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Magnetic Resonance Spectroscopy of siRNA-Based Cancer Therapy

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Abstract

Small interfering RNA (siRNA) is routinely used as a biological tool to silence specific genes, and is under active investigation in cancer treatment strategies. Noninvasive magnetic resonance spectroscopy (MRS) provides the ability to assess the functional effects of siRNA-mediated gene silencing in cultured cancer cells, and following nanoparticle-based delivery in tumors in vivo. Here we describe the use of siRNA to downregulate choline kinase, a critical enzyme in choline phospholipid metabolism of cancer cells and tumors, and the use of ^1H MRS of cells and ^1H magnetic resonance spectroscopic imaging (MRSI) of tumors to assess the efficacy of the downregulation.

Keywords

Choline metabolism; Dual-phase extraction; Magnetic resonance spectroscopy (MRS); Magnetic resonance spectroscopic imaging (MRSI); Nanoplex; Small interfering RNA (siRNA); Transient transfection

1 Introduction

Small interfering RNA (siRNA)-mediated silencing of specific targets has significant potential in cancer therapy to downregulate pathways that are frequently up-regulated in cancer cells, but not in normal tissue, achieving cancer cell-specific treatment [1]. A consistent metabolic hallmark observed in multiple cancers is the increase of cellular phosphocholine (PC) and total choline-containing compounds (tCho), which is closely related to malignant transformation, invasion, and metastasis [2–5]. Enzymes in choline phospholipid metabolism present attractive targets to exploit for treatment. Choline kinase- α (Chk- α) is an enzyme in the Kennedy pathway that phosphorylates free choline (Cho) to phosphocholine (PC), and its up-regulation in several cancers is a major contributor to increased PC levels. Here, we describe the use of siRNA targeting Chk- α and evaluate its functional effects on PC, phosphatidylcholine (PtdCho), and fatty acid levels using *in vitro* MRS and *in vivo* MRSI. MRS and MRSI are widely used to characterize tumor metabolism in both preclinical and clinical settings. With high-resolution ^1H MRS, it is possible to quantify the concentrations of free choline (Cho), PC, GPC, PtdCho and fatty acids. With ^1H MRSI, we can assess *in vivo* the efficacy of the treatment with siRNA directed against Chk- α by measuring the level of tCho that includes the PC signal. In ^1H MRSI, chemical information is spatially encoded, and the localized spectra can be processed to obtain images of metabolites. As MR methods are noninvasive, they can be translated easily from

preclinical research to clinical applications. Here, we have described steps to downregulate Chk- α in human breast cancer MDA-MB-231 cells in culture, and in MDA-MB-231 tumors in mice.

2 Materials

2.1 Cell Culture, Transfection, and siRNA

RPMI medium (Sigma-Aldrich, St. Louis, MO).

Fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA).

DharmaFECT4 (Thermo Fisher Scientific Inc., Waltham, MA).

siRNA against human Chk- α (5'-CAUGCUGUUC CAGUGCUC-3') (Thermo Fisher Scientific Inc., Waltham, MA).

2.2 Dual-Phase Extraction and High-Resolution ^1H MRS

Cold methanol, chloroform, and water.

Chelex bead (Sigma-Aldrich, St. Louis, MO).

High-speed centrifuge, rotary evaporator, lyophilizer.

5 mm NMR tubes (Wilmad Labglass, Buena, NJ).

Deuterated water (D_2O) (Sigma-Aldrich, St. Louis, MO).

3-(Trimethylsilyl) propionic 2,2,3,3- d_4 acid sodium salt (TSP) (Sigma-Aldrich, St. Louis, MO).

Chloroform- D (CDCl_3) and methanol- D_4 (CD_3OD) (Cambridge Isotope Laboratories Inc., Tewksbury, MA).

Tetramethylsilane (TMS; Cambridge Isotope Laboratories Inc., Tewksbury, MA).

2.3 Nanoplex Synthesis

Bacterial cytosine deaminase, bCD.

Polyethyleneimine, PEI (Sigma-Aldrich, St. Louis, MO).

Poly-L-lysine, PLL (Sigma-Aldrich, St. Louis, MO).

Methyl polyethyl glycol succinimide ester (Nanocs. Inc., NY).

PEG-NHS ester (2 kDa) (Nanocs. Inc., NY).

N-succinimidyl-*S*-acetylthiopropionate, SATP (Pierce, Rockford, IL).

Rhodamine succinimide ester, rhodamine-NHS (Sigma-Aldrich, St. Louis, MO).

1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid mono-N- hydroxysuccinimide ester, DOTA-NHS (Macrocyclics Dallas, TX).

Succinimidyl 4-formylbenzoate, SFB (Pierce, Rockford, IL).

HEPES buffer (pH 8.4).

Sodium acetate buffer (pH 4.6–5.5).

Cy5.5-NHS ester (GE Healthcare, Piscataway, NJ).

Succinimidyl 6-hydrazinonicotinamide acetone hydrazine, SANH (Pierce, Rockford, IL).

N-[ϵ -Maleimidocaproyloxy]succinimide ester, EMCS (Pierce, Rockford, IL).

3 Methods

3.1 In Vitro Studies

3.1.1 Cell Transfection

1. Seed cells in a flask or dish. Cell density should be ~70–80 % confluent at the time of collection. In our studies, MDA-MB- 231 breast cancer cells are seeded at 5×10^6 cells in 150 mm dish.
2. Twenty-four hours later, cells are transfected with 25–100 nM siRNA targeted against human Chk- α (5'-CAUGCUGUUC CAGUGCUCC-3') using DharmaFECT4.
3. To transfect the cells, prepare two 15 mL Falcon tubes per transfection. In the first tube, add 1.8 mL of serum-free media followed by 200 μ L of siRNA of desired concentration (diluted in $1 \times$ siRNA buffer) and mix well.
4. In the second tube, add 28 μ L of DharmaFECT4 to 2 mL of serum-free media. Incubate both tubes for 5 min.
5. Mix the contents of both tubes and incubate this complex for an additional 20 min.
6. Add 18 mL of antibiotic free RPMI media supplemented with 10 % FBS to this complex, mix well, and add gently to the 150 mm dish containing MDA-MB-231 cells.
7. Incubate the cells at 37 °C incubator supplied with 5 % CO₂ for 48 h.

3.1.2 Dual-Phase Extraction

1. As described in Subheading 3.1.1, cells are plated, cultured for 24 h, and transfected with siRNA for 48 h.
2. Adherent cells are collected by trypsinization and counted using a hemocytometer. Trypan blue is added to cells during counting to correct for dead cells. More than 10^7 cells are needed for cell extraction to achieve good signal-to-noise ratio (S/N) for MRS. Water-soluble as well as lipid extracts are obtained using the dual-phase

extraction method. Dual phase extraction has the advantage that it does not significantly change the pH of the extracted material, and provides both the water-soluble and the lipid fractions of the cells, thereby leaving the lipids intact.

3. Pelleted cells are mixed with 4 mL of ice-cold methanol and vigorously vortexed.
4. After keeping the samples on ice for 10 min, 4 mL of chloroform are added.
5. The sample is vortexed vigorously and kept on ice for 10 min.
6. Finally, 4 mL of water are added and the sample is shaken well.
7. The sample is stored at 4 °C overnight for phase separation and then centrifuged at $20,000 \times g$ at 4 °C for 30 min.
8. The water-methanol phase containing the water-soluble cellular metabolites can be treated with ~100 mg chelex beads for 10 min on ice to remove divalent cations, followed by removal of the chelex beads.
9. After removing the beads, methanol is evaporated using a rotary evaporator.
10. The remaining water phase is lyophilized and can be kept at -20 °C until analysis.
11. The chloroform phase containing the cellular lipids is dried in a stream of N₂ and stored under N₂ (i.e., the tube is “filled” with N₂) at -20 °C.

3.1.3 High-Resolution ¹H MRS

1. Water-phase cell extracts are resuspended in 0.6 mL deuterated water (D₂O) including 5 µL of 0.75 % (w/w) 3-(trimethylsilyl) propionic 2,2,3,3-d₄ acid sodium salt (TSP) in D₂O, which is used as an internal standard for concentration calculation and chemical shift assignment.
2. Lipid-phase cell extracts are resuspended in 0.4 mL of chloroform-D (CDCl₃) with 0.05 v/v % tetramethylsilane (TMS), as a concentration and chemical shift reference. 0.2 mL of methanol-D₄ (CD₃OD) with 0.05 v/v % TMS are then added.
3. The samples are then ready to be analyzed using a high-field MR spectrometer. Conventional high-resolution ¹H MRS typically utilizes 3 mm to 5 mm probes to cover the sensitive volume of a sample size of 0.6 mL.
4. After placing the tube in the magnet, the probe is tuned and matched, the signal is then locked with D₂O for the water phase and CD₃OD for the lipid phase. Careful shimming is of utmost importance to obtain narrow line widths to resolve the peaks.
5. Fully relaxed high-resolution ¹H MR spectra are acquired using the following acquisition parameters: flip angle, 30°; sweep width, 10,000 Hz; repetition time, 11.2 s; time domain size, 32 K; 128 scans.
6. For metabolite quantification, the signals from fully relaxed MR spectra are integrated and the concentration of each detected metabolite of interest quantified based on the concentration standard. Signal integrals of the -N-(CH₃)₃ resonances

of PC at ~3.225 ppm, GPC at ~3.235 ppm, and Cho at ~3.208 ppm are determined (Fig. 1).

In the lipid phase, the integrals of phosphatidylcholine at 3.220 ppm, and of the methylene groups in fatty acids (F_{mix}) at 1.245 to 1.364 ppm are measured, and related to the integral of the reference peak [1] (Fig. 2).

To quantify the concentrations, the peak integration (I_{met}) from ^1H spectra for PC, GPC, and Cho are compared to that of the internal standard TSP (I_{TSP}) according to the equation

$$[\text{metabolite}] = A_{\text{TSP}} \frac{I_{\text{met}}}{I_{\text{TSP}} N_{\text{cell}} V_{\text{cell}}}$$

In this equation, [metabolite] represents the intracellular concentration of the metabolite of interest expressed in mmol/L (mM). A_{TSP} is the number of moles of TSP in the sample, N is the cell number, and V is cell volume. I represents the integral of the selected peak from the MR ^1H spectra. Because the number of protons contributing to the signals of PC, GPC, and Cho and the TSP peak is the same, a correction for differences in the number of protons is not required. The total choline (tCho) concentration is obtained by adding values for PC, GPC, and Cho. If the cell count is known, but not the cell volume, it is possible to quantify metabolites as fmol/cell. Cell volume, calculated as $[(4\pi/3) \times (d/2)^3]$, is determined by measuring the diameter (d) of 100 randomly selected cells using an optical microscope following cell trypsinization.

3.2 In Vivo Studies

3.2.1 siRNA Delivery In Vivo—We have used both non-targeted [6] and targeted [7] siRNA delivery in our in vivo studies. A brief synthesis of the non-targeted nanoplex (NP) carrying optical and MR imaging reporters is outlined in Fig. 3. In addition to choline kinase siRNA, the NP also carries a prodrug enzyme, bacterial cytosine deaminase (bcd) that converts nontoxic 5-fluorocytosine (5FC) to cytotoxic 5-fluorouracil (5FU). The non-targeted NP is delivered to the tumor through the enhanced permeability retention (EPR) effect. The targeted NP has been shown by us to act as a “theranostic agent” that binds to prostate-specific membrane antigen (PSMA) [7].

1. Initially, a *N*-hydroxysuccinamide (NHS) ester of polyethylene glycol (PEG, 2 kDa) is conjugated with polyethylenimine (PEI, 25 kDa) to form compound **2**.
2. Compound **2** is labeled with NHS-rhodamine to form compound **3**.
3. Compound **3** is conjugated with succinimidyl 4-formylbenzoate (SFB) in HEPES buffer at pH 8.4 to form compound **4**.
4. Poly-L-lysine (PLL, 6 kDa) is labeled with Cy5.5-NHS, DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid)-NHS to produce compound **5**.
5. The DOTA on compound **5** reacts with $\text{Gd}_2(\text{CO})_3$ to form compound **6**.

6. Compound **6** with the imaging reporter is labeled with *N*-succinimidyl-*S*-acetylthiopropionate (SATP) and succinimidyl 6-hydrazinonicotinamide acetone hydrazine (SANH) to produce compound **7**.
7. Conjugation of **4** and **7** at pH 7.4 produces the PEI-PLL copolymer, which is reduced to form compound **8** that contains a free sulfhydryl group.
8. Treatment of bCD with *N*-(*ε*-maleimidocaproyloxy)-succinimide ester (EMCS) produces compound **9**.
9. Equimolar amounts of compounds **8** and **9** are cross-linked through the reaction of maleimide and sulfhydryl to provide the bCD-PLL-PEI.
10. Finally, electrostatic binding of siRNA with compound **10** gives the bCD-PLL-PEI/siRNA NP termed nanoplex **1**.
11. The NP is resuspended in 0.2 ml of PBS and injected intravenously at a concentration of 300 mg/kg.

3.2.2 Tumor Implantation

1. For the in vivo studies, 2×10^6 MDA-MB-231 human breast cancer cells are inoculated orthotopically in female severe combined immunodeficient (SCID) mice, in compliance with institutional guidelines established by the Institutional Animal Care and Use Committee of Johns Hopkins University. The mice are injected with the nanoplex and scanned when the tumors reach approximately 300 mm³.
2. Noninvasive in vivo MRI and MRS studies are performed on a 9.4 T MR spectrometer (Bruker) using a solenoid coil placed around the tumor.
3. During the MR experiment, the animal being scanned is stably anesthetized, and the body temperature maintained by using a blanket circulating with warm water. Physiological parameters, such as body temperature, heart and breathing rates can be followed during the acquisition using specific equipment compatible with high magnetic field. Different anesthetics can be used to perform MRS studies, which are typically a gas anesthetic such as isoflurane, or injectable anesthetics such as a mixture of ketamine and acepromazine or xylazine.
4. Anatomic images are acquired using a multi slice multi echo T₁- weighted sequence, with an echo time of 15 ms, and a repetition time of 500 ms (Fig. 4). We usually acquire 8–12 consecutive 1 mm thick slices to visualize the whole tumor and a 4 mm thick slice localized generally within the center of the tumor. Before acquiring MRSI on the selected slice, we acquire first an anatomic image of the slice.
5. The geometry of the selected 4 mm thick slice is imported into a 2D chemical shift imaging (CSI) sequence. After shimming carefully, water-suppressed MRSI is performed, with an in-plane resolution of 1 mm × 1 mm per pixel with VAPOR water suppression. The following parameters are used: echo time (TE) of 120 ms, repetition time (TR) of 1000 ms, field of view of 1.6 cm × 1.6 cm, phase encode

steps of 16, number of scans (NS) 8 for 2D CSI, block size 1024, and sweep width of 4000 Hz.

6. Water-unsuppressed reference MRSI is acquired from the same tumor slice as the water-suppressed MRSI, with a TE of 20 ms and an NS of 2. All other parameters are kept the same.
7. Spectroscopic images of the tCho signal at 3.2 ppm and the water signal at 4.7 ppm are generated from the MRSI data sets using an in-house IDL program and analyzed using the freeware program ImageJ 1.37v (<http://rsb.info.nih.gov/ij/>).

4 Notes

To minimize spectral line width, only clear solutions should be measured as particulate matter can result in field inhomogeneities that widen line widths. Any particles left in the sample should be spun down before transferring the sample into the NMR tube.

The in vivo studies can be performed with a targeted NP as well, as shown in Fig. 5. In this example, we used a human prostate xenograft model, PC3-PIP, that overexpresses PSMA [7]. We used subcutaneously implanted male SCID mice for the study. The NP was targeted by incorporating a PSMA-specific targeting moiety, by conjugating initially a *N*-hydroxysuccinamide (NHS) ester of the low-molecular-weight urea-based PSMA inhibitor (PI) (2-(3-[1-carboxy-5-[7-(2,5-dioxo-pyrrolidin-1-yloxycarbonyl)-heptanoylamino]-pentyl]-ureido)-pentanedioic acid; MW 572.56) with maleimide-PEG-NH₂ (3.4 kDa) to form PI-PEG-maleimide. Detailed synthesis of the targeted NP can be found in [7].

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Fig. 1. (a) Expanded regions of ^1H MR spectra and (b) quantitation of ^1H MR spectra ($n = 6$) from cell extracts of nonmalignant MCF-12A cells and malignant MDA-MB-231 breast cancer cells following 48 h of transient siRNA-Chk treatment. Abbreviations: Cho, free choline; GPC, glycerophosphocholine; PC, phosphocholine; tCho, total choline-containing compounds; ns, not significant. Columns, mean; bars, $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$. Adapted with permission from ref. 1

Fig. 2.
 (a) Representative ¹H MR spectra and (b) quantitation of the F_{mix} /phosphatidylcholine ratio from these ¹H MR spectra ($n = 3$) obtained from lipid cell extract fractions of nonmalignant MCF-12A cells and malignant MDA-MB-231 breast cancer cells treated with siRNA-Chk. Abbreviation: PtdCho, phosphatidylcholine. Columns, mean; bars, $\pm$ SEM. * $P < 0.05$. Adapted with permission from ref. 1

Fig. 3. Synthetic procedure to generate non-targeted NP loaded with siRNA and multiple imaging reporters

Fig. 4. Representative in vivo tCho maps and color-coded tCho intensity maps overlaid on corresponding T₁-weighted images of a tumor before and at 24 and 48 h after nanoplex injection (300 mg/kg, i.v.). Adapted with permission from ref. 6

Fig. 5.

(a) In vivotCho density maps from 2D CSI data sets acquired from a representative PC3-PIP tumor ($\sim 400 \text{ mm}^3$) before and 48 h after i.v. injection of the PSMA-targeted nanoplex (150 mg/kg). Parameters used were TE = 120 ms, TR = 1000 ms, 4 scans per phase encode step. CSI spectra were acquired at 9.4 T with an in-plane spatial resolution of $1 \text{ mm} \times 1 \text{ mm}$ from a 4 mm thick slice. (b) Corresponding in vivo tCho maps from the same 2D CSI data sets. (c) Representative one voxel spectra from 2D CSI represented in A and B. (d) tCho concentration calculated in arbitrary units before and at 48 h after injection of the nanoplex. Values represent median $\pm$ SEM ($n = 3$, $*P < 0.05$). Adapted with permission from ref. 7