Changes in Gene Transcription Underlying the Aberrant Citrate and Choline Metabolism in Human Prostate Cancer Samples

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Abstract

**Purpose:** Low concentrations of citrate and high concentrations of choline-containing compounds (ChoCC) are metabolic characteristics observed by magnetic resonance spectroscopy 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. Histopathologic evaluation was carried out for each sample before a metabolic profile was obtained with high-resolution magic angle spinning (HR-MAS) spectroscopy. Following the HR-MAS, RNA was extracted from the same sample and quality controlled before carrying out microarray gene expression profiling. A partial least square statistical model was used to integrate the data sets 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 $\geq 8$), $n = 33$. RNA quality was high with a mean RNA Integrity Number score of 9.1 (SD 1.2). Gene products predicting significantly a reduced citrate level 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 histologic, transcriptomic, and metabolic data, our study has contributed to an expanded understanding of the mechanisms underlying aberrant citrate and ChoCC levels in prostate cancer. 

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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 analyzed in vivo using magnetic resonance spectroscopy (MRS) during an MRI examination (3, 4). Thus, reduced citrate and increased ChoCC metabolite levels can aid discrimination of cancerous from noncancerous areas (5), and studies indicate that they also have the potential to identify tumors 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 ChoCCs, observed as a broad signal in vivo, are separated into choline (Cho), phosphocholine (PC), and glycerophosphocholine (GPC) in the HR-MAS spectra. The ChoCCs 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 tumor 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

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The metabolic alterations including low citrate and high choline levels in prostate cancer are increasingly applied in diagnostic tools like magnetic resonance spectroscopy 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 were carried out 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. Because these are the 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.

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 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 were carried out 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. Because these are the 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.

HR-MAS MR spectroscopy
The frozen slice was laid on an aluminum plate in contact with liquid nitrogen and 3 × 2 mm samples (weight 16–20 mg) were removed from the slice with 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 D$_2$O (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 drill. 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 carried out in all cases of malignancy. In 2 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 7 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.
HR-MAS analysis, the sample was immediately refrozen before RNA isolation.

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

Gene expression profiling
An Illumina TotalPrep RNA Amplification Kit (Ambion Inc.) was used to amplify RNA for hybridization on Illumina BeadChips. To synthesize first-strand cDNA with reverse transcription, total RNA from each sample was used. Following the second-strand cDNA synthesis and cDNA purification steps, cRNA was synthesized via in vitro transcription for 12 hours. The gene expression profiles were measured with Illumina Human HT-12 v4 Expression BeadChip (Illumina), 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 $P > 0.01$ for all samples were filtered out. The remaining 23,444 transcript values were log$_2$ transformed and quantile normalized to adjust for technical artifacts between samples (17). This normalization will enable differentiation between biological variations in gene expression levels and variation due to measurement processes. The microarray data were prepared in a format that conforms to the Minimum Information About a Microarray Experiment (MIAME) guidelines and published in Array Express with accession number: E-MTAB-1041.

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, Cho, PC, and GPC (found at 2.73, 3.04, 3.22, 3.23, and 3.24 ppm, respectively) was used as dependent values in the PLS model, whereas the gene expression data were used as independent values. This model identifies 2 orthogonal planes (PC1 and PC2) which maximize the variance between the samples. Individual genes, whose expression levels 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.

Results
Patient and sample characteristics
Patient characteristics and histopathologic 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 tumor (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 of 280 ratio of 2.0 (SD 0.03) and 260 of 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.

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

| Table 1. Patient characteristics and histopathologic evaluation of the prostate gland |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Age (mean)       | Preop PSA (mean) | Tumor volume (mean) | pT-stage     | $n$      | Gleason score | $n$      |
| 62.6 (SD 5.5)   | 10.4 (SD 6.5)   | 21.1% (SD 15.3)   | pT2a         | 2       | 3 + 3       | 1       |
|                 |                 |                  | pT2b         | 1       | 3 + 4       | 17      |
|                 |                 |                  | pT2c         | 25      | 4 + 3       | 13      |
|                 |                 |                  | pT3a         | 6       | 4 + 5       | 8       |
|                 |                 |                  | pT3b         | 7       | 5 + 3       | 1       |
|                 |                 |                  |              |         | 5 + 4       | 1       |
of citrate and an increased ChoCC level compared with normal samples (Fig. 3B and 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 3þ4 and 4þ3. The MR spectra show decreasing citrate signals when going from a less aggressive Gleason grade (3þ4) to a more aggressive variant (4þ3) 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.

Gene expression changes underlying aberrant levels of citrate and ChoCC

We identified a 3-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 downregulation 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 ACLY P = 0.003 and m-acotinase (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 α (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 Fig. 6 with accompanying P values.

Discussion

Benefits of integrating transcriptomic and metabolic data

This study shows the successful integration of histologic, metabolic, and transcriptomic data from the same human prostate tissue samples. Even though the RNA extraction was carried out 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 because the level of expression is often only slightly changed compared with the expression level in a normal...
state. A small increase (2-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.

**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 and colleagues (20) and exemplified by the enzyme 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 ACLY. ACLY is the key enzyme of de novo lipogenesis as it converts citrate to cytosolic acetyl CoA thus linking tumor-associated glycolysis to enhanced lipogenesis, as the acetyl-CoA is utilized in fatty acid and cholesterol synthesis (13). Inhibition of this gene by siRNA has been shown to suppress the growth and survival of tumor cells (14). ACLY inhibition resulted in the inhibition of tumor cell growth in vitro and in vivo and the effect seems to be enhanced by combining ACLY knockdown and statin treatment (22). ACLY overexpression is correlated with poorer prognosis of non–small cell lung carcinoma 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 tumors 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 and colleagues showed that PLA2G7 was highly overexpressed in prostate cancer, especially in Ets
Related Gene (ERG) oncogene-positive cancers suggesting that PLA2G7 could be a therapeutic target in these cancers (27). CHKA \((P = 0.002)\) was the second most significant gene to explain the increase in ChoCC, and it has been shown to be overexpressed 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 noninvasive method to monitor changes in choline levels and potential tumor response following treatment. However, results from in vitro experiments must always be corroborated using other methods 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.

**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, this 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" physiologic phenotypes (30). However, the histopathologic evaluation was carried out on cryosections of each sample and was somewhat impaired by freezing artifacts, 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 tumor, so to minimize this possible effect we selected our "normal"
samples in the radical prostatectomy specimens as far away from the tumor 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 Fig. 2.

Tissue heterogeneity and tumor 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 toward 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).

Conclusion

In summary, our study has unraveled gene transcripts that expand the understanding of the mechanisms underlying aberrant citrate (ACLY and ACON) and ChoCC levels (PLA2G7 and CHKA) in prostate cancer, by integration of data from histologic, 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.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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