Choline kinase silencing in breast cancer cells results in compensatory upregulation of phosphatidylcholine-specific phospholipase C

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Introduction

Choline kinase (Chk) is overexpressed in several cancers, such as breast [1], colorectal [2], prostate [2], and lung [3] cancer. Increased Chk protein levels and activity contribute to the elevated tumoral phosphocholine (PCho) and total choline (tCho) levels in breast cancers, which can be detected by MRS [1, 4]. Chk is associated with tumor aggressiveness, and can be used as target for anticancer therapies [1]. We have previously used RNA interference, which is a powerful technique to silence genes in a sequence specific manner, to downregulate Chk with small interfering RNA specific to Chk (siRNA-chk) [1]. Chk downregulation with siRNA-chk decreased PCho and tCho levels as detected by MRS, which was accompanied by reduced proliferation and increased differentiation in breast cancer cells [1]. We also demonstrated that siRNA-chk mediated downregulation of Chk increased the effect of 5-fluorouracil treatment in breast cancer cells, but not in nonmalignant cells [5]. However, although siRNA-chk targeting of Chk efficiently decreased cellular Chk protein and the resulting PCho metabolite levels in breast cancer cells, there were still substantial amounts of PCho remaining in siRNA-chk-treated breast cancer cells [1, 5]. Therefore, we have investigated for the first time the expression and subcellular localization of phosphatidylcholine-specific phospholipase C (PC-PLC) as a possible compensatory mechanism that could be responsible for the remaining PCho levels in siRNA-chk-treated breast epithelial cells. Increased PC-PLC expression and/or activity was previously demonstrated in *H-ras* oncogene transformed NIH 3T3 fibroblasts [6], as well as ovarian tumor progression [7].

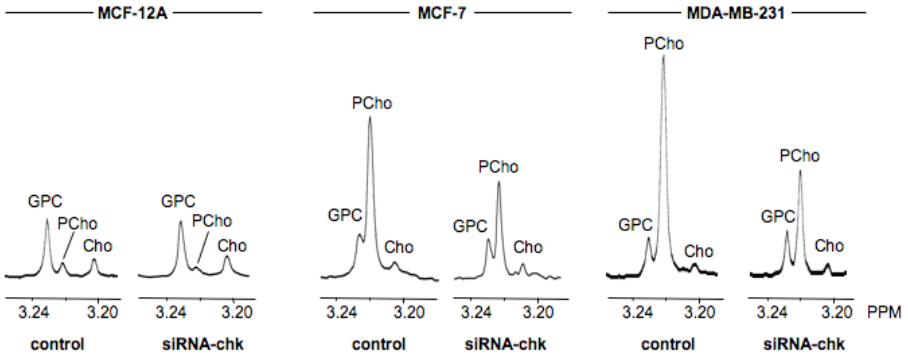

Figure 1: Representative expanded ^{1}H MR spectra of water-soluble cell extracts from controls and siRNA-chk-treated MCF-12A, MCF-7, and MDA-MB-231 cells. Chk knockdown with siRNA-chk resulted in decreased PCho levels in MCF-7 and MDA-MB-231 breast cancer cells, but not in nonmalignant MCF-12A breast epithelial cells. Cho, free choline; GPC, glycerophosphocholine; PCho, phosphocholine.

Methods

Nonmalignant MCF-12A human breast epithelial cells, and MCF-7 and MDA-MB-231 human breast cancer cells were cultured and transfected with siRNA-chk as previously described [1, 5]. Controls were treated with the transfection agent oligofectamine alone [1, 5]. PC-PLC antibody used for immunofluorescence and immunoblotting was raised in rabbit against bacterial PC-PLC, and allowed identification of a human PC-PLC at 66 kDa [6, 8]. Confocal laser-scanning immunofluorescence microscopy studies and gel electrophoresis followed by immunoblotting with PC-PLC antibody were performed as previously described [6, 8]. Following treatment with siRNA-chk or oligofectamine alone for 48 h, water-soluble cell extract fractions were obtained by dual-phase extraction, and fully relaxed ^{1}H MR spectra were acquired on Bruker Avance 500 NMR spectrometer, using 3-(trimethylsilyl)propionic-2,2,3,3,-d_4 acid (TSP) as an internal concentration standard [1, 5].

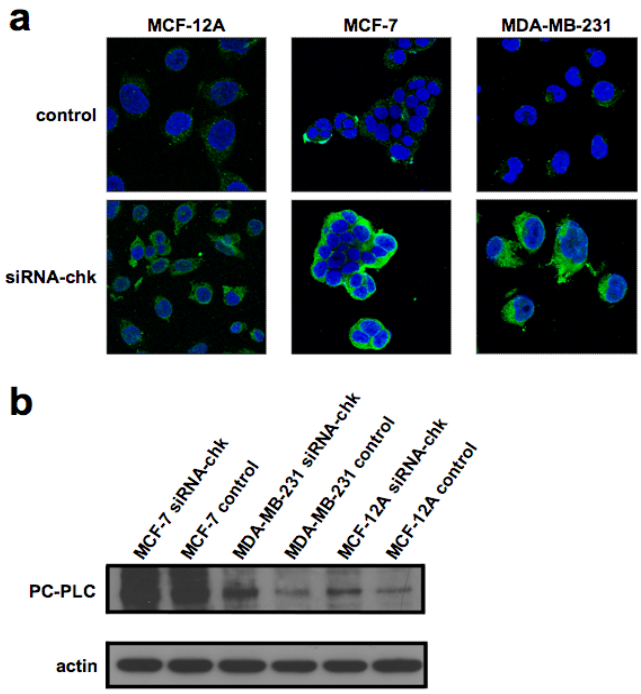

Figure 2: (a) Representative confocal immunofluorescence microscopy images of control and siRNA-chk-treated MCF-12A, MCF-7, and MDA-MB-231 cells. Cells were immunostained with PC-PLC antibody (displayed in green) and counterstained for nuclei (displayed in blue). (b) Immunoblots of control and siRNA-chk-treated MCF-12A, MCF-7, and MDA-MB-231 cells. Immunoreactive bands at 66 kDa were detected with PC-PLC antibody. Bottom panels show the corresponding actin loading controls.

Results

Chk downregulation with siRNA-chk resulted in significantly reduced cellular PCho concentrations in MCF-7 and MDA-MB-231 breast cancer cells, but not in nonmalignant MCF-12A cells (Fig. 1). Although Chk protein expression levels (data not shown) and the corresponding PCho levels were significantly decreased in these breast cancer cell lines upon Chk downregulation, their cellular PCho concentrations were not reduced to as low a level as observed in control MCF-12A cells (Fig. 1). Chk downregulation with siRNA-chk increased PC-PLC protein expression levels in MCF-12A, MCF-7, and MDA-MB-231 breast cancer cells as demonstrated by confocal immunofluorescence microscopic and immunoblot detection of PC-PLC (Fig. 2). PC-PLC was localized in the cytoplasm, nuclei, and membrane of siRNA-chk-treated breast epithelial cells (Fig. 2a). Immunoblotting with PC-PLC antibody demonstrated that siRNA-chk treatment increased PC-PLC protein levels by 1.5-, 1.2-, and 2.0-fold in MCF-12A, MCF-7, and MDA-MB-231 cells, respectively, compared to their respective controls (Fig. 2b, densitometry results).

Discussion

Because Chk downregulation in breast cancer cells decreased proliferation and increased differentiation, we concluded that it is a good target for anticancer therapy [1]. Subsequent studies revealed that Chk silencing increased the effect of 5-fluorouracil treatment in breast cancer cells, but not in nonmalignant cells [5]. Here we have shown that the cellular PCho concentrations that remain in siRNA-chk-treated breast cancer cells in spite of efficient Chk silencing are most likely due to an upregulation of PC-PLC protein expression in these cells. These findings indicate that breast cancer cells may compensate for their loss of Chk protein levels with an increase in PC-PLC protein expression, to maintain a somewhat elevated PCho level. Elevated cellular PCho levels may hence confer a growth advantage to breast cancer cells. In terms of using Chk silencing as anticancer therapy, it may be necessary to silence or inhibit compensatory enzymes, such as PC-PLC, along with Chk to achieve a more dramatic cell kill. Proton MRS of the choline metabolite profile proved extremely helpful in these studies to evaluate the functional response of Chk silencing, and will, in the future, be a valuable clinical tool to monitor the response to anticancer therapies that target enzymes in choline phospholipid metabolism.

References: [1] Glunde et al, *Cancer Res* 65, 11034-43 (2005) [2] Ramirez de Molina A et al, *Biochem Biophys Res Commun* 296, 580-3 (2002) [3] Ramirez de Molina A et al, *Lancet Oncol* 8, 889-97 (2007) [4] Glunde K et al, *Expert Rev Mol Diagn* 6, 821-9 (2006) [5] Mori N et al, *Cancer Res*, in press (2007) [6] Podo F et al, *Anticancer Res* 16, 1399-412 (1996) [7] Iorio E et al, *Cancer Res* 65, 9369-76 (2005). [8] Ramoni C et al, *J Immunol* 167, 2642-50 (2001). This work was supported by NIH P50 CA103175 (JHU ICMIC Program) and Accordo di Collaborazione Italia-USA N. 530F/0F29.