Choline kinase knock-down in human breast cancer xenografts monitored by ^{31}P MR Spectroscopy

K. Glunde[1], V. Raman[1], N. Mori[1], Z. M. Bhujwalla[1]

[1]Radiology Department, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States

Introduction

Elevated phosphocholine (PC) and total choline (tCho) levels in breast cancer cells, frequently detected by ^{1}H and ^{31}P MRS [1], may be caused by an increased expression of choline kinase (ChK). A correlation between increased ChK activity and high tumor grade has been demonstrated in clinical tumor samples [2]. To test the relationship between ChK expression and corresponding MR-detectable choline metabolite levels *in vivo*, we generated stable clones of highly metastatic MDA-MB-231 breast cancer cells expressing small interfering RNA (siRNA) specific for ChK under the control of a U6-promoter (siRNA-chk). This molecular biology technique allows the stable reduction of messenger RNA (mRNA) levels of a gene of choice. ChK mRNA levels were characterized by quantitative real-time reverse transcription-polymerase chain reaction (Q-RT-PCR) analysis, and ChK protein levels were probed for by choline kinase antibody. The PC and tCho levels of siRNA-chk expressing MDA-MB-231 cells were also monitored using ^{1}H MR spectroscopy. An siRNA-chk knock-down clone with efficiently reduced Chk mRNA levels (Fig. 1) and an empty-vector control clone were inoculated into the mammary fat pad of severe combined immunodeficient (SCID) mice. Phosphorus MR spectroscopy was performed *in vivo* and of tumor extracts to monitor changes in global tumoral phospholipid metabolite levels.

Methods

A small interfering hairpin sequence specific for ChK was ligated into the pSHAG vector [3] containing the U6 promoter for RNA Polymerase III catalyzed transcription. The U6 promoter plus hairpin-siRNA for ChK were cut from this vector using restriction endonucleases and ligated into the multiple cloning site of a pCR3.1 vector (Invitrogen) devoid of any promoter. This final construct was transfected into human MDA-MB-231 breast cancer cells, referred to as siRNA-chk, was transfected into human MDA-MB-231 breast cancer cells, selected with G418 (Gibco), and analyzed using Q-RT-PCR analysis with the iQ SYBR Green Supermix and the iCycler (BIO-RAD). Low cellular PC levels were verified by fully relaxed ^{1}H MR spectroscopy from dual-phase extracts [4] of siRNA-chk clones. The MDA-MB-231 breast cancer cell clone, which contained the most significantly reduced ChK mRNA, protein, and PC levels, was chosen for inoculation into the mammary fat pad of SCID mice. Control mice were inoculated accordingly with empty-vector control cells, or MDA-MB-231 wild-type cells. *In vivo* ^{31}P MRS was performed in 6 siRNA-chk expressing tumors and 6 empty-vector control tumors on a Bruker Biospec Avance 4.7T spectrometer. For ^{31}P MR spetra, 1024 scans were acquired at 81 MHz using 45 degree pulses, 5000 Hz sweep width, data block size of 2K with 1s repetition time. Tumor pH was calculated using the ppm-difference (δP_i) between the inorganic phosphate (P_i) signal and the reference signal phosphocreatine (PCr) according to $pH = 6.66 + \log((\delta P_i - 3.079)/(5.57 - \delta P_i))$[5]. Tumors were freeze-clamped after reaching a tumor volume of about 600 mm^3, and extracted by dual-phase extraction [4]. Fully relaxed ^{1}H and ^{31}P MR spectra were obtained on a Bruker Avance 500 spectrometer. Absolute metabolite concentrations were quantified using concentration standards and the respective tumor weight.

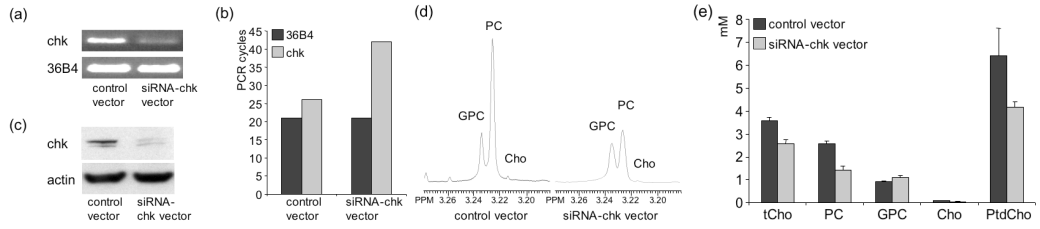

Figure 1: MDA-MB-231 breast cancer cells expressing siRNA-chk vector. (a) Q-RT-PCR of clones containing either empty vector or siRNA-chk vector. ChK message is significantly decreased in siRNA-chk vector cells compared to empty-vector control. (b) Real-time RT-PCR of empty-vector control and siRNA-chk vector cells carried out using the iCycler from BIO-RAD. The number of cycles required to amplify ChK message was higher in siRNA-chk cells than in empty-vector control validating an effective decrease of ChK mRNA in siRNA-chk vector cells, while the internal control 36B4 was amplified equally in both samples. (c) Western blot using ChK antibody demonstrates low ChK protein levels in siRNA-chk cells. Empty-vector control contains ChK levels comparable to wild-type MDA-MB-231 cells (not shown). (d) Corresponding expanded regions of ^{1}H MR spectra show that siRNA-chk cells (right) contain significantly decreased PC levels compared to empty-vector cells (left). (e) Corresponding quantitation of ^{1}H MR specra from water-soluble and lipid phase (n=3).

Results

MDA-MB-231 breast cancer cells expressing siRNA-chk vector contained significantly decreased ChK mRNA and protein levels reflected by significantly decreased tCho, PC, and phosphatidylcholine (PtdCho) levels (Fig. 1). Tumoral PC levels were significantly decreased in siRNA-chk knock-down tumors as demonstrated in Figure 2, which was comparable to data obtained from the respective cell cultures of siRNA-chk expressing MDA-MB-231 cells. However, tumoral glycerophosphocholine (GPC) levels were significantly increased in siRNA-chk tumors, as compared to empty-vector or wild-type MDA-MB-231 tumors (Fig. 2). This did not match the data obtained from the respective cell cultures, where no significant difference was detected between GPC levels in siRNA-chk expressing MDA-MB-231 cell cultures and empty-vector or wild-type MDA-MB-231 cell cultures. Tumoral phosphoethanolamine (PE) concentrations remained constant. *In vivo* ^{31}P MR spectra of siRNA-chk tumors demonstrated a significantly decreased phosphomonoester (PME) to phosphodiester (PDE) ratio compared to empty-vector tumors. We observed a small decrease in tumor pH in siRNA-chk tumors, which was 7.0 ± 0.05 in siRNA-chk expressing tumors compared to 7.1 ± 0.05 in volume-matched empty-vector control tumors.

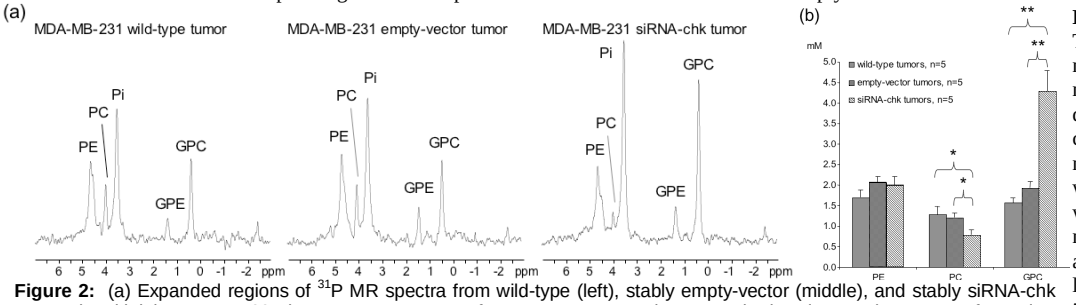

Figure 2: (a) Expanded regions of ^{31}P MR spectra from wild-type (left), stably empty-vector (middle), and stably siRNA-chk expressing (right) MDA-MB-231 breast cancer xenografts. Tumors were volume matched and extraction was performed at tumor sizes of about 600 mm^3. (b) Corresponding quantitation of the ^{31}P MR spectra of 5 tumors each. Values are mean ± standard error. * represents p<0.05, ** represents p<0.01

Discussion

These data indicate that although ChK mRNA levels and expression directly matched the cellular PC and tCho levels detected by ^{1}H MR spectroscopy in breast cancer cells, additional phospholipid metabolite concentrations were changed when these stable MDA-MB-231 clones were grown as solid tumors in the mammary fat pad of SCID mice. In addition to significantly decreased PC levels, we observed a significant increase in tumoral GPC levels not detected in the corresponding cell culture. This increase in tumoral GPC levels may be related, in part, to the decrease in tumor pH detected in the siRNA-chk tumors. As reported previously in a bioreactor system, an increase of cellular GPC levels correlated with intracellular acidosis [6]. Thus, the tumor microenvironment appears to have an important additional impact on tumoral phosphometabolite levels. ^{1}H and ^{31}P MRS proved useful in delineating the functional outcome of ChK knock-down clones of human breast cancer cells and tumor xenografts.

References & Acknowlegdements [1] Katz-Brull R et al, *JNCI* 94, 1197 (2002) [2] de Molina AR et al, *Oncogene* 21, 4317 (2002) [3] Paddison PJ et al, Genes & Dev 16, 948 (2002) [4] Tyagi RK *et al*, *MRM* 35, 194 (1996) [5] Prichard JW et al, *Proc Natl Acad Sci USA* 80, 2748 (1983) [6] Galons JP et al, *MRM* 33, 422 (1995)
This work was supported by NIH 1R01 CA82337 and P50 CA103175 (JHU ICMIC Program). We thank Mr. Gary Cromwell for maintaining the cell lines.