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Effect of sarcosine dehydrogenase knockdown on sarcosine metabolism-related genes expression

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Abstract: Sarcosine is extensively discussed as potential prostate cancer oncometabolite. Sarcosine dehydrogenase (SARDH) is one of the key enzymes involved in sarcosine metabolism that catalyses oxidative demethylation of sarcosine to glycine in mitochondrion. This study investigates the effects of small interfering RNA (siRNA) mediated *SARDH* knockdown on gene expression of sarcosine metabolism related enzymes: glycine N-methyltransferase (GNMT), peroxisomal sarcosine oxidase (PIPOX) and dimethylglycine dehydrogenase (DMGDH). The effect of SARDH knockdown was studied in three human prostate cell lines (PNT1A, DU-145, PC3) and gene expression was evaluated by quantitative real-time PCR (qPCR). Although the lowest *SARDH* knockdown was achieved in cancer DU-145 cell line, the highest changes in expression of other sarcosine metabolism genes were detected. In particular, the significant increase in *DMGDH* and *PIPOX* expression was observed. Our findings revealed potential stimulation effect of *SARDH* knockdown on *DMGDH* and *PIPOX* expression in prostate cancer cell line DU-145.

Key Words: knock-down, prostate cancer, sarcosine dehydrogenase, sarcosine metabolism

INTRODUCTION

Prostate cancer is the second most commonly diagnosed cancer and fifth leading cause of death in men worldwide (Bray et al. 2018). Early diagnosis of prostate cancer is essential step for subsequent successful treatment of this morbidity. In 2009, small non-proteinogenic amino acid sarcosine (N-methylglycine) was identified as a potential biomarker of prostate cancer (Sreekumar et al. 2009). Elevated sarcosine levels in urine have been associated with prostate cancer progression and metastatic processes (Sreekumar et al. 2009). *In vitro* experiments shown that elevated sarcosine levels induce proliferation, invasion and intravasation potential in human prostate cell lines (Khan et al. 2013).

Cancer cells are generally characterized by altered metabolism including biosynthetic and degradation pathways of amino acids (Ananieva 2015). The key enzymes involved in sarcosine metabolism are sarcosine dehydrogenase (SARDH; EC: 1.5.8.3), glycine N-methyltransferase (GNMT; EC: 2.1.1.20), peroxisomal sarcosine oxidase (PIPOX; EC: 1.5.3.1) and dimethylglycine dehydrogenase (DMGDH; EC: 1.5.8.4). Sarcosine can be formed in the cell either from glycine via the enzyme GNMT, which in cytosol catalyzes the transfer of the methyl group from S-adenosyl methionine (SAM) to glycine or from dimethylglycine via the mitochondrial enzyme DMGDH (Khan et al. 2013). On the other hand, degradation of sarcosine can be mediated by two enzymes, by PIPOX in peroxisomes or by mitochondrial SARDH that catalyze the oxidative demethylation of sarcosine to glycine. It was shown that up- or downregulation of sarcosine metabolism enzymes could affect prostate cancer progression, i.e. lowering the levels of GNMT or overexpression of SARDH reduced tumour growth in prostate cancer xenografts (Khan et al. 2013). Understanding the role of sarcosine metabolism related enzymes in prostate cancer metabolism may bring valuable information on tumorigenesis of prostate cancer.

The aim of this study was to investigate the effects of siRNA mediated *SARDH* knockdown on gene expression of sarcosine metabolism related enzymes in normal and prostate cancer cell lines.

MATERIAL AND METHODS

Chemicals

All used chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) unless otherwise noted.

Cell lines

Three different human prostatic cell lines were used. Firstly, PNT1A immortalized normal prostatic epithelial cell purchased from HPA Culture Collections (Salisbury, Great Britain). Secondly, DU-145 prostate cancer cell line derived from brain metastasis purchased from ATCC (Manassas, Virginia, USA). Thirdly, PC3 prostate cancer cell line established from fourth grade of prostatic adenocarcinoma purchased from ATCC (Manassas, Virginia, USA).

Cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum and supplemented by two antibiotics penicillin (100 U/mL) and streptomycin (0.1 mg/mL). All cell lines were maintained in incubator Galaxy 170 R (Eppendorf, Hamburg, Germany) at 37 °C with 5% CO_2. Transfection of siRNA (100 nM) was initiated after cells reached 50–60% confluence.

Transfection

Lipid-based transfection reagent METAFECTENE® SI+ from Biontex (Munich, Germany) was used for siRNA transfection. Transfection was performed according to the manufacturer's instructions. Transfection efficiency was verified using fluorescently labelled siRNA-siFAM (ABM, Richmond, Canada). The evaluation was performed using a fluorescence microscope EVOS FL Auto Imaging System from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

RNA isolation

Cells were harvested 48 hours after initiating transfection and were utilized in RNA isolation. High Pure RNA Isolation Kit was purchased from Roche (Basel, Switzerland) for RNA isolation and the integrity of the isolated RNA was verified using a Bleach gel. Afterwards, the purity and concentration of the isolated RNA was spectrophotometrically verified by Infinite® M200 PRO from Tecan (Männedorf, Switzerland).

Reverse transcription

The First Strand cDNA Synthesis Kit from Roche (Basel, Switzerland) was used for the reverse transcription. 500 ng of isolated RNA was transcribed by application of random hexamers.

Gene expression analysis

Quantification of gene expression was performed by Luna® Universal qPCR Master Mix (New England Biolabs, Ipswich, Massachusetts, USA) and Mastercycler® ep Realplex real-time PCR instrument (Eppendorf, Hamburg, Germany). Each 20 µl qPCR mix contained 25 ng cDNA and set of primer with 250 nM final concentration. Threshold cycle (CT) was determined by noiseband with automatic baseline drift correction using Realplex software (Eppendorf, Hamburg, Germany). The relative expression levels of sarcosine metabolism (*SARMET*) genes were normalized to *HPRT1* reference gene ($\Delta C_T = C_{T\ HPRT1} - C_{T\ SARMET}$). ΔC_T values are used for presentation of basal expression sarcosine metabolism genes. Relative gene expression between cells transfected with negative control siRNA (siNeg) and siRNA targeting SARDH (siSARDH) was calculated according to formula $\Delta\Delta CT = \Delta C_{T\ SARMET siSARDH} - C_{T\ SARMET\ siNEG}$. Data are expressed as $\Delta\Delta C_T$ mean±SD from tree biological replicates.

Descriptive statistics

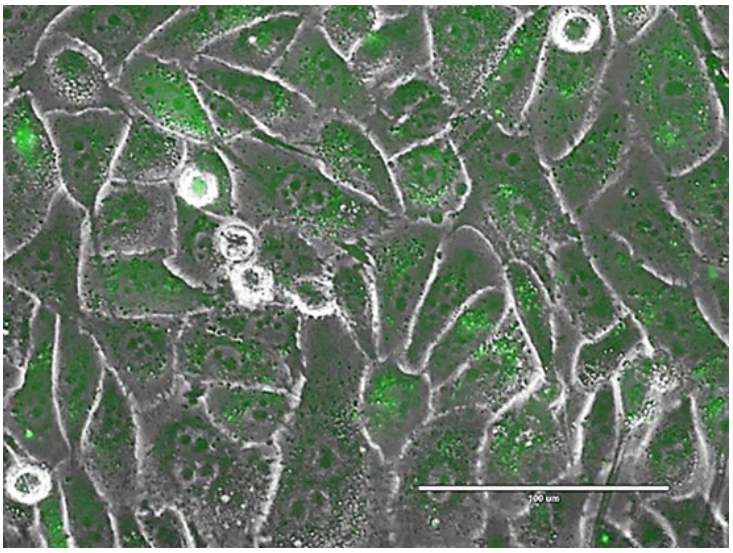
Statistically significant differences between individual results of gene expression were evaluated by unpaired t-test. The threshold for significance was $p < 0.05$. Statistical evaluation was performed in GraphPad Prism (GraphPad Software, San Diego, California, USA).

RESULTS AND DISCUSSION

Verification of transfection efficiency

Prior to the experiments with siSARDH, the efficacy of the selected transfection protocol had to be verified. Transfection efficacy was verified by fluorescently labelled siRNA (siFAM) and analyzed by fluorescence microscopy. Figures 1 showed that >70% of the PC3 cells was transfected by siFAM molecules which confirmed efficacy of transfection protocol.

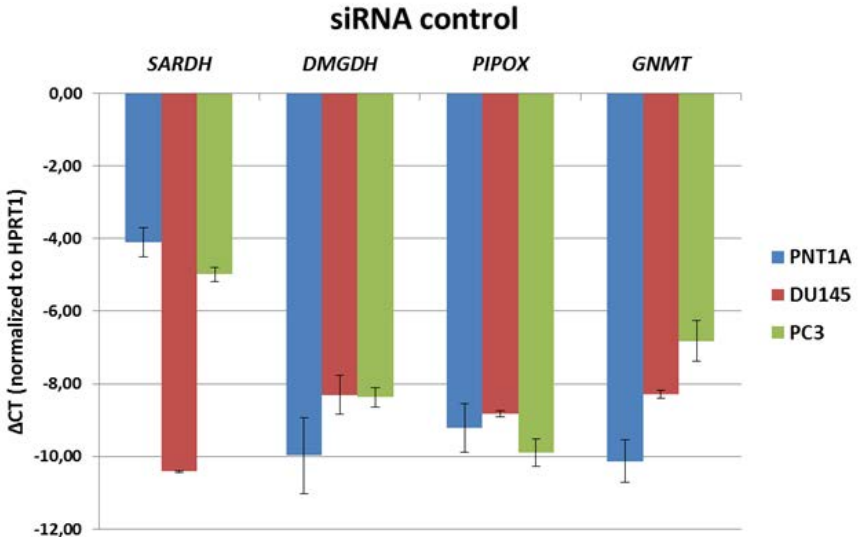
Figure 1 Transfection efficiency assay using fluorescently labelled siFAM analysed by fluorescence microscopy (PC3 cell line, magnification 40×, scale 100 µm)



Determination of basal gene expression

First, the basal expression of sarcosine metabolism genes *SARDH*, *DMGDH*, *PIPOX* and *GNMT* was determined. Figure 2 represents relative gene expression after 48 hours transfection with negative control siRNA. The lowest *GNMT* expression was observed in normal cell line PNT1A compared to both prostate cancer cell lines DU-145 and PC3. Simultaneously the normal cell line PNT1A has the highest *SARDH* expression from all tested cell lines. These results were in agreement with other publications (Khan et al. 2013, Song et al. 2011).

Figure 2 Basal expression of sarcosine metabolism genes (SARDH, DMGDH, PIPOX, GNMT)

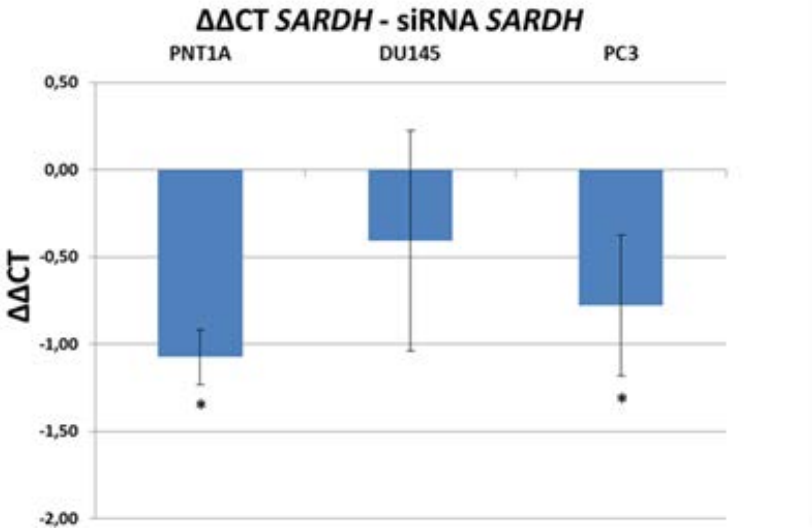


Evaluation of knockdown efficiency

Subsequently, *SARDH* knockdown efficiency was evaluated (Figure 3). The most significant decrease in *SARDH* expression was achieved in the normal prostate cell line PNT1A where the average

$\Delta\Delta C_T$ value was -1.07 corresponding to 52% knockdown efficiency. PC3 cell line also showed a statistically significant decrease in *SARDH* expression with a $\Delta\Delta C_T$ value -0.78, which corresponds to 52% knockdown efficiency. The decrease in *SARDH* expression in DU-145 cell line was not statistically significant which could be probably caused by very low basal *SARDH* expression in this cell line.

*Figure 3 SARDH expression 48 hours after transfection siSARDH (*p≤0.05)*



Changes in expression of sarcosine metabolism genes

Finally, *DMGDH*, *PIPOX* and *GNMT* gene expression after *SARDH* knockdown was evaluated (Figure 4). The most pronounced and statistically significant changes in expression were observed in the cancer cell line DU-145 in which the expression of *DMGDH* and *PIPOX* genes increased after 48h *SARDH* knockdown. This is in contrary with Green et al. 2013 who described the inhibition effect of *SARDH* knockdown to *DMGDH* (Green et al. 2013). DMGDH is an enzyme that commonly catalyses the conversion of dimethylglycine to sarcosine, but it has been found that it can also convert sarcosine to glycine (Wittwer and Wagner 1981a, Wittwer and Wagner 1981b). This could explain the increase in *DMGDH* expression that probably substitutes the function of downregulated *SARDH* in mitochondrion. Similar effect was observed in case of PIPOX that also probably replaced the missing catalytic activity of SARDH in DU-145 cells. No significant changes were observed in the case of *GNMT* expression.

*Figure 4 Change in expression of DMGDH 48 hours after siSARDH transfection (*p≤0.05)*

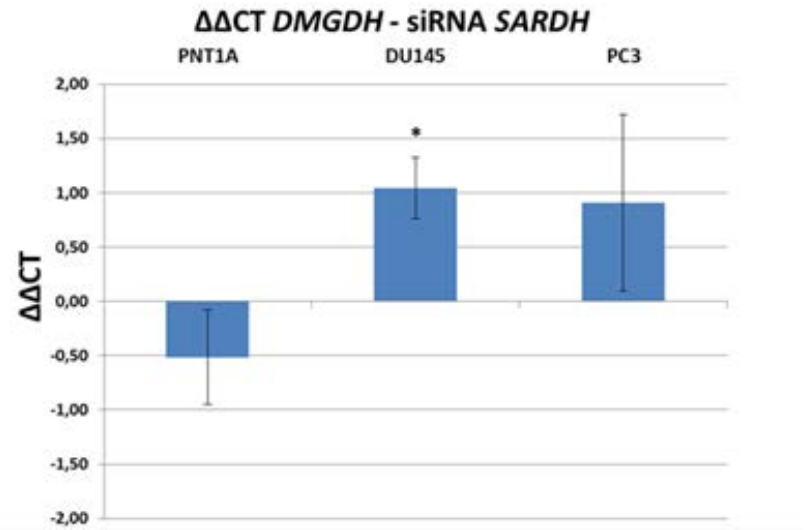


*Figure 5 Change in expression of PIPOX 48 hours after siSARDH transfection (*p≤0.05)*

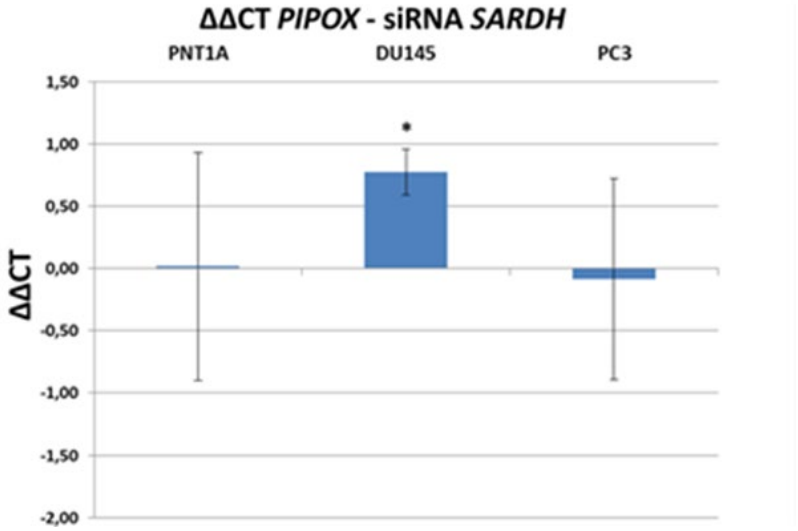
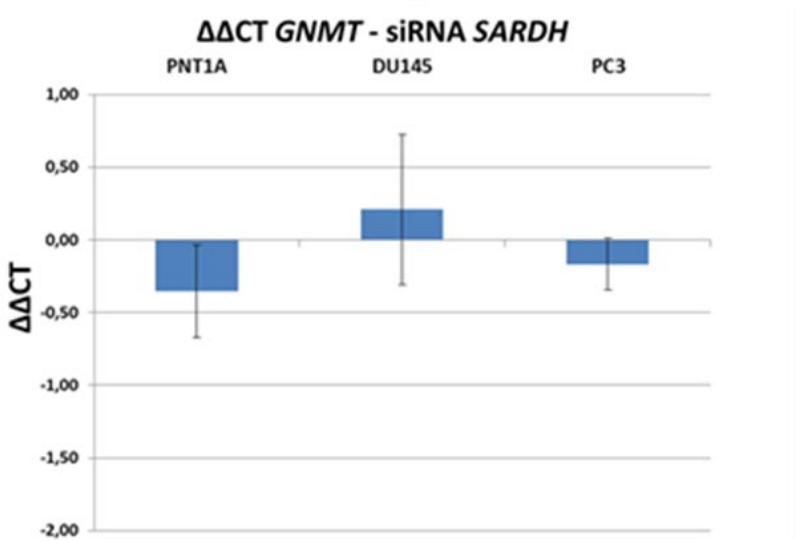


Figure 6 Change in expression of GNMT 48 hours after siSARDH transfection



CONCLUSION

We found a significant increase in the expression of *DMGDH* and *PIPOX* genes after *SARDH* knockdown in the DU-145 cell line, both of which are capable of converting sarcosine to glycine. These results provide new information on sarcosine metabolism that is potentially useful for further investigations regarding therapeutic agents.

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