Changes in gene transcription underlying the aberrant citrate and choline metabolism in human prostate cancer samples

Helena Bertilsson1,2*, May-Britt Tessem3, Arnar Flatberg4, Trond Viset5, Ingrid Gribbestad3, Anders Angelsen2, Jostein Halgunset1,5

1Department of Laboratory Medicine and Children’s and Women’s Health, Norwegian University of Science and Technology (NTNU), Trondheim, Norway
2Department of Urology, St Olavs Hospital, Trondheim University Hospital, Trondheim, Norway
3Department of Circulation and Medical Imaging, NTNU, Trondheim, Norway
4Department of Cancer Research and Molecular Medicine, NTNU, Norway
5Department of Pathology and Medical Genetics, St Olavs Hospital, Trondheim University Hospital, Trondheim, Norway

HB: helena.bertilsson@ntnu.no
MBT: may-britt.tessem@ntnu.no
TV: trond.viset@stolav.no
AF: arnar.flatberg@ntnu.no
ISG: ingrid.s.gribbestad@ntnu.no
AA: anders.angelsen@ntnu.no
JH: jostein.halgunset@ntnu.no

Running title: Genes underlying aberrant metabolism in prostate cancer

Word count of text: 3197, Word count of the abstract: 246, Translational relevance: 158, References: 33, Numbers of figures and tables: 7

Keywords: Gene expression profiling, High resolution magic angle spinning, Metabolic profiling, Prostate cancer

*Corresponding author:
Department Laboratory Medicine, Children’s and Women’s Health
Norwegian University of Science and Technology
and Department of Urology
St Olavs Hospital, Trondheim University Hospital
N-7006 Trondheim
Phone: +47 72 57 31 03
Fax: +47 73 86 74 28
e-mail: helena.bertilsson@ntnu.no
**Translational relevance:**

The metabolic alterations including low citrate and high choline levels in prostate cancer are increasingly applied in diagnostic tools like magnetic resonance spectroscopy (MRS) and positron emission tomography to improve characterization of the disease and to monitor the response to treatment. The study was designed to investigate the genetic alterations behind these metabolic aberrations by integrating histopathology, metabolic and transcriptomic profiles of the same samples extracted from radical prostatectomy specimens. Genome-wide transcription analysis (23,444 transcripts) and high resolution magic angle spinning (HR-MAS) were performed on 133 samples. ATP citrate lyase (p = 0.003) and aconitase (p < 0.001) covaried most significantly with the citrate levels, whereas phospholipase A2 group VII (p < 0.001) and choline kinase alpha (p = 0.002) were the transcripts most significantly accompanying the rise in choline levels. Since these are key regulatory enzymes for important metabolic pathways in prostate cancer progression, our results indicate that these gene products deserve attention as possible targets for prostate cancer specific therapy.
Abstract

Purpose:
Low concentrations of citrate and high concentrations of choline containing compounds (ChoCC) are metabolic characteristics observed by magnetic resonance spectroscopy (MRS) of prostate cancer tissue. The objective was to investigate the gene expression changes underlying these metabolic aberrations to find regulatory genes with potential for targeted therapies.

Experimental design:
Fresh frozen samples (n=133) from 41 patients undergoing radical prostatectomy were included. Histopathological evaluation was performed for each sample before a metabolic profile was obtained using high-resolution magic angle spinning (HR-MAS) spectroscopy. Following the HR-MAS, RNA was extracted from the same sample and quality controlled prior to performing microarray gene expression profiling. A partial least square (PLS) statistical model was used to integrate the datasets to identify genes whose expression show significant covariance with citrate and ChoCC levels.

Results:
Samples were classified as benign, n=35, cancer of low grade (Gleason score 6), n=24, intermediate grade (Gleason score 7), n=41 or high grade (Gleason score ≥8), n=33. RNA quality was high with a mean RNA Integrity Number (RIN) score of 9.1 (S.D 1.2). Gene products predicting significantly a reduced citrate levels were acetyl citrate lyase (ACLY, p=0.003) and m-aconitase (ACON, p<0.001). The two genes whose expression most closely accompanied the increase in ChoCC were those of phospholipase A2 group VII (PLA2G7, p<0.001) and choline kinase alpha (CHKA, p= 0.002).

Conclusions:
By integrating histological, transcriptomic and metabolic data, our study has contributed to an expanded understanding of the mechanisms underlying aberrant citrate and ChoCC levels in prostate cancer.
1. Introduction

Altered metabolism is a hallmark of cancer cells [1]. In prostate cancer, metabolic changes present as a reduction in citrate and an increase in choline-containing compound (ChoCC) compared with normal prostate cells [2]. These metabolites can be analysed in vivo using magnetic resonance spectroscopy (MRS) during a magnetic resonance imaging (MRI) examination [3, 4]. Thus, reduced citrate and increased ChoCC metabolite levels can aid discrimination of cancerous from non-cancerous areas [5], and studies indicate that they also have the potential to identify tumours with varying levels of aggressiveness [6].

The application of a high magnetic field strength (14.1 T) to a tissue sample using high-resolution magic angle spinning (HR MAS) MRS may be used for ex vivo assessment of the metabolic status [7]. This technique gives a more detailed metabolic profile compared with in vivo MRS, and the samples remain intact, allowing for further molecular analyses. The ChoCC, observed as a broad signal in vivo are separated into choline (Cho), phosphocholine (PC) and glycerophosphocholine (GPC) in the HR MAS spectra. The ChoCC constitute important building blocks in cell membranes and are elevated, in several malignancies [8, 9], including prostate cancer [10], due to altered phospholipid metabolism. When using gene therapy to silence the enzyme choline kinase converting Cho to PC, a reduction of cell proliferation and tumour growth was shown in breast cancer cell lines [11]. The prostate is highly specialized for the production and secretion of citrate, which is used as an energy supply for sperm. Thus, the normal prostate contains high levels of citrate, with decreasing levels accompanying the malignant transformation [12]. For the malignant cells to proliferate, they must adapt their metabolism and use the citrate for de novo research.
lipid synthesis [13]. Inactivation of enzymes, such as ATP citrate lyase (ACLY), has been shown to limit cell proliferation in vitro and to reduce tumour growth in vivo in numerous tumours including the prostate cancer cell line PC3 [14].

A better understanding of the underlying mechanisms governing the citrate and ChoCC metabolism has the potential of revealing novel therapeutic targets in prostate cancer. To integrate information from several sources could be a key element in achieving this goal [15]. Since expression of genes involved in intermediate metabolism is small compared to the expression levels of structural or secretory genes, such an integrated approach will contribute in finding genes whose altered expression, although perhaps small, will lead to important metabolic deviations. The main objective of the present study was to explore the molecular mechanisms underlying the changes in citrate and ChoCC metabolism in prostate cancer. We combined data from histopathological evaluation, gene expression analysis and metabolic profiling from the same sample to identify those genes whose expression covariates with the altered levels of citrate and ChoCC.

2. Patients and methods

2.1. Patients and tissue samples
At our institution, a transversal tissue slice (thickness 2 mm) is routinely removed for research from all patients undergoing radical prostatectomy, provided the appropriate consent form has been signed. The slice is removed from the middle of the gland using a double bladed knife and immediately fresh-frozen in liquid nitrogen. The two remaining halves of the prostate gland are stitched to a cork board with the cut surface downwards, as this minimizes the tendency for the capsule to retract during the fixation period in 4 % buffered formaldehyde. After fixation the prostate is covered
with India ink and cut in 4 mm thick transversal slices which are subsequently embedded in paraffin and cut into 4 µm thick sections for H&E staining and microscopic examination. The present study comprises fresh frozen samples from 48 patients operated with open radical prostatectomy, fulfilling the following criteria: no prior treatment for prostate cancer, an estimated tumour volume of > 5% of the gland and tumour represented in both hematoxylin-eosin-saffron (HES) sections adjacent to the frozen slice. Several samples from each slice were selected from locations corresponding to cancerous and non-cancerous areas identified in the adjacent paraffin sections. For finding these areas a fused image of the digital photo of the fresh slice and the adjacent HES section are overlaid the frozen slice [16]. Cryosections (4 µm) from each sample were stained with HES and evaluated by a pathologist trained in uropathology. The cellular composition of the section (percentage of area occupied by benign epithelium, stroma and cancer) was visually assessed, and Gleason grading was performed in all cases of malignancy. In two slices, no cancer was found in any of the extracted samples, and in 5 additional slices the RNA extracts showed low concentration or reduced quality (low RIN score), both supposedly due to a high share of stroma. These seven slices were therefore excluded. Thus, the statistical analysis is based on the remaining 133 samples from 41 patients, all of which gave rise to successful gene expression profiling after HR-MAS MRS.

2.2. HR MAS MR spectroscopy

The frozen slice was laid on an aluminium plate in contact with liquid nitrogen and 3x2 mm samples (weight 16-20 mg), were removed from the slice using a specially designed drill. Cryosections were cut for histopathologic evaluation from each sample before HR MAS. Thereafter, the sample was transferred to a disposable insert for the zirconium HR MAS rotor (50 µl, 4 mm diameter) together with 3 µl D₂O (deuterium
oxide) containing TSP (sodium-3'-trimethylsilylpropionate-2,2,3,3-d4). To fit the
insert the sample was cut into smaller pieces using a punch biopsy tool. HR MAS was
performed using a 14.1 T (600 MHz for 1H) Bruker Avance DRX spectrometer
(Bruker Biospin GmbH, Germany) equipped with a 4-mm 1H/13C HR MAS probe.
Spectra were acquired at 4°C with a spin rate of 5000 Hz. A Carr-Purcell-Meiboom-
Gill (CPMG) spin echo sequence [90º- (τ-180º- τ)n – acquisition] was acquired to
suppress signals from overlapping lipids and macromolecules (128 transients,
effective echo time=60ms, acquisition time=3.27s). A spectral region of 10 KHz was
collected into 64,000 data points, and a line broadening of 0.30 Hz was applied to the
raw data before Fourier transformation. After 36 minutes of HR MAS analysis, the
sample was immediately refrozen prior to RNA isolation.

2.3. RNA isolation
After HR MAS analysis, the sample was homogenized for 10-20 seconds in 400µl
tissue lysis buffer using a rotor-stator homogenizer (Omni TH, Tissue Homogenizer,
Omni International, Marietta, GA, USA). The RNA was extracted manually using a
mirVana™ miRNA Isolation Kit (Ambion). The RNA concentration and purity were
determined using a NanoDrop Spectrophotometer (NanoDrop Technologies,
Wilmington, DE, USA). The RNA integrity was determined using a 2100 Bioanalyzer
(Agilent Technologies, Santa Clara, CA USA), according to the manufacturers’
instructions and the results are presented as RIN (RNA Integrity Number) scores.

2.4. Gene expression profiling
An Illumina TotalPrep RNA amplification Kit (Ambion Inc., Austin, TX, USA) was
used to amplify RNA for hybridization on Illumina BeadChips. To synthesize first
strand cDNA using reverse transcription, total RNA from each sample was used.
Following the second strand cDNA synthesis and cDNA purification steps, cRNA
was synthesized via \textit{in vitro} transcription for 12 hours. The gene expression profiles were measured using Illumina Human HT-12 v4 Expression BeadChip (Illumina, San Diego, CA, USA), which enables genome-wide expression analysis (more than 47,000 transcripts) of 12 samples in parallel on a single microarray. The individual transcripts with a detection p-value above 0.01 for all samples were filtered out. The remaining 23,444 transcript values were log2 transformed and quantile-normalized to adjust for technical artefacts between samples [17]. This normalization will enable differentiation between biological variations in gene expression levels and variation due to measurement processes. The microarray data was prepared in a format that conforms to the Minimum Information About a Microarray Gene Experiment (MIAME) guidelines and published in Array Express with accession number: E-MTAB-1041.

2.5. Statistics
Partial least square (PLS) multivariate regression was used as the statistical model to investigate the relationship between the HR MAS spectra and Gleason score and for finding genes whose expression level covariate with the selected metabolites. Each metabolite intensity value was found on the HR MAS spectrum at the specific ppm value for the included metabolites. For the integration of metabolite and gene data, the highest intensity spectral value for citrate, creatine, choline, PC and GPC (found at 2.73, 3.04, 3.22, 3.23, 3.24 ppm respectively) were used as dependent values in the PLS model, whereas the gene expression data were used as independent values. This model identifies two orthogonal planes (PC1 and PC2) which maximize the variance between the samples. Individual genes, whose expression level covariate with the above defined metabolites, were identified from the size and variance of the regression coefficients. Testing whether regression coefficients were significantly
different from zero gave a p-value for the association of each gene to each of the
metabolites.

3. Results

3.1. Patient and sample characteristics
Patient characteristics and histopathological evaluations of the prostate gland are
presented in Table 1. The percentage of the different tissue types within each sample
is presented in Fig 1. In 35 of the samples only benign histology was found. Of the
remaining 98 samples containing cancer, 24 showed low-grade tumour (Gleason score
= 6), whereas 41 samples showed intermediate grade (Gleason score = 7), and 33
samples contained highly aggressive cancer (Gleason score ≥ 8). RNA extracts with
high purity, indicated by a 260/280 ratio of 2.0 (SD 0.03) and 260/230 ratio of 1.94
(SD 0.39), were isolated from all samples. The average RIN score was 9.1 (SD 1.2).
All gene transcripts (n=23,444) and selected metabolites (n=5) serve as variables for
finding genes whose expression level significantly can predict a response in the
metabolites. To illustrate the variance of the samples included in the PLS model
integrating genes and metabolites, a score plot depicting each sample (n=133) as a pie
chart is shown in Fig 2. The first principal component (PC1) distinguishes malignant
from benign samples, whereas the second principal component (PC2) separates the
cancer samples according to their aggressiveness in terms of Gleason score.

3.2. Metabolic profiling
Results from HR-MAS MRS analysis are shown in Fig 3. The PLS model (Fig 3a)
shows HR-MAS spectra (region 3.25-2.5 ppm) with a strong correlation to the
Gleason score ($R^2 = 0.52$), thereby discriminating benign from cancerous samples.
Cancer samples have a reduced level of citrate and an increased ChoCC level
compared with normal samples (Fig 3b, d). In Fig 3d, the samples with a Gleason
score of 7 and an equal amount of benign and stromal tissues are divided between
Gleason grades 3+4 and 4+3. The MR-spectra show decreasing citrate signals when
going from a less aggressive Gleason grade (3+4) to a more aggressive variant (4+3)
within the same Gleason score. Prostate stromal cells only produce citrate to a small
extent, and samples with a high proportion of stroma (in Fig 3c selected as samples
with >70% stroma) mimic a malignant spectrum in this respect. However, they are
separated from the latter by their lower level of ChoCC. The mean ChoCC level is
increased in malignant samples compared with normal and all the individual
components of the ChoCC contribute to the increased signal.

3.3 Gene expression changes underlying aberrant levels of citrate and ChoCC
We identified a three-dimensional predictive subspace, as visualized in Fig 2, using
gene expression data, metabolic profiling and sample histopathology. The PLS
regression model was used to find gene expression with high covariance with citrate
and ChoCC levels. To evaluate the possible influence of gene expression changes on
the selected metabolites in this study we do not present the results as up or down-
regulation of the gene, but as a measurement of how these variables change together,
their covariance. We explored all gene expression values involved in the metabolic
pathways of citrate and ChoCC to find out which genes significantly predict a
response in the selected metabolites. The enzymes with possible regulatory effects on
citrate production (Fig 4) with significant covariance were ATP citrate lyase (ACLY)
p= 0.003 and m-aconitase (ACON) p= < 0.001 (Fig 6). ACON also has a significant
covariance with GPC (p=0.003). The ChoCC and their metabolic pathway are more
complex and involve several enzymes in the regulation of the different metabolites
(Fig 5). The gene transcripts with the most significant covariance to GPC were
PLA2G7 (p< 0.001), a member of the arachidonic acid releasing PLA2 family, and
choline kinase alpha (CHKA) p= 0.002. PLA2G7 (p= 0.05) and cholinephosphotransferase 1 (CHPT1, p=0.05) showed significant covariance with the increased Cho levels. To illustrate the covariance between the genes ACON, ACLY, PLA2G7, CHKA and the metabolites citrate, Cho and GPC the results are presented in figure 6 with accompanying p-values.

4. Discussion

4.1 Benefits of integrating transcriptomic and metabolic data

This study demonstrates the successful integration of histological, metabolic and transcriptomic data from the same human prostate tissue samples. Even though the RNA extraction was performed after HR MAS MRS, the RNA integrity was very high, which is important for generating a robust integrated model. We identified genes whose expression showed a high covariance to the aberrant levels of citrate and ChoCC metabolites and which can thereby possibly act as molecular targets for therapy. A small alteration in the expression of a gene which codes for a rate limiting enzyme can result in significant changes in the activity of the corresponding metabolic pathway and thereby a change in the levels of the affected metabolite [18]. Finding genes with profound effects on cellular and metabolic functions is highly relevant for the development of targeted therapies and for improving diagnostics [19]. Metabolic genes are at greater risk of being erroneously eliminated in microarray analyses, since the level of expression is often only slightly changed compared to the expression level in a normal state. A small increase (two-fold) in a metabolic regulatory gene can be of greater importance for cancer progression than a 50-fold change in expression of a structural protein.
4.2 Genes with significant covariance to low citrate and high ChoCC levels

The concern of missing important metabolic genes by simply studying the level of gene expression has been addressed by Costello et al. [20] and exemplified by the enzyme m-aconitase (ACON), which is a key enzyme for converting citrate to isocitrate. There is no detectable difference in the expression of ACON between normal and malignant tissue by immunohistochemistry [21]. Whereas in normal cells ACON is effectively inhibited by zinc (Fig 4), malignant cells have a reduced capability to store zinc, leading to a reduced inhibition of ACON and thus citrate is oxidized in the citric cycle and consequently citrate levels will sink. The importance of ACON in prostate cancer metabolism could have been missed by a pure genetic/proteomic approach. However, in our study, ACON is a highly significant predictor of the citrate level in cancer samples. The other gene transcript that showed significant covariance with citrate levels was acetyl citrate lyase (ACLY). ACLY is the key enzyme of de novo lipogenesis as it converts citrate to cytosolic acetyl CoA thus linking tumour-associated glycolysis to enhanced lipogenesis, as the acetyl-CoA is utilised in fatty acid and cholesterol synthesis [13]. Inhibition of this gene by siRNA has been shown to suppress the growth and survival of tumour cells [14]. ACLY inhibition resulted in the inhibition of tumour cell growth in vitro and in vivo and the effect seems to be enhanced by combining ACLY knockdown and statin treatment [22]. ACLY over expression is correlated with poorer prognosis of non-small cell lung cancer and is therefore suggested to be an attractive target for RNA interference in a clinical setting [23]. Studies suggesting obesity being a risk factor for the development of larger tumours and more aggressive cancers also support the involvement of lipogenic enzymes in prostate cancer pathophysiology [24, 25]. Rapidly proliferating cancer cells require phospholipids for cell membrane synthesis, which may explain the increase in ChoCC in many cancers. The gene most
significantly predicting an increase in ChoCC, especially an increase in GPC levels, was PLA2G7, a member of the PLA2 gene family. The arachidonic acid pathway is implicated in prostate carcinogenesis [26]. Vainio et al. demonstrated that PLA2G7 was highly over-expressed in prostate cancer, especially in ERG (Ets Related Gene) oncogene-positive cancers suggesting that PLA2G7 could be a therapeutic target in these cancers [27]. Choline kinase alpha (CHKA p=0.002) was the second most significant gene to explain the increase in ChoCC and it has been shown to be over-expressed in prostate cancer [28]. CHKA protein expression levels have been shown to decrease following treatment with PI-103 (class 1 PI3K and mTOR inhibitor) in prostate and colon carcinoma cell lines [29] and inhibition of CHKA was identified as a factor for the observed decrease in phosphocholine levels. The authors examined the response in choline metabolism by MRS of aqueous extracts from the cells concluding that MRS can provide a non-invasive method to monitor changes in choline levels and potential tumour response following treatment. However, results from in vitro experiments must always be corroborated using other methods in order to assess their validity in the intact organism. Our study, using fresh frozen human tissue samples, confirms CHKA as a relevant gene to target in prostate cancer treatment.

4.2 Limitation and strength of the study

The strength of our study is the stringent and validated method of fresh tissue harvesting resulting in material of high molecular quality, allowing microscopic examination, gene expression mapping and analysis of small molecular metabolites to be carried out using the same sample. Although many prostate biobanks storing fresh tissue have been established, the present paper is the first to present results from an integrative design bringing together transcript and metabolite profiles. The benefit of
using human tissue is the possibility to overcome the limitations of using immortalized cell models with “normal” physiological phenotypes [30]. However, the histopathologic evaluation was performed on cryosections of each sample and was somewhat impaired by freezing artefacts, which were prominent in some samples. Another limitation is the definition of a sample with benign histopathology as normal although it has been removed from a slice containing cancer. There may well be molecular changes in the surroundings of a tumour, so in order to minimize this possible effect we selected our “normal” samples in the radical prostatectomy specimens as far away from the tumour area as possible. In addition, some low aggressive samples were extracted from a slice with a higher total Gleason score, which might conceivably affect the metabolism in that sample. Nevertheless, there was a clustering of low aggressive samples, as visualized in figure 2.

4.3 Tissue heterogeneity and tumour aggressivity assessment

Although not the main objective of this study, we present results supporting the inverse correlation between the citrate signal and the Gleason score [6, 31, 32]. In cancer samples with equal amounts of benign epithelium and stroma, we observed lower citrate levels in high-risk samples (Gleason score >8) than in low-risk samples (Gleason score= 6) (Fig 3d). Furthermore, there is a trend towards a difference of citrate within the intermediate-risk group (Gleason score= 7), separating the Gleason grade 3+4 from 4+3, a separation which is clinically highly relevant due to its impact on the prognosis [33].

4.4 Conclusion

In summary, our study has unravelled gene transcripts that expand the understanding of the mechanisms underlying aberrant citrate (ACLY, ACON) and ChoCC levels (PLA2G7, CHK A) in prostate cancer, by integration of data from histological,
transcriptomic and metabolomic analysis of the same samples removed from radical prostatectomy specimens. These genes deserve attention as possible prostate cancer specific targets for therapy.
References


**Text to figures**

**Figure 1 - Ternary graphs illustrating the cellular composition of the samples.**

The ternary graph to the left shows the distribution of the samples (n=133) with respect to the volume percentage of benign epithelium (BE), stroma (S) and cancer (Ca) content. The coloured triangles represent 100% of the specific tissue type and the gray shade of the circles indicates the number of samples sharing the same tissue composition. Each coloured line represents 10% of the specific tissue type so that samples with equal parts of BE, S and Ca will be placed in the centre of the triangle. The three graphs to the right show in an analogous manner the distribution of the cancer-containing samples with respect to the volume percentage of different Gleason grades. In these graphs, benign epithelium and stroma are combined in the common category N (normal).

**Figure 2 - Score plot illustrating the relationship between the prostate samples.**

The PLS multivariate regression model is based on gene expression data (expression values for each gene as independent variables) and MR data (spectral value for Citrate, Creatine, Cho, PC and GPC as dependent variables). Each sample in the study is represented by one pie chart, showing the relative amount of the respective tissue type (stroma, benign epithelium and in cancer in samples, and the Gleason grades). The figure illustrates the variance of the samples and the direction which maximizes the variance is called the principal components. The first principal component (PC1) is the direction in the dataset which maximize the variance. This component separates benign from malignant samples. The amount of stroma in the benign samples widens the variation of these samples. The second principal component (PC2) is the direction which maximizes variance among all directions orthogonal to PC1. PC2 indicates a
clustering of malignant samples depending on cancer aggressiveness. The low
aggressive cancer samples (Gleason score 3+3) cluster mostly in the upper left
quadrant and highly aggressive samples (Gleason score ≥ 8) cluster with some
exceptions in the lower left quadrant of the figure. The intermediate aggressive
samples (Gleason grade 3+4 and 4+3) are not clearly clustered, but there is a trend to
more Gleason grade 4+3 samples clustering towards the highly aggressive samples.

Figure 3 - HR MAS MRS spectra from benign and malignant prostate samples.
11
HR MAS spectra (region 3.25-2.5ppm, n=133) correlated to Gleason score where a)
the PLS score plot of PC1 and PC2 (describing 72.4% of total variation) shows a
discrimination of benign samples (blue, n=35) from cancer samples (orange, red,
green, black, n=98) and the model shows a strong correlation to Gleason score (R² =
0.52). 3b) The loading weights on PC1 (explaining 60.3% of the total variation) show
an increased level of ChoCC and a decreased level of citrate in cancer samples
compared with benign samples, and likewise in samples with higher Gleason scores
compared with lower Gleason scores. Fig c) and d) share the same Y-axis showing
mean spectra of benign samples in c) with less citrate in samples with high stromal
content (> 70%) compared with samples with mostly benign epithelium (>70%). d)
Mean spectra of cancerous samples with equal amount of stromal and benign
epithelium indicate reduced citrate levels with increasing aggressiveness. The dashed
lines in the spectra are areas not included in the statistical model and are therefore
excluded.
Figure 4 - Important components of the citrate metabolism in a prostate cell

Citrate produced in the mitochondria, can either be used for ATP production or transported into the cytoplasm for fatty acid production. In normal prostate cells, a large amount of citrate is produced and secreted into the prostatic fluid where it supplies the sperm with energy. The accumulation and secretion of citrate is possible due to the high intracellular zinc level, which inhibits the enzyme m-aconitase (ACON) thereby preventing citrate oxidation. In prostate cancer, citrate is used for fatty acid production and citrate oxidation via the citric acid cycle, providing more readily available energy to the malignant cells. In our study ACLY (p=0.003) and ACON (p<0.001) were the gene transcripts (encircled by an orange line) that had significant covariance with the reduced citrate levels in the metabolic spectra of the cancerous samples.

Figure 5 – Important components of the choline-containing compound metabolism (ChoCC) in a prostate cell.

Abnormal ChoCC metabolism is a consistent feature of prostate cancer, being firmly linked to increased cell proliferation and is advantageous for cell survival. The genes in the yellow boxes marked with a bolder orange line are significantly correlated with the metabolic response of choline, phosphocholine (PC) or glycerophosphocholine (GPC) in our study. Measured metabolites in this study are marked with a bold green line. Genes with significant covariance to increased levels of GPC are PLA2G7 (p<0.001), CHKA (p=0.002), LYPLA2 (p=0.008), LYPLA1 (p=0.026) and CHDH (p=0.018). LYPLA1 was also significantly correlated to an increase in PC (p=0.002). The increased level of choline was an effect of increased levels of CHPT1 (p=0.05) and PLA2G7 (p=0.05).
Figure 6: Scatterplots illustrating the relationship between genes and metabolites

The figure illustrates the integration of transcriptomic and metabolic data from a subset of gene transcripts and metabolites in the study namely, ACON, ACLY, PLA2G7, CHKA and the metabolites citrate, choline and GPC. Each circle in the plot represents a sample (n=133). Testing whether the regression coefficients are different from zero gives a p-value for the relation between the gene and the metabolite. Citrate shows significant covariance with ACON (p<0.001), ACLY (p=0.003) and PLA2G7 (p=0.009). PLA2G7 (p=0.05) shows significant covariance with Cho levels and GPC with ACON (p=0.003), PLA2G7 (p<0.001) and CHKA (p=0.002).

Acknowledgements

The authors thank medical laboratory technicians Toril Rolfseng, Unn Granli and Borgny Ytterhus for excellent assistance with the laboratory work, Jørn-Ove Sæternes for the design and development of equipment used to harvest and handle frozen prostate tissue and urologists Jan Gerhard Mjønes and Dag Halvorsen for an essential contribution to the material collection.

Disclosure of potential conflicts of interest

The authors have no potential conflicts of interest to disclose.

Funding

The study was supported by grants from the Cancer Foundation of St. Olavs Hospital, University Hospital of Trondheim, Unimed Innovation St. Olavs Hospital, the Norwegian Cancer Society and the Functional Genomics Program (FUGE) in the Research Council of Norway. The Illumina service was provided by NMC (Norwegian Microarray Consortium) at the National Technology Platform, and supported by FUGE.
Fig. 3

a) PC1 vs. PC2

Gleason Score
- Blue: 0
- Orange: 6
- Red: 7
- Green: 8
- Black: 9

b) Loadings (PC1)

PC: Phosphocholine
GPC: Glycophosphocholine
Cho: Choline
Creatine
Citrate

ppm

3.2 3.1 3.0 2.9 2.8 2.7 2.6 2.5

3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6 2.5

Intensity

C) Stroma > 70%
Benign epithelium > 70%
Excluded parts

PC Cho
Creatine
GPC
Citrate

D) Gleason score 6
Gleason score 7 (3+4)
Gleason score 7 (4+3)
Gleason score >= 8
Excluded parts

ppm
Fig. 6

Gene expression

ACON
P-value: < 0.001

ACL
P-value: 0.24

PLA2G7
P-value: 0.003

P-value: 0.009

P-value: 0.05

P-value: < 0.001

CHKA
P-value: 0.15

P-value: 0.67

P-value: 0.002

Citrate
Choline
GPC
Table 1. Patient characteristics and histopathologic evaluation of the prostate gland

<table>
<thead>
<tr>
<th>Age (mean)</th>
<th>Preop PSA (mean)</th>
<th>Tumor volume (mean)</th>
<th>pT-stage</th>
<th>n</th>
<th>Gleason score</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>62.6 (SD 5.5)</td>
<td>10.4 (SD 6.5)</td>
<td>21.1 % (SD 15.3)</td>
<td>pT2a</td>
<td>2</td>
<td>3+3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pT2b</td>
<td>1</td>
<td>3+4</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pT2c</td>
<td>25</td>
<td>4+3</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pT3a</td>
<td>6</td>
<td>4+5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pT3b</td>
<td>7</td>
<td>5+3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5+4</td>
<td>1</td>
</tr>
</tbody>
</table>
Changes in gene transcription underlying the aberrant citrate and choline metabolism in human prostate cancer samples

Helena Bertilsson, May-Britt Tessem, Amar Flatberg, et al.

Clin Cancer Res Published OnlineFirst April 17, 2012.