth of liver cancer cells in a nude mouse xenograft model. The proteolipid prepared directly from Tf enhances the therapeutic gene into the hepatoma cells and is a potential liver cancer delivery system. In conclusion, the strategy of combining transferrin liposome and AChE gene delivery provides a new idea for gene therapy of liver cancer.

Declarations

Credit authorship contribution statement

Kai Wang and, Methodology, Investigation, Formal analysis, Writing-original draft. Fusheng Shang, Dagui Chen and Jianpeng Jiao: Resources, Data curation, Writing - review & editing. Tieliu Cao: Resources, Investigation. Xiaofei Liang, Shengli He & Xiaowei Wang: Conceptualization, Methodology, Supervision, Writing-review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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Figures
Gene-carrying transferrin liposome preparation procedure (A) and delivery strategy of transferrin liposome-carrying gene-targeted transferrin receptor-positive cells (B). The proteoliposome interacts with the surface TfR of the targeted cell (1), and then enters the cytoplasm by endocytosis (2), released by lysosomal digestion (3), carries the target gene into the nucleus (4), and integrates Overexpression of the gene of interest into the genome (5).
Figure 2

Performance characterization of transferrin liposomes. A: Ultraviolet absorption spectrum; B: SDS-PAGE analysis of protein bands; C: liposome atomic force microscopy, C1 (PL/AchE), C2 (TF-PL/AchE); D: dynamic light scattering analysis of particle size distribution of protein liposomes in aqueous solution, D1 (PL/AchE), D2 (TF-PL/AchE); E: dynamic light scattering analysis of potential of liposomes in aqueous solution, E1 (PL/AchE), E2 (TF-PL/AchE).
Figure 3

Transferrin receptor as a drug delivery target for liver cancer cells. A: Western Blot analysis of Tf expression in different cells; B: Flow cytometry analysis of Tf expression on different cell surfaces; C: In vitro cellular uptake study.
Figure 4

In vitro cytotoxicity and cell migration of AChE treatment study. AChE treatment study on 293T cells (A) and SMMC-7721 Cells (B), Inhibition of cell migration in SMMC-7721 cells following Ache treatment (C), representative images of the Transwell assay showing the inhibitory effect of AChE on cell migration following treatment with Control, Free AChE, PL/ AChE, Tf-PL/ AChE for 2 h at an equivalent AChE concentration of 2.5 μg/mL. (D) Quantification showing the migration inhibition rates in SMMC-7721 cells following the treatments.
Cell apoptosis analysis. (A) Flow cytometric analysis and (B) quantification of cell cycle distribution in SMMC-7721 cells following treatment with (a) Control, (b) Free AChE, (c) PL/AChE, (d) Tf-PL/AChE for 24, 48 and 72 h at an equivalent AChE concentration of 2.5 μg/mL.
Figure 6

Cell cycle analysis. (A) Flow cytometric analysis and (B) quantification of cell cycle distribution in SMMC-7721 cells following treatment with Control, Free Ache, GL/Ache, Tf-PL/Ache for 24, 48 and 72 h at an equivalent Ache concentration of 2.5 μg/mL.
Figure 7

In vivo distribution of Tf-PL loaded with Cy5.5 or GFP. A: fluorescence distribution at 24 h; B: fluorescence distribution at 72 h; C: fluorescence intensity reference
Figure 8

Analysis of tumor treatment effect
A: The relative tumor size of the subcutaneous model changed with time
B: Image of tumor size in each group
C: Relative tumor volumes as a function of time
D: Relative tumor weight as a function of time
Figure 9

HE staining of tumor tissue from different tumor treatment groups

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