Molecular imaging of lentiviral-mediated silencing of choline kinase as gene therapy in a human breast cancer xenograft

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Introduction

Choline kinase (Chk), which is overexpressed in several cancers, increases the tumoral phosphocholine (PC) and total choline (tCho) levels in breast cancers [1-2]. Increased Chk activity correlates with high tumor grade in clinical samples [2]. Chk is associated with tumor aggressiveness, and can be used as target for anticancer therapies [1]. RNA interference, which results in sequence specific gene silencing, has emerged as a powerful technique to regulate the gene expression of specific genes of interest. Lentiviral vectors have emerged as vectors of choice for long-term, stable *in vitro* and *in vivo* gene transfer. In the present study, we have used an HIV-based lentivirus to target Chk *in vitro* in MDA-MB-231 breast cancer cells and *in vivo* in MDA-MB-231 breast cancer xenografts. We constructed a lentivirus containing a pol III promoter, which enables efficient gene delivery for stable integration, producing double-stranded short hairpin RNA (shRNA) specific to Chk (shRNA-chk). Gene delivery to cells and tumor xenografts was monitored using fluorescence microscopy of enhanced green fluorescent protein (EGFP) expression, which was also encoded in the lentiviral construct. The effects of efficient lentiviral-mediated Chk silencing were confirmed using quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Chk downregulation in breast tumor xenografts following systemic injection of the lentivirus was monitored using single-voxel ^{31}P MRS *in vivo* to detect tumoral phosphomonoester (PME) and PC levels.

Methods

We designed a construct encoding for shRNA-chk expression in the pRRL-pGK-EGFP lentiviral vector [3]. Lentiviral particles mediating shRNA-chk expression were produced in 293T cells transfected with a plasmid containing pRRL-shRNA-chk-pGK-EGFP (transducing vector), δR8.2 (packaging vector), and a plasmid expressing vesicular stomatitis virus G (VSV-G glycoprotein) as shown in Fig. 1a. Forty-eight hours post-transfection, virus-containing supernatant was collected and added to MDA-MB-231 cells. Controls were transduced with virus expressing shRNA against Luciferase (shRNA-luc). Total mRNA was isolated from transduced MDA-MB-231 cells using a Qiagen kit (Qiagen, Chatsworth, CA), and subjected to qRT-PCR analysis using SYBR Green supermix (Bio-Rad, Richmond, CA) and appropriate Chk primers, to validate the effectiveness of Chk silencing in the transduced cells. Expression of 18s ribosomal RNA was used as internal control for qRT-PCR.

For *in vivo* experiments, human MDA-MB-231 breast cancer cells were orthotopically inoculated into the mammary fat pad of severe combined immune suppressed (SCID) mice. Large-scale preparation of virus was performed for systemic delivery *in vivo*, concentrating the virus-containing 293T cell supernantant ~130 fold. An average of 2.7×10^7 lentiviral particles in 200 µl phosphate-buffered saline per mouse was injected into the tail vein of MDA-MB-231 breast tumor bearing SCID mice. *In vivo* single-voxel ^{31}P MRS was performed on a 4.7T Bruker Biospec spectrometer to dynamically monitor tumoral PME and PC levels pre-and post-lentiviral gene therapy. Correlative low power brightfield and flurorescence microscopy of 1-mm thick freshly cut tumor and organ sections was performed at the treatment endpoint of 4 days following lentiviral particle injection. Phosphorus MR spectra were processed and analyzed with an in-house IDL program (Dr. D. C. Shungu), using gaussian multiplication and a combination of linear and nonlinear least-square fitting [4].

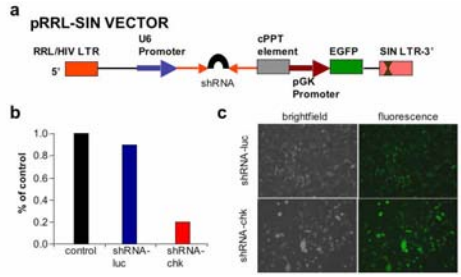

Figure 1: (a) Lentiviral shRNA vector map. (b) Chk mRNA expression measure by qRT-PCR in control (black bar), shRNA-luc control (blue bar), and shRNA-chk (red bar). (c) Brightfield and fluorescence microscopy of MDA-MB-231 cells transduced with shRNA-luc or shRNA-chk lentiviral vectors.

Results

MDA-MB-231 breast cancer cells transduced with lentiviral particles encoding for shRNA-chk and EGFP demonstrated 100% transduction efficiency as demonstrated by fluorescence microscopy (Fig. 1). qRT-PCR showed that Chk mRNA was silenced following lentiviral treatment in cell culture (Fig. 1). Following systemic administration of lentiviral particles into the tail vein of MDA-MB-231 breast tumor xenograft bearing SCID mice, sufficient gene delivery into the tumor occurred as shown by correlative brightfield and fluorescence microscopy (Fig. 2). *In vivo* ^{31}P MRS revealed that tumoral PC levels decreased in shRNA-chk treated tumors compared to shRNA-luc treated controls, which was also reflected by a decrease in phosphomonoester (PME) levels (Fig. 3). Phosphodiester levels decreased as well following treatment with shRNA-chk expressing lentiviral particles (Fig. 3).

Discussion

We have previously shown that Chk downregulation in breast cancer cells decreases proliferation and increases differentiation, and, therefore, is a good target for anticancer therapy [1]. Here we have demonstrated that Chk silencing in existing can be achieved *in vivo* through intravenous injection of lentiviral particles, which are capable of transducing cells to generate shRNA-chk. These lentiviral particles achieved efficient gene delivery as shown by fluorescence microscopy. Systemic gene delivery of the shRNA-chk generating construct resulted in silencing of Chk, which decreased the tumoral PC, PME, and PDE levels as monitored by *in vivo* ^{31}P MRS. Decreased PC and PME levels following shRNA-chk delivery are in good agreement with our previous studies [1]. This novel approach of Chk-targeted gene therapy is feasible for clinical translation, and can be monitored by single-voxel ^{31}P MRS *in vivo*.

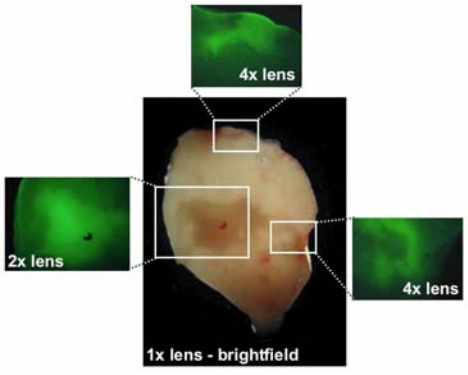

Figure 2: Brightfield and fluorescence microscopy of a representative MDA-MB-231 tumor following systemic gene therapy with lentiviral particles encoding for shRNA-chk and EGFP, demonstrating good gene delivery and transduction.

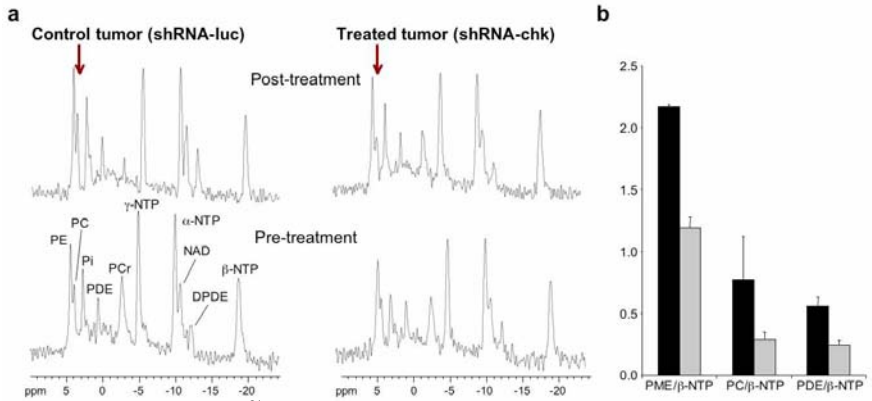

Figure 3: (a) *In vivo* single-voxel ^{31}P MR spectra of a representative control (left) and shRNA-chk-treated (right) tumor pre- and post-treatment (day 3) following intravenous injection of lentiviral particles. (b) PME/β-NTP, PC/β-NTP, and PDE/β-NTP ratios at three days following injection of viral particles. Black bars are shRNA-luc treated control tumors (n=2) and gray bars are shRNA-chk treated tumors (n=3). DPDE, diphosphodiester; NTP, nucleoside triphosphate; PC, phosphocholine; PDE, phosphodiester; PCr, phosphocreatine; PE, phosphoethanolamine; PME, phosphomonoester.

References: [1] Glunde K et al, *Cancer Res* 65, 11034 (2005) [2] de Molina AR et al, *Oncogene* 21, 4317 (2002) [3] An DS et al, *Hum Gene Ther* 14, 1207 (2003) [4] Glunde K et al, *Magn Reson Med* 48, 819 (2002) This work was supported by NIH 1R01 CA82337 and P50 CA103175 (JHU ICMIC Program).