^{31}P Magnetic Resonance Spectroscopy study on the effects of stably silencing the glycerophosphocholine phosphodiesterase GDPD5 in a breast cancer model in vivo

Jannie P. Wijnen[1,2], Lu Jiang[1], Tiffany R. Greenwood[1], Maria D. Cao[3], Balaji Krishnamachary[1], Dennis W.J. Klomp[2], and Kristine Glunde[1]

[1]Johns Hopkins University In Vivo Cellular and Molecular Imaging Center,Russell H. Morgan Department, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States, [2]Department of Radiology, University Medical Centre Utrecht, Utrecht, Netherlands, [3]Department of Circulation and Medical Imaging, Norwegian University of Science and Technology, Trontheim, Norway

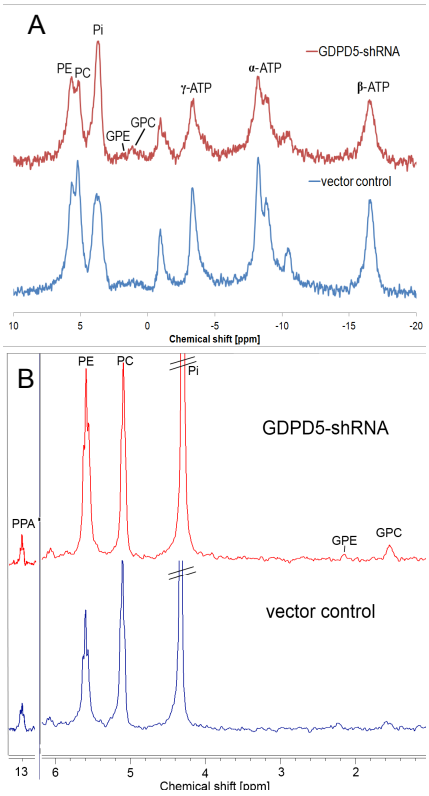

Figure 1: A) *In vivo ^{31}P MR spectra of a mouse with MDA-MB-231-GDPD5-shRNA tumour and a mouse with an MDA-MB-231-control vector tumour.* ***B)*** *^{31}P HR MR spectra of the same tumours shown in (A).*

Introduction: An increase in cellular phosphocholine (PC) and total choline-containing compounds (tCho) is a hallmark of cancer that accompanies malignant transformation, invasion and metastasis [1]. Enzymes in choline phospholipid metabolism present attractive targets that can be exploited for treatment [2]. Glycerophosphocholine phosphodiesterase (E.C. 3.1.4.2; GPC-PDE) is an enzyme in choline phospholipid metabolism that catalyzes the degradation of glycerophosphocholine (GPC) to free choline (Cho) and glycerol-3-phosphate. We recently demonstrated that the glycerophosphodiester phosphodiesterase domain containing 5 (GDPD5) gene expresses an enzyme with GPC-PDE activity that is partly responsible for the relatively low GPC concentration in breast cancer cells [3, 4]. In the present study, we stably silenced GDPD5 using short hairpin RNA (shRNA) against GDPD5 and investigated the effects of this stable GDPD5 silencing in a breast tumour xenograft model with ^{31}P Magnetic Resonance Spectroscopy (MRS). Besides detecting the resonances arising from choline phospholipid metabolism, ^{31}P MRS also enabled us to study the analogous ethanolamine phospholipid metabolites, phosphoethanolamine (PE) and glycerophosphoethanolamine (GPE).

Methods: We stably silenced GDPD5 using shRNA against GDPD5 (GDPD5-shRNA) delivered by lentiviral transduction in MDA-MB-231 breast cancer cells. These cells were orthotopically inoculated in 6 athymic nude mice (2 million cells in 50 µl Hank's buffer). Another 6 mice were inoculated with MDA-MB-231 cells that were transduced with a control vector. We monitored tumour growth using standard calipers. We performed *in vivo* ^{31}P MRS on a Bruker 9.4T small animal MR scanner. A home build solenoid coil tuned to ^{31}P and ^{1}H frequency, with an inner diameter of 12 mm was used. Animals were anesthetized by breathing a mixture of isofluorane and air, and a heating pad with circulating water was used to maintain normal body temperature. A 3D RARE image from the tumour was acquired and shimming of the main magnetic field was done manually for the 1st order shim fields. Non-localised ^{31}P MR spectra were obtained using an adiabatic excitation (BIR4, 45°, 200us, 120ppm BW, TR 1sec, NSA 1800) and a saturation slab covering the mouse body (adiabatic full passage pulse driven at half the power to achieve excitation with fully dispersed phase [5]). Lorentzian lines were fitted to the data using JMRUI4.0 software. Metabolite levels were corrected for differences in T1 relaxation, metabolite T1 values were measured *in vivo* in normal MDA-MB-231 tumours (n=4). Following *in vivo* measurements, mice were sacrificed and tumours were freeze-clamped and dual phase extraction was performed to separate the water and lipid soluble phase [6]. Fully relaxed high-resolution (HR) ^{1}H and ^{31}P MR spectra of the extracted water-phase were measured on a Bruker Avance 500 MR Spectrometer and analyzed using the MestReC 4.9.9.6 software. Quantitative RT-PCR (qRT-PCR) was performed to detect GDPD5 mRNA levels in stably transduced MDA-MB-231 cells using iCycler (Bio-Rad) and iQ SYBR Green (Quanta BioSciences).

Results: The *in vivo* ^{31}P MR spectra had sufficient spectral resolution for fitting the individual resonances of PE, PC, GPE and GPC (Fig. 1A). We detected higher levels of GPC and PE in the *in vivo* MR spectra of MDA-MB-231-GDPD5-shRNA tumours compared to the control vector tumours (Fig. 2). This observation was confirmed and reached statistical significance by corresponding ^{31}P HR MR spectra (Fig. 1B) of the tumour extracts (p=0.03 for both GPC and PE, Fig. 3). We did not observe any significant changes in the PC and GPE levels. The free choline level, measured from the 3.206 ppm resonance in the ^{1}H HR MR spectrum, was lower in the MDA-MB-231-GDPD5-shRNA tumours (p=0.03). qRT-PCR analysis of GDPD5 mRNA expression on stably transduced cells prior to orthotopic inoculation showed significant silencing of GDPD5-shRNA compared to control vector cells (p=0.0027 data not shown). The volume of the MDA-MB-231-GDPD5-shRNA tumours after 22 days was larger than the volume of control vector tumours (Fig. 4), which was opposite of what we have previously observed for MCF-7-GDPD5-shRNA tumors.

Discussion and Conclusion: As expected, stable silencing of the GDPD5 gene caused an increase in GPC and a decrease in free choline in breast tumour xenografts. This can be explained by the reduction in GDPD5 expression in GDPD5-silenced tumours, which leads to less degradation of GPC into free choline and glycerol-3-phosphate as GDPD5 confers GPC-PDE activity. This observation is in agreement with our previous study of GDPD5 silencing in MCF-7 breast cancer cells [3]. The increase in PE could be caused by phosphorylation of ethanolamine by choline and/or ethanolamine kinase as a compensatory mechanism to maintain high levels of phosphomonoesters in the cells. Silencing GDPD5 resulted in an accumulation of GPC, which may disturb choline phospholipid metabolism through inhibition of membrane breakdown and turnover. It is not clear at this point as to how GDPD5 silencing affects tumour growth as opposing effects were observed in two different breast tumour xenograft models. Additional studies are necessary to clarify the role of GDPD5 in cancer. As the choline-containing metabolite signals in the 3.2 ppm region in the ^{1}H MR spectrum overlap, we could not detect a significant difference in PC and GPC levels by their ^{1}H resonances between these two groups. Therefore, using *in vivo* and *ex vivo* ^{31}P MRS is advantageous as it provides resolved signals of PE, PC, GPE and GPC, to further investigate the role of GDPD5 expression in choline and ethanolamine phospholipid metabolism.

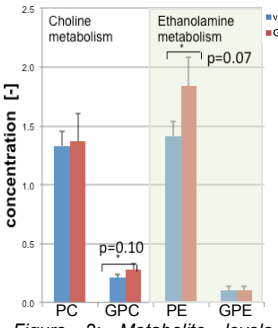

Figure 2: Metabolite levels quantified from in vivo ^{31}P MR spectra. Metabolite levels are normalised to β-ATP. Mean and standard error of 6 mice per group.

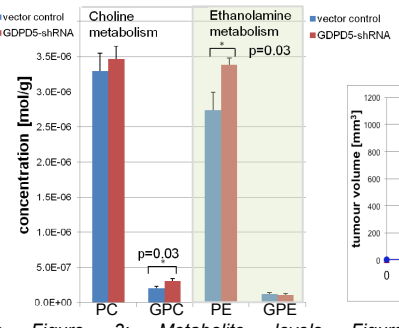

Figure 3: Metabolite levels quantified from ^{31}P HR MR spectra. Phenylphosphonic acid was used as reference for quantification. Mean and standard error of 6 mice per group.

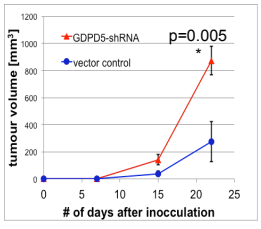

Figure 4: Tumour volume in MDA-MB-231-GDPD5-shRNA and vector control tumours. Mean and standard error of 6 mice per group.

References: 1. Glunde, K. et al. Semin Oncol, 2011; 2. Bañez-Coronel M. et al. Curr Cancer Drug Targets, 2008; 3. Doepkens M. et al, ISMRM 2010; 4. Cao MC. et al. NMR in biomed in press; 5. Choi I. et al. MRM, 2000; 6. Tyagi, RK. et al. MRM, 1996; *Acknowledgements: This work was supported by NIH R01 CA134695 and the Niels Stensen foudation.*