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Noninvasive Detection of Lentiviral-Mediated Choline Kinase Targeting In a Human Breast Cancer Xenograft

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Abstract

Elevated phosphocholine (PC) and total choline metabolites are widely established characteristics of most cancer cells including breast cancer. Effective silencing of choline kinase (chk), the enzyme that converts choline to phosphocholine, is associated with reduced tumor growth. The functional importance and down regulation of chk using RNAi has been previously established. Here, we report on the preclinical evaluation of lentiviral vector-mediated downregulation of choline kinase (chk) using short hairpin RNA (shRNA), in established tumors derived from human breast cancer cells. Concentrated lentivirus expressing shRNA against chk was injected intravenously in the tail vein of MDA-MB-231 tumor bearing female severe combined immunodeficient (SCID) mice. Transduction efficiency in cells and tumors *in vivo*, was assessed optically by enhanced green fluorescent protein (EGFP) expression and additionally from chk mRNA and protein levels. An 80% reduction in chk mRNA and protein was achieved following almost 100% transduction efficiency in cells. Post-transduction with chk-shRNA, ¹H magnetic resonance spectroscopy (MRS) of cell and tumor extracts showed decreases in PC and total choline levels ($P < 0.01$ and 0.05 respectively) in comparison to controls. PC levels were monitored non-invasively by ³¹P MRS in tumors, and by ¹H MRS in cell and tumor tissue extracts. Noninvasive ³¹P MR spectra of chk-shRNA transduced tumors *in vivo* showed lower PC and phosphomonoester (PME) levels that were associated with reduced tumor growth and proliferation. This study demonstrates the use of lentiviral vectors to target chk in a human breast cancer xenograft, and noninvasive MRS detection of this targeting.

Keywords

choline kinase; breast cancer; magnetic resonance imaging; lentivirus; shRNA

Introduction

Increased phosphocholine (PC) is one of the hallmarks of cancer and several studies have established a strong correlation between increased PC and malignant progression (1,2). One of the major causes of high PC in tumors is the increase in expression and activity of choline kinase (chk), the rate-limiting enzyme that phosphorylates and converts choline to phosphocholine (1–3). Chk has been previously targeted with novel pharmacological inhibitors (4–6) and post-transcriptional gene silencing (7). Pharmacological inhibition of chk in cancer cells resulted in growth arrest and apoptosis (5). Downregulation of chk with plasmid-based shRNA decreased cellular PC and resulted in a reduction of proliferation and induction of differentiation of breast cancer cells (7). An additional advantage of targeting chk is that

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noninvasive ^{31}P or ^1H magnetic resonance spectroscopy (MRS) can be used to detect the decrease of PC and total choline (tCho) resulting from its downregulation (4).

Gene therapy holds significant promise in the prevention and treatment of many diseases. The advantages of gene silencing over chemical inhibitors are the long-term effect and specificity, and the ability to efficiently regulate various isoforms of target genes (8). Some of the problems that currently face conventional gene therapy are the lack of an efficient and reliable delivery system, stability of vectors (both viral and non-viral), the associated immunogenic response, problems associated with integration, and the inability to noninvasively detect the outcome of integration (9). Of the delivery mechanisms, viral vectors, and in particular lentivirus vectors, have potential for use in for gene therapy (10).

Here we have used a lentivirus vector to deliver chk shRNA in established tumors and demonstrated, for the first time, the therapeutic potential of chk downregulation *in vivo* in a human breast cancer xenograft model. We cloned the shRNA against chk into a lentivirus vector with enhanced green fluorescent protein (EGFP) as a reporter gene and transduced MDA-MB-231 breast cancer cells in culture to test the efficacy of the knock down. We subsequently transduced the virus *in vivo*, in an established breast cancer xenograft model derived from MDA-MB-231 cells, by systemically injecting concentrated virus through the tail vein. Transduction of chk-shRNA *in vivo* reduced tumor growth due to decreased proliferation, as detected by Ki-67 staining (11). The decrease in chk mRNA and protein levels and the downregulation of chk in tumors was detected noninvasively by a reduction of PC and PME in ^{31}P MR spectra, and of PC and total choline in cell and tumor tissue in ^1H MR spectra.

Materials and Methods

Cells and cell culture conditions

The MDA-MB-231 breast cancer and the human embryonic kidney (HEK) 293T cell lines were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were cultured in RPMI 1640 and Dulbecco's Modified Eagles' Medium (Mediatech, USA) respectively and supplemented with 10% FBS (SIGMA, USA), 100 units/ml of penicillin and 100 $\mu\text{g}/\text{ml}$ of streptomycin (Invitrogen Corp., Carlsbad, CA). Cells were maintained in a humidified atmosphere of 5% CO_2 in air, at 37° C.

Cloning, virus preparation, and titration

shRNA against chk in a PCR3.1 basic vector (7) was digested and cloned between NdeI and PstI (New England Biolabs, Beverly, MA, USA) into a human U6 promoter driven pRRL vector (Irvin S.Y. Chen, UCLA,) containing a reporter gene driven by a phosphoglycerate kinase promoter (PGK promoter, Figure 1A). A vector with shRNA against luciferase (luc-shRNA) was used as control (12). Infectious viral supernatants (DMEM media with 1% FBS) were derived by transient co-transfection of 293T (6×10^6 in 100mm^3 petri-plates) cells using lipofectamine 2000. A total of 19.5 μg of plasmid in the proportion of 12 μg of lentiviral vector carrying shRNA, 6 μg of packaging plasmid pCMV Δ R8.2 DVPR (VPR deleted) (13) and 1.5 μg of pCMV-VSVG were used, and viral supernatant collected at 48, 72 and 96 h post transfection. Pooled supernatants were concentrated using an Amicon Ultra-15 100K cutoff filter device (Millipore Billerica, MA, USA). The viral titer of the concentrated supernatant was determined by performing a p24 ELISA kit (Cell BIOLABS, INC. San Diego, USA) to detect the HIV-p24 core protein of the vector.

Establishing breast cancer xenografts

Approximately 2×10^6 MDA-MB-231 cells in 50 μl of Hanks buffered salt solution (HBSS, Mediatech, USA) were inoculated in the upper right thoracic mammary fat pad of age-matched

female severe combined immune deficient (SCID) mice. All surgical procedures and animal handling, including viral vector delivery, were performed in accordance with protocols approved by the Johns Hopkins University Institutional Animal Care and Use Committee, and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes for Health.

Cell and in vivo tumor transduction

For cell studies, 2×10^6 MDA-MB-231 cells were plated in 100 mm³ dishes and 5 ml of viral supernatant with 1 mg/ml of polybrene were added for 4–5 h. This procedure was repeated for three days. The efficiency of transduction was assessed and photomicrographs of EGFP expression were recorded using a Nikon TS100 inverted microscope (Nikon, USA) equipped with a charged coupled device (CCD) camera. Images were processed using Image Pro Plus 5.1 (MediaCybernetics, Silver Spring MD) software.

Lentiviral transduction of tumors *in vivo* was achieved in established MDA-MB-231 xenografts. Briefly, once tumor sizes reached 50 mm³, *in vivo* transduction of lentivirus was achieved through tail vein injections of 0.2 ml of concentrated viral suspension with a viral titer of 6.2×10^{11} -lentiviral particles/ml ($\sim 5 \mu\text{g}$ of p24/ml) in HBSS, twice a week for up to 30 days.

Tumoral phosphomonoester (PME) and PC levels were noninvasively monitored *in vivo* using single-voxel ³¹P MRS. MRS scans were performed 5 days after each injection on a 4.7T Bruker Biospec spectrometer (Bruker, Billerica, USA), over 30 days. At the end of the imaging studies, the kidney, liver and tumor were excised from each animal and 1-mm thick sections of the tissue samples were observed under a fluorescence microscope to detect EGFP expression. Tumor volumes were measured over the course of the injections. Tumor samples and other harvested tissues were processed for molecular analysis by snap freezing. For histochemical analysis, frozen tissues were embedded in OCT for cryosectioning, and fresh tissues were fixed in 10% formaldehyde.

Noninvasive in vivo ³¹P MRS studies

In vivo single-voxel ³¹P MRS was performed on a 4.7T Bruker Biospec spectrometer to dynamically monitor tumoral PME and PC levels through the time-course of chk-targeted lentiviral gene therapy. Mice were anesthetized with an intraperitoneal injection of ketamine (25 mg/kg; Phoenix Scientific, Inc., St Joseph, MO) and acepromazine (2.5 mg/kg; Aveco, Phoenix Scientific) diluted in saline. Body temperature was maintained during the experiment by using a blanket circulating with warm water as previously described (14). Single-voxel ³¹P MR spectra were acquired with a home-built surface coil, using a single-pulse sequence and the following parameters: pulse width of 45 degree, sweep width of 5000 Hz, data size of 2048 points, repetition time of 1 second, and 1024 scans. Spectra were processed and analyzed with an in-house IDL program (Dr. D. C. Shungu, Hatch MR Research Center, Columbia University, NY), using gaussian and exponential multiplication (exponential line broadening = -15 Hz, gaussian broadening = 0.03 Hz) to achieve the spectral resolution necessary to analyze the PC signal, or exponential multiplication (exponential line broadening = 20 Hz) for analysis of PME, which is relevant for spectra obtained at the lower field strengths used clinically, and a combination of linear and nonlinear least-square fitting in the time domain as previously described (4). The α -nucleoside triphosphate (NTP) signal was set to -10 ppm, and the signals were normalized and scaled to the β -nucleoside triphosphate (NTP) signal at -18.6 ppm, which remained constant during the course of the experiment.

RNA isolation, cDNA synthesis, and qRT-PCR

Total RNA was isolated from freeze-clamped tumor tissue and frozen MDA-MB-231 breast cancer cells transduced with lentivirus using QIAshredder and RNeasy mini kits (Qiagen, Chatsworth, CA). Finely powdered tumor tissues were further homogenized in RLT buffer (Qiagen mini Kit) before passing through the shredder. cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad, Richmond, CA). cDNA samples were diluted 1:10 and real-time PCR was performed using IQ SYBR Green supermix and gene specific primers in the iCycler real-time PCR detection system (Bio-Rad). All primers were designed using Beacon designer software 5.1 (Premier Biosoft International, Palo Alto, California USA). The expression of target RNA relative to the house keeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT1) was calculated based on the threshold cycle (C_t) as $R=2^{-\Delta(\Delta C_t)}$, where $\Delta C_t = C_t$ of target – C_t HPRT1. $\Delta(\Delta C_t) = \Delta C_t$ Chk shRNA transduced – ΔC_t luciferase shRNA.

Protein isolation and western blots

Total protein from tumor tissue or transduced cells was extracted using 1X cracking buffer (100mmol/L Tris(pH6.7), 2% glycerol) containing a protease inhibitor (Sigma, St.Louis, MO) @ 1:200 dilution, and resolved on 10% SDS-PAGE and immunoblot analysis, incubated using custom-made polyclonal antibody against choline kinase @ 1:100 dilution in 5% non-fat dry milk overnight at 4°C. A mouse monoclonal antibody against β -actin (Sigma) @ 1:10,000 was used as control. Appropriate HRP-conjugated secondary antibody, either anti-mouse or -rabbit (Amersham Biosciences) was used @ 1:2,500 dilution in milk. Immunoblots were developed using supersignal West Pico chemiluminescent substrate kit (Pierce Biotechnology Inc., IL, USA).

Dual-phase extraction and high-resolution MRS studies

Dual-phase extraction and MR studies were carried out with tumor tissue (<0.3g) or $\sim 2 \times 10^7$ cells from water-soluble fractions using methanol/chloroform/water(1:1:1 v/v/v) as previously described (7). Briefly, tumor samples were freeze-clamped, pulverized with liquid N_2 , and homogenized in 4 ml of ice-cold methanol. Pelleted cells were mixed with 4 ml of ice-cold methanol and vigorously vortexed. After keeping tumor and cell samples on ice for 10 minutes, 4 ml of chloroform were added, vortexed vigorously and incubated on ice for additional 10 minutes. Finally, 4 ml of water were added and the samples were vigorously shaken. All procedures were performed on ice, and samples were stored at 4 °C overnight for phase separation and later centrifuged at 15,000 $\times g$ at 4 °C for 30 minutes. The water/methanol phase containing the water-soluble cellular metabolites such as choline (Cho), PC and glycerophosphocholine (GPC) was treated with ~ 100 mg of chelex beads (Sigma-Aldrich, USA) to remove any divalent cations. After removing the beads, methanol was evaporated using a rotary evaporator. The remaining water phases were lyophilized. Cell extracts were resuspended in 0.6 ml deuterated water (D_2O) for MRS analysis. Five μl of 0.75% (w/w) 3-(trimethylsilyl) propionic 2,2,3,3- d_4 acid sodium salt (TMSP) in D_2O was used as an internal standard. Fully relaxed 1H MR spectra of the water-soluble extracts were acquired on a Bruker Avance 500 spectrometer (Bruker BioSpin Corp., Billerica, MA) as previously described (7). Signal integrals of N-(CH_3) $_3$ of Cho, PC, and GPC from within the 3.20–3.24 ppm region were determined and normalized to cell numbers or tumor weight, and compared to the standard. To determine concentrations of cell samples, peak integration (I_n) from 1H spectra for PC, GPC, Cho, and tCho (PC + GPC + Cho) were compared to that of the internal standard TMSP according to the equations:

$$[\text{metabolite}_{\text{cell}}] = A_{\text{TMSP}} \cdot \frac{I_{\text{metabolite}}}{I_{\text{TMSP}} \cdot N_{\text{cell}}} \quad (1)$$

$$[\text{metabolite}_{\text{tumor}}] = A_{\text{TMSP}} \cdot \frac{I_{\text{metabolite}}}{I_{\text{TMSP}} \cdot W_{\text{tumor}}} \quad (2)$$

In the equations, $[\text{metabolite}_{\text{cell}}]$ is the molar concentration per cell of the metabolite expressed as mol/cell, and $[\text{metabolite}_{\text{tumor}}]$ is the molar concentration in tumor samples. A_{TMSP} is the number of moles of TMSP in the sample, N_{cell} is the cell number. W_{tumor} is tumor volume (based on 1 g = 0.001 liter). Because the number of protons contributing to the signal of all the choline metabolites at 3.20–3.24 ppm and to the TMSP peak at 0 ppm is the same, correction for differences in the number of protons was not required. Cell culture data are the mean of 3 independent transduction experiments. *Ex vivo* tumor extract data are from an average of 4 animals with tumor weight $\leq 300\text{g}$.

Immunohistochemistry

Formalin-fixed, 5 μm thick sections were stained for hematoxylin and eosin (H&E) using a standard protocol. OCT embedded, frozen tissue samples were cut at 5 μm thickness on a cryotome and stained for Ki-67 using DAKO Rapid EnVision system (DAKO, Carpinteria, CA, USA) as published elsewhere (15). Cut sections were fixed in acetone for one minute and incubated with primary antibody (Ki-67 rabbit polyclonal antibody, 1:20 dilution, Novus Biologicals, Co, USA) for three minutes. Following this treatment, sections were incubated with the EnVision complex at 37 °C for an additional three minutes before incubation with substrate solution (DAB, 3,3'-diaminobenzidine). After each incubation, sections were washed three times with Tris Buffered saline (TBS) for 10 s each. Before counterstaining with a hematoxylin quick staining kit (Vector Labs, CA, USA), sections were washed with tap water and mounted on DAKO faramount aqueous mounting solution (DAKO, Carpinteria, CA, USA). Photomicrographs were taken on a Nikon microscope equipped with a CCD camera. Using Image J analysis software, photomicrographs of H&E sections, captured at 4x magnification, were quantified for percentage necrotic area as a ratio of the pixels in the necrotic region divided by the total pixel in the tumor section. Ki-67 quantification was achieved by measuring the ratio of the number of cells positive for brown staining to the total cells in a given field of view at 40x magnification. Five fields of view were analyzed per section.

Statistical Analyses

Data were analyzed using the software package JMP (www.jmpin.com) or a Microsoft excel software (www.microsoft.com) using a two-sided t-test, assuming unequal variances. Values of $P < 0.05$ were considered significant.

Results

Molecular and functional characterization of transduced MDA-MB-231 cells

To determine the efficacy of viral vectors, viral supernatants prepared from either luc-shRNA (control) or chk-shRNA were added to MDA-MB-231 breast cancer cells. EGFP expression in photomicrographs of control, and chk-shRNA transduced cells shown in Figure 1B confirmed ~90% transduction efficiency. To evaluate the silencing efficiency, transduced cells were characterized for endogenous chk mRNA by qRT-PCR using specific primers against endogenous chk, and for protein expression in immunoblots obtained using a custom-made rabbit polyclonal antibody against chk (Figure 1C and D). MDA-MB-231 cells transduced with chk shRNA showed approximately 80% reduction in chk mRNA and protein relative to control cells. Wild-type and luciferase transduced control cells had comparable expression levels of chk mRNA and protein.

To detect the efficiency of transduction by lentivirus expressing chk-shRNA in cells, ^1H MRS was performed on water-soluble extracts obtained from transduced cells. Representative fully relaxed ^1H MR spectra from chk-shRNA transduction showed that PC levels were significantly higher in control cells transduced with luc-shRNA as compared to those transduced with chk-shRNA (compare Figure 2A *versus* B). Levels of PC and, as a result, total choline-containing compounds were found to be significantly lower ($P \leq 0.01$) in chk-shRNA transduced cells (Figure 2C). GPC was not altered in control and chk-shRNA transduced cells, but the decrease of PC resulted in a significantly lower ratio of PC/GPC following chk-shRNA transduction (Figure 2D). These data represent an average of three separate experiments for each cell line.

Evaluation of lentivirus-mediated transduction of tumors *in vivo*

Data characterizing the chk-shRNA transduction of tumors are shown in Figure 3. As shown in Figure 3A, tumor growth in mice injected with chk-shRNA was reduced compared to control tumors. There was no evidence of weight loss or physical distress resulting from the treatment protocol. At the end of the intravenous injection protocol, the tumor and other organs such as, kidney, lung and liver were harvested. Representative photomicrographs of EGFP expression in 2-mm thick sections of control and chk-shRNA transduced tumor tissue are shown in Figure 3B. EGFP was not observed in other organs (data not shown). A representative immunoblot shown in Figure 3C (upper panel) demonstrates the maximum chk protein downregulation that was achieved following treatment. Reduction of protein expression was primarily due to the efficient downregulation of chk mRNA as shown in Figure 3C (lower panel). Compared with control, chk mRNA was downregulated on average by approximately 35% in tumors transduced with chk-shRNA (Figure 3C lower panel). q-PCR of genomic DNA from tumor and organ tissue detected expression of EGFP as well as lentivirus vector markers such as the ampicillin resistant gene LV1 and LV2 in tumor tissue only (Figure 3D).

In vivo and *ex vivo* spectroscopic analyses of transduced tumor xenografts

To validate the efficiency of virus-mediated gene targeting, noninvasive *in vivo* ^{31}P MRS was performed to detect tumoral phosphomonoester (PME) and PC levels. A decrease in both PC and PME levels in tumors of animals injected with chk-shRNA, compared to those injected with luc-shRNA was evident in the *in vivo* single-voxel ^{31}P MRS spectra (Figure 4A). As shown in Figure 4B and C, by day 14 of the treatment protocol or after five intravenous chk-shRNA injections, tumoral PME/ β -NTP and PC/ β -NTP levels were significantly ($P < 0.01$ and $P < 0.05$, respectively) decreased. This decrease in PME and PC levels was consistent with the observation of regions of EGFP expression in the tumor tissue. To further substantiate this finding, water-soluble extracts from tumor tissue were analyzed for PC, GPC and tCho levels by ^1H MR spectroscopy. Four tumors with matching volumes (tumor weight $\leq 300\text{mg}$) were selected from each group for this analysis. Representative, fully-relaxed ^1H MR spectra from a luc-shRNA and chk-shRNA transduced tumor are shown in Figure 5A and B. A decrease of the PC peak is evident in the chk-shRNA transduced tumor. Quantitative data of PC, GPC, and tCho levels in luc-shRNA and chk-shRNA transduced tumors are shown in Figure 5C. Consistent with the reduction of PC in chk-shRNA transduced cells, a significant decrease of PC and tCho was detected in the chk-shRNA transduced tumors. However the PC/GPC ratio was not significantly different (Figure 5D) because of the slight decrease of GPC in the chk-shRNA transduced tumors.

Tumor associated phenotypic changes

Both anatomical and morphological differences were observed between the luc-shRNA and the chk-shRNA transduced tumors. To further evaluate the effects of chk-shRNA transduction, formalin fixed tumor samples were evaluated for necrosis, and immunohistochemistry of frozen OCT-embedded tissue was performed for the proliferation marker Ki-67.

Representative photomicrographs of H&E stained sections from luc- and chk-shRNA transduced tumors are shown in Figure 6A at 4x (i, ii), and 20x (iii, iv). There was a trend towards increased necrosis in the chk-shRNA transduced tumor (compare i vs. ii, and iii vs. iv). On average, however, the increase in necrosis in chk-shRNA transduced tumors was not statistically significant (Figure 6B). Cells in the Ki-67 immunostained sections obtained from luc-shRNA transduced control tumors showed intense brown staining in the nucleus, compared to the chk-shRNA transduced treated tumors due the presence of the Ki-67 antigen in the granular compartment of the nucleolus region. Representative photomicrographs of Ki-67 antigen stained sections from luc- and chk-shRNA transduced tumors shown in Figure 6C at 20x (i, ii), and 40x (iii, iv), reveal a decrease of Ki-67 staining in chk-shRNA transduced tumors compared to luc-shRNA transduced control tumors. Summarized data are presented in Figure 6D and demonstrate significantly lower Ki-67 positive cells in chk-shRNA treated tumors compared to luc-shRNA control tumors (35% vs. 64%, $P \leq 0.005$)

Discussion

Here we have demonstrated the ability of a viral based delivery system to downregulate chk expression in established tumors. Chk downregulation was detected noninvasively by MRS, and resulted in reduced tumor growth together with decreased proliferation.

Virus based therapy, especially lentivirus based therapy, has shown promising results in the treatment of both inherited and acquired diseases (16). The use of HIV-1 based lentivirus as a mode of gene delivery was envisaged almost a decade ago (17) and its transduction efficacy demonstrated in over 42 different cell lines representing 10 different human tumor types (18). A careful dissection of various elements in the virus and the elimination of sequences not required for transduction and integration has led to a safer self-inactivating (SIN) type vector system. The incorporation of elements such as a central polypurine tract (cPPT) and post transcriptional regulatory elements (PRE) to the vector backbone has resulted in better transduction efficiency and transgene expression (18). The packaging vector used in this study (Δ R8.2 DVPR) has the accessory element VPR deleted (19) and thus incorporates safety into the system without any negative effects on yield or infection efficiency. We used the VSV-G protein to pseudotype the envelope of the virus to increase the transduction efficiency of the transgene. Such a modified virus has been previously used to successfully transduce dividing and non-dividing cells in culture (17) as well as *in vivo* (20,21). The pRRL vector used in our study incorporated the required safety elements, and showed over 95% transduction efficiency upon addition of the pseudotyped viral supernatant to MDA-MB-231 cells.

The robust EGFP expression observed in tumors was consistent with the reduction of chk expression at both mRNA and protein levels, and resulted in a significant decrease of PC. A similar reduction in mRNA and protein with a corresponding decrease of PC has been previously reported in MDA-MB-231 (7) and MCF-7 cells transfected transiently with siRNA (22) and in MDA-MB-231 cells stably expressing U-6 driven chk-shRNA (7).

An important aspect of gene therapy is the mode of injection (8). Viruses can be administered by an intratumoral injection of the viral supernatant, the intratumoral implantation of a polymeric device that can release viral vectors locally, or by an intravenous injection of the viral supernatant through the tail vein. The intravenous administration protocol used in our study resulted in robust EGFP expression in transduced tumors and an efficient knockdown of chk. The biodistribution and rate of accumulation of virus following an intravenous injection will depend on the virus type, total surface area of the microvessel, microvascular permeability and rate of cellular uptake (8). Following intravenous administration, lentiviruses disperse within the vascular space and are filtered by the liver (23) where they are scavenged by Kupffer cells (8). Since these viral particles are of the order of 10 nm or larger, they will not leak out

easily through normal tissue vasculature into normal tissues, but will leak out into the tumor interstitium from the permeable vasculature found in tumors (24). In a previous study involving BALB/c mice, intravenous injection of lentivirus through the tail vein, generated using a first generation packaging construct, resulted in GFP expression that was sustained for 40 days in bone marrow. Additional organs such as liver and spleen showed GFP expression to a lesser extent (23). However, after 40 days GFP expression diminished in liver and spleen. Although in the present study we did not look for GFP expression in bone marrow, the lack of EGFP detection in the lung, kidney, and liver in our study was most likely due to the inability of viral particles to permeate through normal vasculature, and due to their degradation in the liver. The expression of EGFP was more robust in chk-shRNA transduced tumors compared to luc-shRNA transduced control tumors. These differences may be attributed to the reduced proliferation and tumor growth in the chk-shRNA transduced tumors.

One of the major challenges facing cancer gene therapy is the ability to detect the response of the tumor to the treatment. Chk is an attractive target for gene therapy as its downregulation has been demonstrated to reduce proliferation (this study, (7) and increase differentiation, as well as to increase cell kill in combination with conventional chemotherapy (22). A major advantage of chk targeting is the ability to detect viral transduction noninvasively with ^1H or ^{31}P MRS. Our data demonstrate that the reduction in tumoral chk protein levels through lentiviral-mediated chk-shRNA delivery resulted in decreased PC levels, which is the metabolic product of the chk enzyme reaction. The decrease in PC, and hence PME levels could be dynamically monitored over a time period of 30 days in our preclinical studies. Residual PC observed in our *in vivo* ^{31}P MRS studies may be attributed to the, possibly even compensatory, action of other PC-producing enzymes, such as phosphatidylcholine-specific phospholipase C. A decrease of total choline was also observed in *ex vivo* studies of tissue extracts. Future clinical assessment of successful chk-shRNA gene therapy could be performed with single-voxel ^{31}P MRS, or with ^1H MRS, which detects the total choline signal with high detection sensitivity.

In summary, these preclinical data demonstrate that intravenously administered lentivirus expressing chk-shRNA can be used to target chk in established tumors. The downregulation of chk, which can be detected noninvasively with ^{31}P or ^1H MRS, results in a reduction of cell proliferation and tumor growth. Our data suggest that chk downregulation with this system resulted in a cytostatic rather than cytoreductive effect. Future studies using virus with increased plasma half-life can enhance the rate of accumulation and be more effective in target-gene silencing *in vivo*. A comparable lentivirus dose used in the mouse studies may be possible to achieve in humans by injecting a larger volume and increasing the titer. In spite of safety concerns involving use of lentivirus as a mode of gene delivery, these studies are an important forerunner of future gene therapy trials targeting chk in tumors.

Abbreviations

Cho, choline
 chk, choline kinase
 DPDE, diphosphodiester
 EGFP, enhanced green fluorescent protein
 GPC, glycerophosphocholine
 HPRT1, hypoxanthine phosphoribosyltransferase 1
 luc, luciferase
 MRS, magnetic resonance spectroscopy
 NAD, Nucleoside adenosine diphosphate
 NTP, β -nucleoside triphosphate
 PC, phosphocholine

PCr, phosphocreatine
 PDE, phosphodiester
 PE, phosphoethanolamine
 PME, phosphomonoester
 shRNA, short hairpin RNA
 TMSP, 3-(trimethylsilyl) propionic 2,2,3,3-d₄ acid sodium salt
 tCho, total choline

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Figure 1.

Molecular characterization of chk knockdown in MDA-MB-231 cells following *in vitro* transduction. A) Schematic map of the pRRL vector showing the organization of the different components. B) Representative brightfield (i and iii) and fluorescent (ii and iv) photomicrographs showing the transduction efficiency of both luc-shRNA (i and ii) and chk-shRNA (iii and iv) as evident by EGFP expression (ii and iv). C) qRT-PCR for chk mRNA expression in transduced cells (assay performed in triplicates and represented as standard deviation of the mean). Values were obtained relative to the house keeping gene hypoxanthine phosphoribosyltransferase-1 (HPRT-1). D) Representative immunoblot for chk protein. Lane 1: wild-type MDA-MB-231 cells; lane 2: MDA-MB-231 cells transduced with luc-shRNA; lane 3: MDA-MB-231 cells transduced with chk-shRNA. Actin immunoblotting was performed as loading control.

Figure 2.

Functional analysis of chk knockdown in MDA-MB-231 cells following *in vitro* transduction. Representative expanded regions of ^1H MR spectra from (A) MDA-MB-231 cells transduced with luc-shRNA and (B) MDA-MB-231 cells transduced with chk-shRNA. Abbreviations are: GPC, glycerophosphocholine; PC, phosphocholine; tCho, total choline-containing compounds. C) Quantitation of PC, GPC, and tCho from cell extracts of MDA-MB-231 cells transduced with either luc-shRNA (dotted box) or chk-shRNA (diagonal stripes) expressing lentivirus. D) Ratio of PC to GPC in MDA-MB-231 cells transduced with lentivirus expressing luc-shRNA (dotted box) or chk-shRNA (diagonal stripes). Values presented are mean and standard error obtained from three separate experiments for each group; *, $P < 0.05$; **, $P < 0.01$.

Figure 3.

In vivo transduction of lentivirus. A) Tumor growth characteristics with lentivirus-mediated chk targeting. Tumor volume in mm³ of luc-shRNA (gray circles, n=5) and chk-shRNA (black circles, n=5) transduced tumors. Values represent mean and standard error. * P < 0.05, # P < 0.07. B) Representative brightfield and fluorescence photomicrographs of a tumor section showing distribution of EGFP in a luc-shRNA and chk-shRNA transduced tumors. C) Representative immunoblot showing chk protein from tumor samples (upper panel). Actin was used as loading control (upper panel). Quantitative RT-PCR for chk mRNA expression from tumor samples (n=5) (lower panel). D) q-PCR of genomic DNA from tumor tissue to detect viral vector genes to establish the integration of virus in both luc-shRNA tumor (gray box) and chk-shRNA transduced tumors (black box).

Figure 4.

In vivo single-voxel ^{31}P MRS of MDA-MB-231 tumor xenografts. A) Representative pre-treatment (bottom) and post-treatment (top) spectra of a tumor following systemic delivery of lentivirus expressing luc-shRNA (left) or chk-shRNA (right). Chk-shRNA injection resulted in a significant reduction of PC (arrow) as compared to luc-shRNA. Significant differences were detected in (B) PME/ β -NTP (**, $P < 0.01$) and (C) PC/ β -NTP (*, $P < 0.05$) between luc-shRNA (black triangles, $n=5$) and chk-shRNA (gray rectangles, $n=5$) on day 15. Assignments: DPDE, diphosphodiester; NAD, Nucleoside adenosine diphosphate; NTP, Nucleoside triphosphate; PC, phosphocholine; PDE, phosphodiester; PCr, phosphocreatine; PE, phosphoethanolamine; PME, phosphomonoester.

Figure 5.

Functional analyses of chk knockdown of tumors following *in vivo* transduction.

Representative expanded regions of ^1H magnetic resonance spectra from tumors transduced with (A) luc-shRNA and (B) chk-shRNA. Abbreviations are: GPC, glycerophosphocholine; PC, phosphocholine; tCho, total choline-containing compounds. A) Quantitation of PC, GPC and total choline from extracts of luc-shRNA (dotted box) or chk-shRNA (diagonal stripes) transduced tumors. B) Ratio of PC to GPC in extracts of tumors transduced with luc-shRNA (dotted box) or chk-shRNA (diagonal stripes). Values presented are mean and standard error obtained from four tumors in each group; *, $P < 0.05$.

Figure 6.

A) Photomicrographs of representative 5 μ m thick formalin fixed sections stained with hematoxylin and eosin obtained at 4x (i, ii) and 20x (iii, iv) from a luc-shRNA (left) and chk-shRNA (right) transduced tumor. The arrow marks the extensive necrosis (N) evident in the chk-shRNA transduced tumor. B) Quantitation of necrotic area relative to the tumor size upon transduction of luc-shRNA (gray box, n=5) and chk-shRNA virus (black box, n=5). C) Photomicrographs of representative 5 μ m thick frozen sections immunostained with the proliferation marker Ki-67 obtained at 20X (i, ii) and 40X (iii, iv) magnification from a luc-shRNA (left panel) and chk-shRNA (right panel) transduced tumor. The arrow marks dark brown spots that are positively stained for Ki-67. D) Quantitation of Ki-67 positive cells in immunostained sections derived from luc-shRNA (gray box, n=4, five fields of view per sample, total of 20) and chk-shRNA (black box, n=4, five fields of view per sample, total of 20) derived tumors. Values represent percentage of positive cells and standard error. ***; $P \leq 0.005$.