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Increased expression of mitochondrial glycerophosphate dehydrogenase and antioxidant enzymes in prostate cancer cell lines/cancer

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Abstract

The involvement of mitochondrial glycerophosphate dehydrogenase (mGPDH) has previously been established in the production of ROS in prostate cancer cell lines (LNCaP, DU145, PC3 and CL1). The current study demonstrates that the mRNA level of mGPDH in prostate cancer cells is 3.3–8.9-fold higher compared to the normal prostate epithelial cell line, PNT1A. This is consistent with the enzymatic activity and protein level of mGPDH. However, cytochrome *c* oxidase (COX) activity is 2.9–3.2-fold down-regulated in androgen-independent prostate cancer cell lines. The level of antioxidant enzymes, catalase, MnSOD and CuZnSOD are up-regulated in prostate cancer cell lines. Furthermore, it was observed that the activity of mGPDH is significantly higher in liver tissues from all mice with cancer compared to liver tissues from control mice. These data suggest that the up-regulation of mGPDH, due to a highly glycolytic environment, contributes to the overall increase in ROS generation and may result in the progression of the cancer.

Keywords: *Mitochondrial glycerophosphate dehydrogenase, cancer, reactive oxygen species, antioxidant enzymes*

Introduction

The primary metabolic characteristics of cancer cells are the high rate of glycolysis and elevated reactive oxygen species (ROS) production [1,2]. The increased rate of glycolysis up-regulates the activity of mGPDH which is located in the inner mitochondrial membrane and constitutes the rate-limiting part of the glycerophosphate shuttle in prostate cancer cell lines [3]. The glycerophosphate shuttle enables the transfer of electrons from NADH generated from glycolysis to coenzyme Q in the electron transport chain. Histochemical studies for pathways of insulin metabolism demonstrated the importance of mGPDH in hydrogen shuttle system, which reoxidizes the glycolysis-derived NADH [4]. The function of mGPDH and regulation of ROS production may form an important link between metabolic stress and the activation of oxidative stress dependent pathways. ROS play an important role in regulating growth and survival of cancer, as well as mediating damage to cell structures, including lipids, membranes and DNA. In contrast, the beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in cellular response to anoxia and in the function of a number of cellular signalling pathways. The mitogenic responses are presumably interacting

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with nuclear regulatory factors (AP-1, NFκB, APE), regulatory kinases (Src kinase, protein kinase C, mitogen activated protein kinase), receptor tyrosine kinases, protein-tyrosine phosphatases and angiogenic factors [5]. The intracellular balance between oxidizing and reducing equivalents allows ROS to function as second messengers in the control of cell proliferation and differentiation [6].

Mitochondria are a major source of cellular free radicals and contain several redox carriers that can potentially leak single electrons to oxygen and convert it into superoxide anion. The superoxide anion can be converted to a number of molecular species of ROS [7]. Recently, it has been shown that mGPDH is involved in the production of ROS in rat liver mitochondria [8], brown adipose tissue mitochondria [9], as well as in mitochondria from human placenta [10]. Furthermore, we have previously demonstrated that its function is altered substantially in prostate cancer cells [3]. mGPDH has the capacity to generate large amounts of superoxide, much of it is produced on the cytosolic side of the inner membrane, identified as the active site of this enzyme [9]. Additional sources of mitochondrial ROS include Complex I and III [11]. Cytochrome *c* oxidase (COX) itself is not a source of ROS, however, inhibition of COX may facilitate ROS production from other complexes. Mutations of COX have been shown to inhibit OXPHOS and stimulate increased ROS production, resulting in increased tumour growth [12].

In order to clarify the role of mGPDH in ROS production and its role in the progression of tumour formation, we have investigated the expression of mGPDH and the cellular content of antioxidant enzymes such as catalase, MnSOD and CuZnSOD in a high ROS environment characteristic of tumour formation. In order to correlate the function of mGPDH to pathology, we have further compared the enzymatic activity of mGPDH in the liver tissue of C57BL/6 mice with and without cancer.

Materials and methods

Cell cultures and rat liver tissues

The normal immortalized prostate epithelial cell line, PNT1A, was a generous gift of Professor Norman J. Maitland (University of York, UK) [13–15]. This cell line was maintained in RPMI 1640 (Invitrogen, Ontario, Canada) supplemented with 10% foetal bovine serum (Invitrogen), 1% penicillin-streptomycin (Invitrogen) and 1.0 mM glutamine. The androgen-sensitive human prostate cancer cell line, LNCaP and androgen insensitive DU145 and PC3 cell lines were obtained from the American Type Culture Collection (Manassas, VA). The cell lines were maintained in RPMI 1640 supplemented with 10 mM HEPES, 1.0 mM sodium pyruvate, 10% foetal bovine serum and 1% penicillin-streptomycin. The CL1 androgen-independent cells derived from the LNCaP line were a generous gift from Dr A. S. Belldegrun (University of California Los Angeles Medical School, Los Angeles, CA) and were maintained in RPMI 1640 supplemented with 10% charcoal-stripped foetal bovine serum and 1% penicillin-streptomycin. Cell lines were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Using a total of 13 C57BL/6 mice, we divided the group into three categories. Those that were unaffected by cancer, those having detectable tumours in any tissue other than liver (lung, spleen, blood, skin) and those having some form of hepatocellular carcinoma (see Table I). In all cases liver tissues were extracted and homogenized in STE medium (0.25 m sucrose, 10 mM Tris-HCl, 1 mm EDTA, pH 7.4) at 4°C in a Tenbroeck homogenizer (50–80 mg liver/1 ml of STE buffer).

Table I. Enzymatic activity of glycerophosphate cytochrome *c* reductase (GCCR), citrate synthase (CS) and ratio of GCCR and CS in rat liver tissues.

Pathology of mice	Animal identity	GCCR activity	CS activity	GCCR/CS
WT	40	3.05±0.06	88.12±1.61	0.034±0.001
	41	2.17±0.37	82.23±3.67	0.024±0.001
	43	1.83±0.40	83.92±5.23	0.020±0.004
	54	3.93±0.20	83.89±0.36	0.046±0.001
	58	2.68±0.32	92.54±1.67	0.027±0.004
	68	3.21±0.41	90.46±0.45	0.033±0.001
WT+cancer	60	4.77±0.28	93.94±1.10	0.053±0.003
	61	6.92±0.83	100.70±2.86	0.074±0.005
	66	6.79±0.49	65.72±1.05	0.107±0.003
	67	5.90±0.39	89.71±0.44	0.068±0.002
WT+hepatocellular carcinoma	44	4.61±0.33	85.80±0.79	0.056±0.001
	51	6.18±1.03	75.42±1.64	0.090±0.004
	52	4.60±0.65	80.74±1.55	0.061±0.005

WT, WT+cancer and WT+HCC indicate wild-type mice without cancer, with cancer in organs except in liver and with hepatocellular carcinoma, respectively. Number of measurements are four. The statistically significant difference was found among groups using one-way ANOVA and between WT group and WT+cancer or WT+HCC using Student's *t*-test ($p<0.05$).

Spectrophotometric measurement

The enzymatic activity of mGPDH and succinate dehydrogenase was evaluated by monitoring the rate of reduced cytochrome *c* formation using glycerophosphate or succinate, termed as glycerophosphate or succinate cytochrome *c* reductase (GCCR or SCCR), respectively. Activity of GCCR and SCCR was measured in prostate cancer cell lines and rat liver tissues, as described previously [3,16]. Briefly, the reaction mixture contained 10 mM potassium phosphate (pH 7.4), 2 mM EDTA, 0.01% bovine serum albumin (fatty acid free), 0.2 mM ATP, 1 mM KCN, 5 μM rotenone and 20 mM glycerophosphate or 20 mM succinate (Sigma-Aldrich, Ontario, Canada). Sonicated cell lysates (0.2 mg protein) or liver homogenates (0.3 mg protein) were incubated in the reaction mixture for 3 min, after which time 40 μM oxidized cytochrome *c* was added. Changes in absorbance were monitored for 5 min at 550 nm and 30°C. For calculation of enzyme activities, the extinction coefficient, -19.6 cm^2/mol was used. Citrate synthase activity was evaluated in a medium containing 150 mM Tris-HCl, pH 8.2, 8 mg of lauryl maltoside/mg of protein, 0.1 mM dithionitrobenzoic acid and 0.15 mg of liver homogenate. The reaction was initiated with the addition of 300 μM acetyl CoA and changes in absorbance at 412 nm were measured for 1 min. This rate was subtracted from the rate obtained after the subsequent addition of 0.5 mM oxalacetic acid. Absorbance values were measured for a total of 5 min. Cytochrome *c* oxidase was measured at 30°C by following the rate of oxidation of reduced cytochrome *c* at 550 nm. COX activity measurements in cultured cells were performed using 40 μM reduced cytochrome *c*, 20 mM phosphate buffer, 0.1 mg of protein from freshly cultured cells and 8 mg of lauryl maltoside/mg protein (0.16% LM). All measurements for enzymatic activity were performed using a Beckman DU-640 spectrophotometer (Beckman Instruments, Fullerton, CA).

Quantitative RT-PCR of mGPDH mRNA in prostate cancer cell lines

Total RNA was prepared from PNT1A, DU145, PC3, LNCaP and CL1 cell lines using RNeasy Mini Kit (Qiagen Inc, Ontario, Canada) according to the manufacturer's protocol. Real-time quantitative RT-PCR (TaqMan PCR) for *mGPDH* was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) and pre-developed TaqMan assay reagents of human *mGPDH* and *GAPDH* control probe were purchased from Applied Biosystems. Briefly, reactions consisted of 12.5 μl 2X TaqMan Universal PCR MasterMix, 0.625 μl Multiscribe and RNAse Inhibitor Mix, 1.25 μl fluorescently-labelled primer in a total volume of 25 μl with 100 ng RNA. Standard curves for each primer were performed using 0, 12.5, 25, 50 and 100 ng concentrations of RNA, to determine whether the amplification efficiencies of each primer were comparable. All reactions of the samples were performed in quadruplicate. The PCR reaction was carried out according to the manufacturer's protocol. The thermal cycler conditions were 48°C for 30 min, 95°C for 10 min then 40 cycles of 15 s at 95°, and 60°C for 1 min. Data were analysed using the Sequence Detection Software, which calculated the threshold cycle (C_T) values. The expression of mGPDH was normalized to GAPDH and the relative change in gene expression was calculated according to the $2^{-\Delta\Delta C_T}$ method [17].

Western blot analysis

Whole cellular lysates from PNT1, DU145, PC3, LNCaP and CL1 were prepared in the NP40 lysis buffer containing 1% NP40, 150 mM NaCl, 50 mM Tris base (pH 7.4) and 1 mM EDTA. Briefly, cells were washed with ice cold PBS, pelleted through centrifugation and re-suspended in ice-cold NP40 lysis buffer supplemented with 1 × protease inhibitor (Sigma). Cells were extracted with constant rocking at 4°C for 45 min and then centrifuged at 1845 × g for 5 min and supernatant collected. Protein concentrations were determined according to Bradford [18]. 5 μg of protein was separated using a 10% SDS-PAGE gel and the electrophoretogram was transferred onto a nitrocellulose membrane and blocked with 5% skimmed milk in TBST for 1 h. Membranes were then incubated overnight at 4°C with rabbit anti-human catalase (1:2000, AbCam, Cambridge, MA), rabbit anti-human MnSOD (1:2000, Upstate, Chicago, IL) and sheep anti-human Cu-ZnSOD polyclonal antibodies (1:5000, Upstate, Chicago, IL) with 5% skimmed milk in TBST for overnight at 4°C. After three washes in TBST, the membranes were incubated in horseradish peroxidase-linked goat anti-rabbit IgG antibody (1:5000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and the membrane of Cu-ZnSOD was incubated HRP-conjugated anti-sheep antibody (1:10000, Invitrogen Canada Inc., Ontario, Canada) for 1 h and proteins were detected using the enhanced chemiluminescence Western blotting detection kit (Amersham Biosciences).

Densitometric study of Western blotting

'NIH Image Software' has been used to analyse the densitometric intensity of Western blots. The band intensity of the target protein was normalized to the β-actin band.

Statistical analysis

Data are presented as means ± SD. Statistical significance was determined through the use of a Student's *t*-test with two-tailed distribution and one-way single

factor ANOVA test was used among groups. Significance was considered at $p < 0.05$.

Results

Prostate cancer cell lines exhibit increased ratio of mitochondrial glycerophosphate dehydrogenase (GCCR) to succinate dehydrogenase (SCCR) and citrate synthase (CS) activities

In our previous publication [3] we demonstrated that the enzymatic activity, protein level of mitochondrial glycerophosphate dehydrogenase and also oxygen consumption with glycerophosphate was significantly higher in prostate cancer cell lines (DU145, PC3, LNCaP and CL1) compared to normal prostate cell line, PNT1A. We have further examined the activity of mitochondrial glycerophosphate dehydrogenase in relation to another respiratory chain enzyme, succinate dehydrogenase, as well as citrate synthase, a matrix-soluble enzyme. Prostate cancer cells showed 1.8–6.6-fold higher GCCR activity related to SCCR and 2.1–7.4-fold increased GCCR activity related to citrate synthase when compared to normal PNT1A cells (Table II). Although COX itself is not a source of ROS, inhibition or dysfunction of COX may facilitate ROS production from other mitochondrial complexes. COX activity in prostate cancer cells is 2.9–3.2-fold decreased in androgen-independent prostate cancer lines compared to normal prostate cells (Table II). However, COX activity in androgen-dependent LNCaP cells was not changed significantly compared to normal prostate cells. When related to CS activity, the COX activity was found to be very similar in LNCaP and PNT1A (Table II).

Increased mRNA level of mGPDH in prostate cancer cell lines

We wanted to determine if the changes observed with the mGPDH activity were occurring at the transcriptional level or whether they may be due to post-translational modifications. Therefore we evaluated the mRNA level in these prostate cancer cell lines by carrying out RT PCR using the ABI PRISM 7700 Sequence Detection System from Applied Biosystems. Relative mRNA levels of mGPDH were found to be 3.3-fold (LNCaP), 4.5-fold (DU145), 8.2-fold (PC3) and 8.9-fold (CL1) higher than in control prostate cell lines, PNT1A (Figure 1). A strong correlation was observed between mRNA and protein levels of mGPDH as well as its enzymatic activity and ROS production [3].

Prostate cancer cell lines exhibit increased levels of antioxidant enzymes (catalase, MnSOD and CuZnSOD)

The antioxidant system is important for cells in the defense against endogenous and exogenous ROS insult. The antioxidant enzymes work coordinately to achieve an equilibrium between pro-oxidant and antioxidant systems. Protein levels of catalase and MnSOD were significantly elevated whereas cytoplasmic CuZnSOD was altered in prostate cancer cell lines when compared to normal prostate cell line, PNT1A (Figure 2). Having demonstrated in our previous work [3] that these cell lines exhibit elevated levels of ROS, we addressed the effect of the elevated levels of intracellular radicals on the response of the antioxidant defense system. The prostate cancer cells showed 2.44–62.14, 0.93–3.44 and 1.29–2.03-fold elevated protein levels of catalase, MnSOD and CuZnSOD (Figure 2A–C) related to β-actin, respectively, when compared to the normal prostate cell line, PNT1A.

Higher enzymatic activity of mitochondrial glycerophosphate dehydrogenase (GCCR) in mouse liver with cancer

In order to evaluate the utility of mGPDH activity as an *in vivo* marker of oncogenesis, we used C57BL/6 mice that had developed cancer either in liver or in other organs. Similar to the results found in the prostate cancer cell lines model [3], liver homogenates from animals with cancer either in the liver or in other

Table II. Spectrophotometric determination of glycerophosphate cytochrome *c* reductase (GCCR) related to succinate cytochrome *c* reductase (SCCR), citrate synthase (CS) and cytochrome *c* oxidase (COX) activity in normal prostate epithelial cells (PNT1A) and prostate cancer cells (LNCaP, DU145, PC3).

	Enzymatic activity (nmol/min/mg protein)			
Cells	GCCR/SCCR	GCCR/CS	COX	COX/CS
PNT1A	0.193 ± 0.035	0.021 ± 0.003	18.737 ± 0.231	0.240 ± 0.004
LNCaP	0.339 ± 0.026*	0.044 ± 0.003*	20.712 ± 2.460	0.208 ± 0.022
DU145	0.540 ± 0.010*	0.094 ± 0.005*	6.461 ± 0.324*	0.086 ± 0.008*
PC3	1.282 ± 0.054*	0.155 ± 0.011*	5.793 ± 0.866*	0.086 ± 0.022*

Enzymatic activity of GCCR and SCCR was determined with 20 mM glycerophosphate or 20 mM succinate in freshly harvested sonicated cells measuring the reduction rate of cytochrome *c* (nmol of cytochrome *c* oxidized per min per mg protein). The enzyme activity determination methodology of each individual enzyme is described in details in Materials and methods and number of measurements are five.
* $p < 0.05$ with respect to normal prostate epithelial cells, PNT1A.

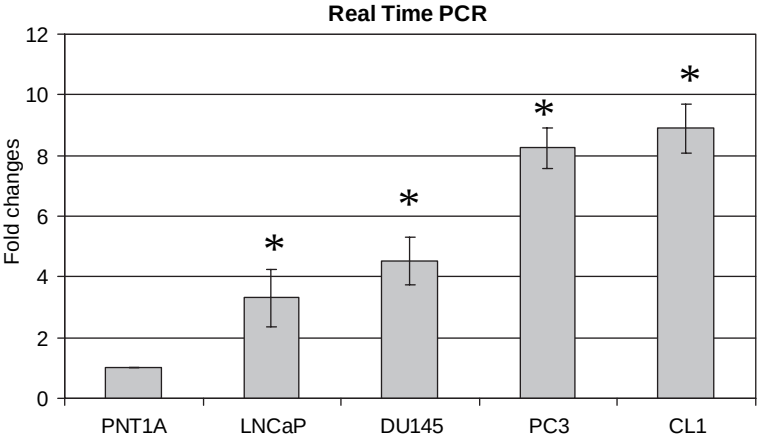

Figure 1. mRNA level of mGPDH in prostate cancer cell lines. Real-time PCR was used to measure the relative abundance of steady-state mRNA levels of mGPDH in prostate cancer cell lines. Real-time PCR was performed using ABI PRISM 7700 Sequence Detection System from Applied Biosystem (see details in Materials and methods). * $p < 0.05$ with respect to normal prostate epithelial cells, PNT1A and number of measurements are four.

organs showed higher GCCR activity when compared to animals with no tumours (Table I, Figure 3A and B). CS activity did not change significantly with tumour development. GCCR/CS demonstrates specific changes in GCCR as opposed to general mitochondrial changes (Table I, Figure 3A and B). Further investigations need to be done to clarify the consequences of mGPDH mediated ROS production during the process of tumour development.

Discussion

A common characteristic of primary and metastatic cancers is up-regulation of glycolysis. The glycolytic phenotype of many cancer cells and tumours has been demonstrated at both the biochemical and molecular levels. In this study we attempted to delineate the mechanism by which a highly glycolytic environment participates in the production of ROS via the glycerophosphate shuttle. mGPDH is the rate-limiting enzyme of this shuttle to transfer electrons from cytoplasm to the electron transport chain. The enzyme activity and protein content of mGPDH in prostate cancer cell lines are distinctly higher as compared to normal prostate cells [3]. We evaluated its activity related to the activity of other respiratory chain enzymes such as succinate dehydrogenase, COX and citrate synthase (Table II). The ratio of mGPDH related to succinate dehydrogenase and CS are significantly higher in prostate cancer lines compared to normal prostate cells. The elevated level of mGPDH likely results from increased transcription as indicated by the elevated level of mRNA for this protein (Figure 1). This suggests that activation of mGPDH transcription may be part of the tumour genesis programme, perhaps in an effort to deal with increased glycolytic flux.

The mitochondrial electron transport chain is the major endogenous source of ROS. For several years a growing body of evidence has accumulated to indicate signalling role of ROS in the pathogenesis of cancer. mGPDH is involved in maintaining the highly glycolytic environment of prostate cancer cell lines. The present study as well as the previous one [3] offers to support the proposal that mGPDH is involved in ROS production by showing evidence that antioxidant enzymes are up-regulated in all prostate cancer cell lines (Figure 2). The high production of ROS can also be further facilitated by the down-regulation of COX in cancer cells which would limit the flux of electrons down the respiratory chain and therefore the generation of superoxide radicals. Androgen-independent prostate cancer cells showed lower COX activity compared to androgen-dependent LNCaP cells which could explain that inactivation of COX might be involved in further increase of ROS production. Tumour cells have been shown to generate endogenous ROS and this elevated level of intracellular ROS production is correlated to the uncontrolled growth of cancer cells [19]. Our data suggest that all tumour cell lines exhibit elevated antioxidant defenses to counterbalance elevated intracellular ROS levels and to mitigate the effects of oxidative stress. Interestingly, antioxidant defenses in the androgen-dependent system (LNCaP cells) seem to respond more robustly to ROS production (Figure 2). The increase in catalase and CuZnSOD is significantly greater in these cells than in their androgen-independent counterparts (PC3, DU145 and CL1). Perhaps these differences contribute, in part, to the aggressiveness of the ensuing tumour [20]. The work of Lim et al. [21] reported that LNCaP cells express higher catalase activity as compared to PC3 cells. Our research confirms this observation as the LNCaP cells were

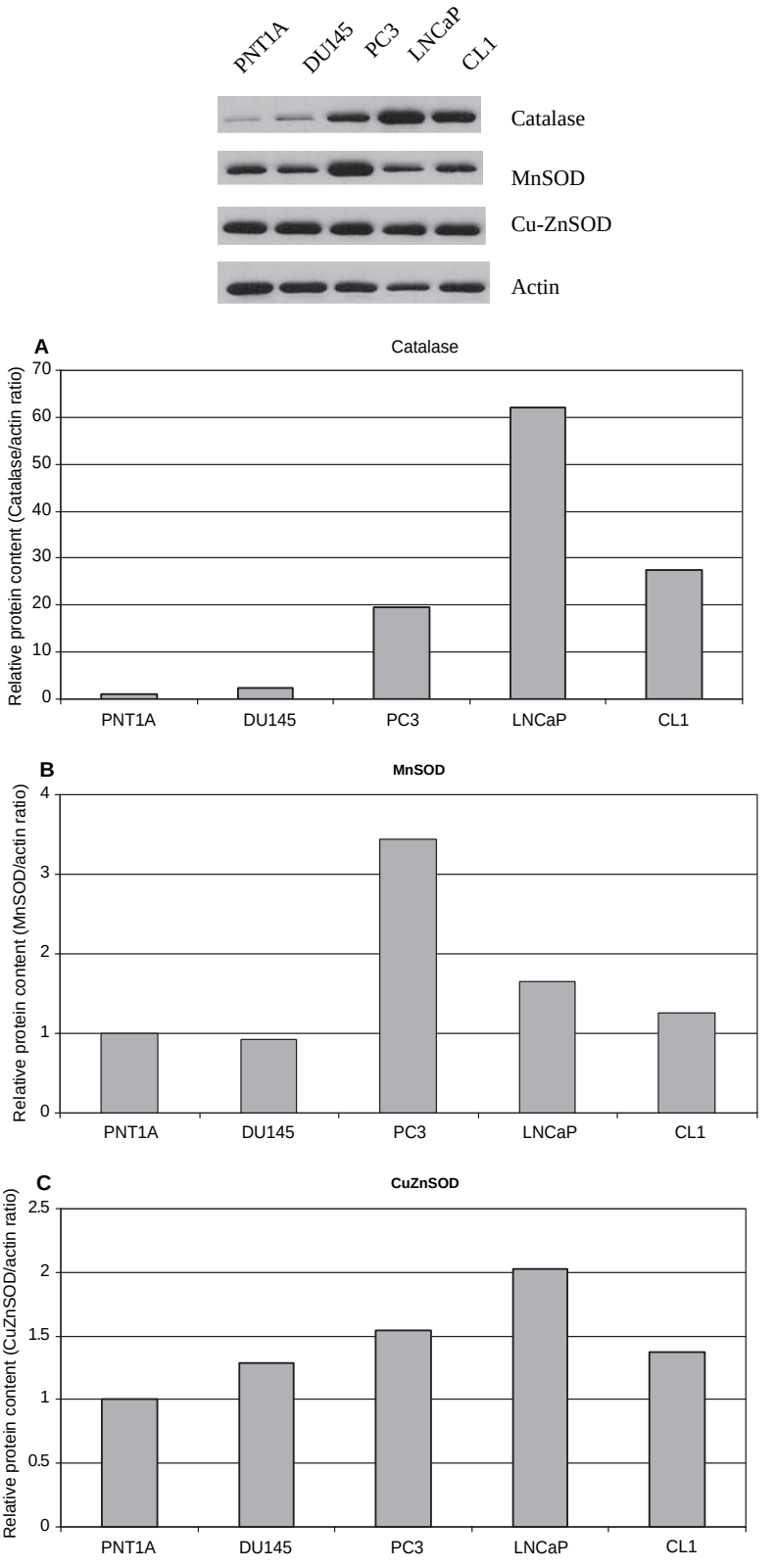

Figure 2. Immunodetection of antioxidant enzymes in prostate cancer cell lines and densitometry of antioxidant enzymes (A–C). Cellular lysates (5 μg/lane) from cultured cell lines PNT1A, DU145, PC3, LNCaP and CL1 were analysed by 10% SDS-PAGE and western blotting using anti catalase (A), anti manganese superoxide dismutase (MnSOD) (B) and anti copper-zinc superoxide dismutase (CuZnSOD) (C). β-actin serves as a control of protein loading and integrity.

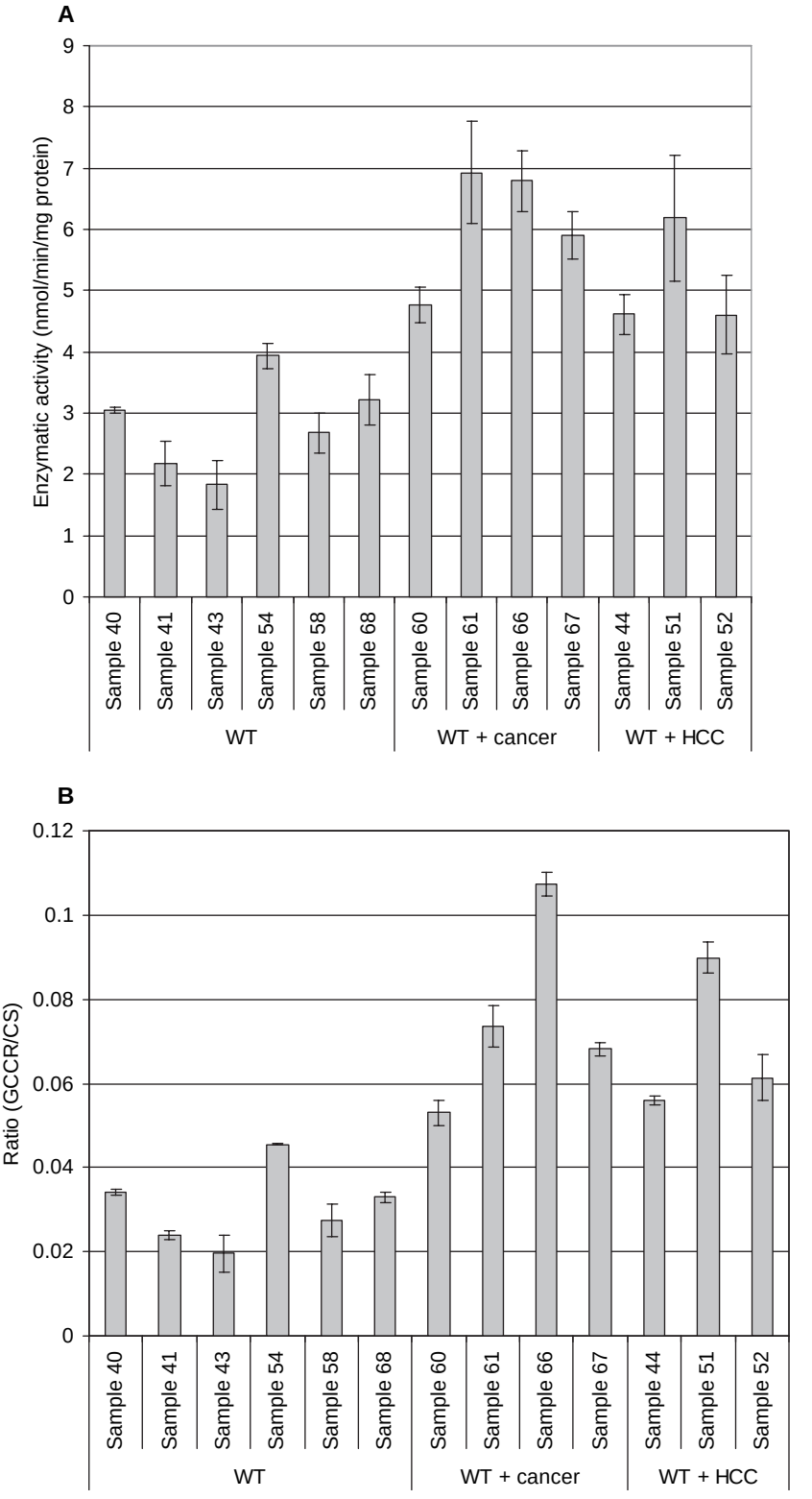

Figure 3. Enzymatic activity of glycerophosphate cytochrome *c* reductase (GCCR) (A) and GCCR activity related to citrate synthase (B) in mouse liver tissues. Enzymatic activity of GCCR and GCCR activity related to citrate synthase were measured in liver tissues from mice with and without cancer either in liver tissue or other organs. WT, WT+cancer and WT+HCC indicate wild-type mice without cancer, with cancer in organs except in liver and with hepatocellular carcinoma, respectively. The statistically significant difference was found among groups using one-way ANOVA and between WT group and WT+cancer or WT+HCC using Student's *t*-test ($p < 0.05$).

found to contain much higher levels of the catalase protein. This may account for the elevated levels of enzyme activities observed by Lim et al. In addition we find that the PC3 cells have a greater level of protein content for MnSOD. This may indicate that the PC3 cells direct a greater proportion of their ROS production into the mitochondrial matrix and this may be triggering increased import of MnSOD into mitochondria. It would be interesting to speculate on the level of mitochondrial DNA damage between these two cell lines. Furthermore, Dakubo et al. [22] have reviewed many of the biochemical changes that occur in mitochondria as a result of prostate cancer and suggest, while it is unlikely that mtDNA mutations may initiate the process of tumour genesis, they could very well compromise oxidative phosphorylation and lead to increased ROS production. Such increases can result in a 'vicious cycle' and the progression of the disease [22].

Therefore the metabolic demands of a cancer cell may cause increased activity of mGPDH, which can potentially result in increased ROS production, thereby facilitating mtDNA damage as well as signalling nuclear gene expression changes. In order to evaluate whether this may be a general oncogenic process, we examined the levels of mGPDH activity in both normal livers and those bearing tumours. Our observation indicates that mGPDH activity is elevated regardless of whether the tumour is hepatic or located peripherally, which suggests that it can be used as a biomarker for the oncogenic process.

We also studied the enzyme activity of mGPDH in human ovarian cancer cells, 2008, and its cis-platin resistant variants, C13 cells with higher ROS production [23]. C13 cells have shown significantly higher activity of mGPDH (data are not shown). These reports suggest that mGPDH is an important site of ROS production in cancer cells.

Although we do not understand, currently, the mechanism behind the changes in the activity of mGPDH in tumour cells, this study provided the support for a role of mGPDH as a link between metabolic distress and ROS signalling.

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