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CORRELATION OF PHOSPHOLIPID METABOLITES WITH PROSTATE CANCER PATHOLOGIC GRADE, PROLIFERATIVE STATUS AND SURGICAL STAGE – IMPACT OF TISSUE ENVIRONMENT

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Abstract

This study investigated the relationship between phospholipid metabolite concentrations, Gleason score, rate of cellular proliferation, and surgical stage in malignant prostatectomy samples by performing one and two-dimensional HR-MAS total correlation spectroscopy (TOCSY), pathology, and Ki-67 staining on the same surgical samples. At radical prostatectomy, surgical samples were obtained from 49 patients (41 with localized TNM stage T1 and T2, and 8 with local cancer spread (TNM stage T3). Thirteen of the tissue samples were high-grade prostate cancer (Gleason score: 4+3 [n=7]; 4+4 [n=6]), 22 low-grade (Gleason score 3+3 [n=17]; 3+4 [n=5]), and 14 were benign prostate tissues. This study demonstrated that high-grade prostate cancer, has significantly higher Ki-67 staining and concentrations of PC and GPC than low-grade prostate cancer ($2.4 \pm 2.8\%$ versus $7.6 \pm 3.5\%$, $p < 0.005$, and 0.671 ± 0.461 versus 1.87 ± 2.15 mmolal, $p < 0.005$, respectively). In patients with local cancer spread, increases in [PC+GPC+PE+GPE] and Ki-67 index approached significance (4.2 ± 2.5 mmolal versus 2.7 ± 2.4 mmolal, $p = 0.07$, and $5.3 \pm 3.8\%$ versus $2.9 \pm 3.8\%$, $p = 0.07$, respectively). PC and Ki-67 were significantly lower and GPC higher in prostate tissues as compared to cell cultures, presumably due to a lack of important stromal-epithelial interactions in cell cultures. The findings of this study will need to be validated in a larger cohort of surgical patients with clinical outcome data, but support the role of *in vivo* ¹H MRSI in discriminating low- from high-grade prostate cancer based on the magnitude of elevation of the *in vivo* total choline resonance.

Keywords

Phospholipid metabolism; prostate cancer; tumor microenvironment; pathologic grade; Gleason score; Ki-67; stage

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INTRODUCTION

The American Cancer Society estimates that 217,730 men will be diagnosed with prostate cancer in the United States in 2010, which is a higher incidence figure than any other non-cutaneous human malignancy(1). The decision on how to manage prostate cancer once detected poses a dilemma for patients and their physicians, because prostate cancers demonstrate a tremendous range in pathologic and biologic diversity, associated risk of progression and are treated with a broad spectrum of approaches from “active surveillance” and minimally invasive focal therapies to more aggressive surgical, radiation-based, and systemic therapies (2,3). Such therapies have tradeoffs in that treatment, no matter how well delivered, is frequently associated with changes in health-related quality of life (4,5). The histology of prostate cancer is variable, ranging from well (Gleason score 3+3) to poorly differentiated (Dominant pattern 4 or 5, i.e. Gleason score 4+3, 8, 9, 10) (6). The evaluation of the dominant histological pattern at biopsy is important, because poorly differentiated tumors (Gleason score 4+3) are predictive of later stage potentially lethal disease, and are treated more aggressively (7,8). Nuclear antigen, Ki-67, is present in proliferating cancer cells, and the presence of high Ki-67 staining has been associated with high pathologic grade and stage prostate tumors (9) and metastases (10). Earlier and more accurate identification of such cancers would allow for better selection of initial treatment, accrual to clinical trials whose goal it is to improve outcomes, and earlier selective application of systemic therapy. This is a critical question, since there is currently no cure for metastatic prostate cancer and an estimated 32,050 men (~15% of incidence rate) will die of the disease in the United States in 2010 (1).

Commercial 1.5T magnetic resonance imaging and three-dimensional proton magnetic resonance spectroscopic imaging (MRI/3-D ^1H MRSI) exams are currently available and published data has demonstrated that the metabolic biomarkers, choline, citrate and polyamines, provided by 3-D ^1H MRSI combined with the anatomical information provided by MRI can significantly improve the detection, and staging of prostate cancer in individual patients (11,12). Through *in vivo* ^1H MRSI studies of prostate cancer patients, the elevation of the *in vivo* total choline resonance has been shown to be a specific biomarker of prostate cancer presence and extent (11), with the degree of elevation of choline roughly correlating with Gleason score (11,13–15). The *in vivo* total choline resonance at 3.2 ppm arises from a number of choline and ethanolamine containing metabolites that are the major cytosolic precursors and degradation products of phospholipid membrane assembly and catabolism (16), as well as other metabolites such as taurine (3.24 ppm). Several *ex vivo* ^1H spectroscopy studies of benign and malignant human prostate cancer cell lines (17–19) and tissues (20–22) have demonstrated that elevated total choline in regions of prostate cancer is predominately due to significant increases in the choline head group singlets (9 equivalent protons) of free choline (Cho), phosphocholine (PC), and glycerophosphocholine (GPC). While PC, GPC and their ethanolamine counterparts phosphoethanolamine (PE), and glycerophosphoethanolamine (GPE), can be spectrally resolved in ^1H NMR spectra of prostate cell and tissue extracts, it is difficult to robustly resolve these in ^1H NMR spectra of intact prostate tissue. HR-MAS two-dimensional total correlation spectroscopy (TOCSY) (20) can be used in a non-destructive manner to quantify the choline and ethanolamine phospholipid metabolites from surgical prostate tissues prior to pathology and immunohistochemistry of the same tissues (23). Additionally, PE and GPE can be resolved using ^{31}P spectroscopy (24) and prior ^1H HR-MAS and ^{31}P spectroscopy studies have demonstrated that PE and GPE are also significantly elevated in human prostate cancer (20). Interestingly, significant differences, possibly associated with the tumor microenvironment, have been reported for the tissue levels of phospholipid metabolites obtained from human prostate cancer cells growing in culture and intact human prostate tissues (17–22).

To date, a systematic correlation of changes in phospholipid metabolite concentrations in human prostate cancer tissues with pathologic grade, rate of proliferation, and surgical stage (TNM classification) has not been performed. The goal of this study was to investigate the relationship between individual and combinations of phospholipid metabolite concentrations, Gleason score, rate of cellular proliferation and surgical stage by performing quantitative HR-MAS spectroscopy, pathology, and Ki-67 staining on the same snap frozen human prostate surgical samples.

MATERIALS AND METHODS

Tissue Samples

49 prostate tissue samples were harvested from 49 patients (Mean age: 65 ± 8 years, low grade 63 ± 10 years and high grade 64 ± 6 years) at surgery. The whole gland was removed, the region of cancer was macroscopically identified by a pathologist, and a core was taken and put on dry ice within 15 minutes. The samples were subsequently placed in cryogenic vials, and stored at -80°C until use. Samples were analyzed using custom designed 20 and 30 μl leak proof zirconium rotors, which contained an oblate spheroid shaped sample chamber to facilitate shimming and an airtight screw top plug to prevent leakage (25). 3.0 μl of $\text{D}_2\text{O}+\text{TSP}$ was pipetted into the bottom of a tared rotor and weighed to 0.01 mg. Tissue cores were then excised from the surgical specimens using a 3.5 mm biopsy punch (Sklar Instruments, West Chester, PA, USA). The balance was tared again and the tissues were weighed to 0.01 mg (mean = 16.98 ± 3.71 mg), then slowly inserted into the rotor. Finally, the top screw was carefully inserted to avoid air bubbles, tightened, and then the rotor was placed in the spectrometer for analysis.

1-D and 2-D HR-MAS Spectroscopy

HR-MAS TOCSY's were acquired from snap frozen tissue samples obtained at radical prostatectomy in order to resolve the choline (choline – Cho, phosphocholine – PC, glycerophosphocholine - GPC) and ethanolamine-containing compounds (ethanolamine - Eth, phosphoethanolamine - PE, glycerolphosphoethanolamine - GPE) that overlap in 1-D ^1H HR-MAS tissue spectra. Data were acquired at 11.7 T (500 MHz for ^1H), 1°C , and a 2,250 Hz spin rate using a Varian INOVA spectrometer equipped with a 4 mm gHX nanoprobe (Varian Inc, Palo Alto, CA).

Prior to beginning each TOCSY experiment, a quantitative 1D “presat” spectrum was acquired with a 2s presaturation delay, 2s acquisition time ($\text{TR} = 4\text{s}$), and 128 transients (22). TOCSY data were acquired using a rotor synchronized WURST-8 adiabatic mixing scheme flanked by two sets of ~ 1.8 ms trim pulses (26). Both, the adiabatic and trim pulses, were rotor synchronized by setting the pulse lengths equal to the reciprocal of the spin rate (0.444 ms). The TOCSY experiment was optimized for tissue studies, and the following parameters provided optimal water presaturation, S/N, and resolution in a total experiment time of 1.08 hrs: $\text{TR} = 1.24$ s (presaturation delay = 1.0 s, $\tau_m = 40$ ms, $\text{AT} = 0.20$ s); $\text{SS} = 4$ (1^{st} increment only); $\text{NT} = 24$; $\text{NP} = 4096$ (complex) and spectral width = 20,000 Hz in F2; $\text{N}_i = 64$ (complex) and spectral width = 6,000 Hz in F1 (20). For the 52 tissue samples studied, the total time for sample preparation, shimming, and 1D and 2D data acquisition was <1.5 hours per sample.

Data Analysis

Data were processed offline using ACD/Labs 1D and 2D NMR processor version 9 (ACD/Labs, Toronto). One-dimensional spectra were processed and quantified using Lorentzian-Gaussian peak fitting as previously described (25). TOCSY data were processed using $4 \times \text{N}$ linear prediction in F1, zerofilled to 4K (F2) and 1K (F1) complex points, apodized using a

Gaussian function and Fourier transformed in both dimensions. TOCSY spectra were then phased by displaying slices containing cross peaks near the beginning, middle, and end of the spectrum in F2 and then F1. Zero and first order phase corrections were applied in each dimension and then to the entire 2D matrix. Finally, the spectra were frequency referenced to TSP at (0.00 × 0.00 ppm).

Phospholipid metabolite concentrations were estimated using the side chain CH₂–CH₂ cross peaks peak volumes of Cho (3.55 × 4.07 ppm), PC (3.62 × 4.17 ppm), GPC (3.71 × 4.33 ppm), Eth (3.14 × 3.82 ppm), PE (3.23 × 3.99 ppm), and GPE (3.30 × 4.12 ppm) relative to the the diagonal of TSP (20). The cross peaks above the diagonal were used for quantitation because they contained less environmental or “t1” noise than the cross peaks below the diagonal (27). Metabolite cross peaks and the TSP diagonal peak were volume integrated by setting a threshold at 0.05% of each cross peak’s maximum intensity, below which the data were considered to be in the noise. Cross peak volumes (V_{met} , V_{TSP}) exceeding the threshold were then numerically integrated and exported to an Excel spreadsheet. Metabolite cross peak to TSP diagonal peak ratios were then calculated and corrected for the magnetic transfer efficiency (K_{MT}) of the CH₂–CH₂ spin systems of the phospholipid metabolites (20). Millimolar (mmolal) metabolite concentrations were then calculated based upon the masses of tissue and D₂O+TSP in the rotor, and the TSP and metabolite cross peak volumes using the following equation:

$$(V_{\text{met}}/V_{\text{TSP}}) \times K_{\text{MT}} \times \frac{\text{Mass } D_2O + \text{TSP} \times 0.75\% \text{ (mg)}}{\text{Molecular weight TSP (172.23 g/mol)}} \times \frac{1000 \text{ g/kg}}{\text{Mass tissue (mg)}} = \text{Metabolite concentration (mol/kg)}$$

where 0.75% is treated as a constant.

Pathologic and Ki-67 Analysis

After the HRMAS study, samples were immediately frozen in optimal cutting temperature (OCT) compound and 5 mm sections were obtained using a Leica CM1850 cryostat. For each sample, adjacent sections were stained with hematoxylin and eosin (H&E) and Ki-67 (25). For Ki-67, the slides were incubated for 60 min with a monoclonal mouse Ki-67 antibody, clone MIB-1 (M7240, Dako, Copenhagen), diluted 1:100 at room temperature. Secondary antibody was peroxidase labeled HRP polymer (Dako, Copenhagen) and incubated for 60 min. The antigen localization was achieved by the DAB-chromogen (Dako, Copenhagen). Nuclei were considered Ki67-positive if any nuclear staining was present, regardless of staining intensity. The pathology slides were reviewed by two trained pathologists (12 and 16 years experience) on two separate days. They estimated the percentage of benign glandular, benign stroma, the percentage and grade of prostate cancer, and the % of the cancer that stained positive for Ki-67. Samples containing more than 5% of other confounding pathologies, including chronic inflammation (prostatitis), high grade prostatic intraepithelial neoplasia (HGPIN), corpora amylacea (inspissated secretions), mucin, and atrophy were excluded from the analysis. A Ki-67 labeling index was defined as the percentage of positively staining cells for each cell type, as determined by counting approximately 1000 cells of that type. Pathology readings and Ki-67 labeling index were then recorded into a database and averaged for both readers and days.

Statistical Analysis

For statistical comparisons, prostate cancer tissues were divided into three groups, benign tissues, low-grade (3+4) and high-grade (4+3) cancer, regardless of relative glandular and stromal composition. Ki-67 results were divided into 4 groups, unstained, < 5%, 5–10%, and > 10% Ki-67 staining. Statistical analyses were performed using JMP 8.0 statistical

analysis software (SAS Institute Inc. Cary, NC). Phospholipid metabolite concentrations (mmol/kg) were statistically compared between benign, high (4+3) and low (3+4) prostate cancer and using a Student's T- test with a p-value of less than 0.05 as significant. Percent staining of prostate tissue using the Ki-67 antigen (unstained, < 5%, 5–10%, >10%) was compared to tissue type (benign, high [4+3] and low [3+4] prostate cancer) using a contingency analysis and the Pearson chi-squared test was used to determine the probability of a significant relationship. Phospholipid metabolite concentrations (mmol/kg) were statistically compared between patients with local cancer spread (stage T3) versus localized disease (stages T1 and T2) at surgery using a Student's T- with a p-value of less than 0.05 as significant. No corrections were made for multiple comparisons.

RESULTS

At radical prostatectomy, 41 of the patients studied had cancer localized to the prostate (TNM stages T1 and T2), 5 patients had extracapsular extension (ECE, stage T3A) and 3 patients had seminal vesicle invasion (SVI, Stage T3B). There was no significant difference serum PSA between patients with localized disease and local metastases (ECE, SVI) (6.07 ± 3.09 and 7.15 ± 3.76 , respectively). A total of 49 surgical samples, one from each patient, were studied; 13 high grade (Gleason score: 4+3 [n=7]; 4+4 [n=6]), 22 low grade (Gleason score 3+3 [n=17]; 3+4 [n=5]), and 14 benign prostate tissues. The percent of the samples that contained cancer, ranged from 5 to 85%, with the remaining tissue being a mixture of benign glandular and stromal tissue. The mean percent of the samples containing cancer in the high- and low-grade groups (43 ± 26 and $31 \pm 20\%$, respectively) was not significantly different. The benign samples consisted of 9 predominately glandular (30% glandular tissue(21)) and 5 predominately stromal tissues, and benign samples were taken from the side opposite of the hemiprostate containing cancer in patients with unilateral disease. Since no significant difference in phospholipid metabolite levels were observed between between predominately glandular and stromal benign tissues, the samples were combined.

Representative fully relaxed ^1H HR-MAS spectra of a benign predominantly glandular prostate tissue (left), low Gleason score (3+3) prostate cancer (middle) and high Gleason score (4+4) prostate cancer (right) are shown in figure 1A. Similar to prior publications, benign glandular tissues demonstrated high levels of citrate (doublet of doublets at 2.55 ppm) and polyamines (PA, broad multiplet at 3.13 ppm) that were dramatically reduced in both low and high grade prostate cancer spectra (refs). There was also a visually apparent increase in the choline (3.21 ppm), PC (3.23 ppm), and GPC (3.24 ppm) head group singlets; however, PC and GPC could not be resolved from each other and from PE (3.22 ppm) in 1-D ^1H HR-MAS prostate tissue spectra. Eth (3.15 ppm) and GPE (3.30 ppm) co-resonated with a number of other metabolites, including polyamines (3.13 ppm), taurine (3.26 ppm) myo-inositol (3.28 ppm) and glucose (≈ 3.23 ppm) in this highly overlapping region of the ^1H spectrum. Additionally, the ethanolamine containing phospholipid metabolites arise from two proton multiplets in the ^1H spectra, leading to a reduction in overall signal intensity relative to the 9 proton singlet head group resonances of PC and GPC. Predominantly benign stromal tissues demonstrated a reduction in citrate and polyamines relative to benign glandular tissues as previously reported (21,22), but there was no difference in the choline containing metabolites (refs).

Figure 1B shows the upper diagonal $\text{CH}_2\text{--CH}_2$ region of the two dimensional TOCSY spectra obtained on the same benign and malignant prostate tissue samples shown in figure 1a. The Cho, PC, GPC, Eth, PE and GPE cross peaks are clearly resolved and their relative concentrations are reflected by the number of contour levels as in a topographic map. The choline cross peak co-resonated with myo-inositol (Cho+mI) and therefore Cho was not

quantified in this study. On visual inspection, PC, GPC, PE, and GPE levels increased in cancer relative to benign TOCSY spectra (Figure 1B). Quantitatively, these observations proved true with all four phospholipid metabolites significantly increasing in prostate cancer relative to benign prostate tissues (Table 1A). Additionally, the concentrations of PE and GPE were 4 fold higher than PC and GPC in both benign and malignant tissues. Specifically, [PC+GPC] increased 4.5 fold from 0.263 ± 0.187 to 1.12 ± 1.45 mmolal, while [PE+GPE] increased 4 fold from 1.03 ± 0.893 to 4.11 ± 3.24 mmolal in benign versus malignant prostate tissues. Due to the large variability, there was overlap in individual [PC+GPC] and [PE+GPE] values between benign and malignant tissues.

Visually, PE and GPE increased most dramatically between benign and low-grade prostate cancer TOCSY spectra, while PC and GPC increased most between low- and high-grade cancer (Figure 1B). A quantitative comparison demonstrated that [PE] and [GPE] were increased by 2.5 and 4 fold between benign and low-grade prostate cancer, but their concentrations were similar between low- and high-grade cancer (Table 1B). [PE+GPE] provided the best discrimination of individual benign and low-grade prostate cancer tissues, but there was still overlap between individual values (Figure 2A). [PC] and [GPC] were dramatically and significantly increased by 27 and 30 fold in high-grade prostate cancer relative to benign tissues. [GPC] was also significantly increased by 2 fold in high- versus low-grade prostate cancer (Table 1B). [PC+GPC] provided the best discrimination of individual high- versus low-grade prostate cancer tissues, but there was overlap between individual values (Figure 2B). The mean total phospholipid concentration, [PC+GPC+PE+GPE] was significantly higher in both low- and high-grade prostate cancers relative to benign tissues (Table 1B), but there was overlap between individual values for all three tissue types (Figure 2C). Correcting phospholipid metabolite concentrations for the percentage of cancer present in the sample was problematic for samples with small percentages of cancer, in that the correction resulted in grossly over estimated values.

Figure 1C shows representative H&E histologic sections and adjacent Ki-67 stained sections from the same tissue samples that the 1- and 2-D HR-MAS spectra was acquired from in Figures 1A and B. A majority (11/14) of the benign prostate samples and 50% (11/22) of the low-grade prostate cancer samples did not stain for Ki-67 (Figure 3). All high-grade cancer samples stained positive for Ki-67 (n=13), with more than half of the tissues having more than 10% of the cancer cells staining positive for Ki-67 (Figure 3). The mean percent of cells staining for Ki-67 was significantly increased between benign tissue and low-grade cancer (0.07 ± 0.27 versus 2.4 ± 2.8 , $p < 0.01$) and between low- and high-grade cancers (2.4 ± 2.8 versus 7.6 ± 3.5 , $p < 0.005$), and there was a significant correlation between tissue type and Ki-67 staining (Figure 3, Chi square = 32.2, $P < 0.005$). However there wasn't a significant correlation (all p values > 0.1) between Ki-67 staining and phospholipid metabolite levels for any of the tissue types.

In patients with local cancer spread (ECE or SVI, Stage T3), increases in [PC+GPC+PE+GPE] and Ki-67 staining approached significance (4.2 ± 2.5 mmolal versus 2.7 ± 2.4 mmolal, $p = 0.07$, and $5.3 \pm 3.8\%$ versus $2.9 \pm 3.8\%$, $p = 0.07$, respectively) as compared to disease localized to the prostate (Stages T1 and T2).

DISCUSSION

The results of this study demonstrate that high grade prostate cancers, i.e. cancers having a dominant Gleason pattern 4 (Gleason score $4+3$), demonstrated significantly higher Ki-67 staining and concentrations of choline and ethanolamine containing phospholipid metabolites than lower grade (Gleason score $3+4$) cancers. Several *in vivo* MRI/ ^1H MRSI studies of prostate cancer patients suggest that ^1H MRSI may be able to provide a non-

invasive assessment of cancer grade for all of the cancer foci within the prostate, potentially with reduced inter-observer variability and grade clustering (13,15,28,29). In these studies, the magnitude of elevation of the choline plus creatine to citrate ratio correlated with Gleason score. The ability of this metabolic ratio to predict the pathologic grade of prostate cancer is due both to a significant reduction in citrate and an elevation of phospholipid metabolites in prostate cancer. Early biochemical studies have demonstrated that citrate levels in prostatic adenocarcinomas are grade dependent, with citrate levels being low in well-differentiated, low-grade prostatic cancer and effectively absent in poorly-differentiated high-grade prostatic cancer (30,31). Proton HR-MAS spectroscopy studies of intact human prostate surgical and biopsy tissues have also demonstrated significant decreases in citrate and polyamines and a significant elevation of choline and ethanolamine containing phospholipid membrane metabolites in prostate cancer relative to benign prostate tissues (20–22,32,33). Similar to a recent HR-MAS TOCSY study of surgical prostate tissues (20), we observed a significant increase in the concentrations of all of the phospholipid metabolites, [PC], [GPC], [PE] and [GPE], in prostate cancer relative to benign prostate tissues. The concentration of the ethanolamine containing phospholipid metabolites were 4 fold higher than the choline containing metabolites in both benign and malignant tissues. This finding is consistent with early *in vivo* and *ex vivo* ^{31}P studies of prostate cancer (24,34–37), and a prior ^1H HR-MAS TOCSY study of snap frozen surgical prostate tissues.

Prior ^1H HR-MAS studies of human prostate cancer tissues obtained at surgery and biopsy suggested that larger increases in total choline, and decreases in citrate and polyamines ($P < 0.05$) were observed with more aggressive cancers (38,39). In this study, the mean total phospholipid concentration, [PC+GPC+PE+GPE], was significantly higher in high- versus low-grade prostate cancer, however there was overlap of individual values between the two Gleason subscores. The increase in ethanolamine phospholipid metabolites, [PE+GPE], provided the best discrimination of individual benign from low-grade prostate cancer tissues. While the increase choline phospholipid metabolites, [PC+GPC], provided the best discrimination of high- and low-grade prostate cancer tissues. Interestingly, while the increase in [PC] in high- versus low-grade prostate cancer approached being significant ($p=0.082$), it was the significant increase in [GPC] that provided the discrimination of high-grade prostate cancer. Since the *in vivo* total choline resonance is dominated by the choline head group singlets (9 equivalent protons) of free choline (Cho), phosphocholine (PC), and glycerophosphocholine (GPC), it is not surprising that high grade prostate cancer can be more accurately detected by *in vivo* ^1H MRSI. Specifically, it has been shown that sensitivity of *in vivo* prostate ^1H MRSI was 56% for tumor detection, increasing from 44% in lesions with Gleason score of 3+3 to 89% in lesions with Gleason score greater than or equal to 4+4. The ability to discriminate between the Gleason subscores, 3+4 versus 4+3, has clinical significance, since it can be used to predict different outcomes in men with clinically localized prostate cancer (40,41).

The [PC]/[GPC] ratio in the human prostate cancer tissues studied was much less than 1 which is in agreement with a prior TOCSY study of surgical human prostate tissues (20) but in disagreement with prior studies of culture immortalized (17) and primary human prostate cancer cells (19), in which [PC] was the dominant phospholipid and [PC]/[GPC] ratio was much greater than 1. The low concentrations of PC relative to GPC observed in high grade human prostate cancer tissues is also consistent with ^1H HR-MAS spectra of low grade glioma tissues in which GPC was the dominate phospholipid as compared to high grade gliomas where PC dominated (42).

The phospholipid metabolite differences observed between studies of cultured human prostate cancer cells versus tissues are due in part to the lack of stromal-epithelial interactions present in intact tissues but absent in cell cultures. Stromal-epithelial

interactions are known to be extremely important in normal prostate biology, in the development and progression of prostate cancer (43,44), and its response to therapy (45). The lack of tumor microenvironmental factors in prostate cancer cell cultures also contribute to their unnaturally rapid mitotic rate relative to the human situation (17,19). Additionally, metabolite levels in cell culture are effected by nutrient supply and culture conditions, which may not reflect the *in vivo* situation (17,19). In this study, the mean percent of cancer cells staining positive for Ki-67 was significantly increased between benign tissue and low-grade cancer (0.07 ± 0.27 versus 2.4 ± 2.8 , $p < 0.01$) and between low- and high-grade cancers (2.4 ± 2.8 versus 7.6 ± 3.5 , $p < 0.005$). However, Ki-67 staining for even high grade prostate cancer was dramatically less than the 50 to 90% Ki-67 staining observed for human prostate cancer cells in culture (17,19,46). This low level and variability of Ki-67 staining in human prostate cancer tissue is consistent with two published studies, in which low- and high-grade prostate cancer tissues had $2.0 \pm 3.3\%$ and $3.0 \pm 3.1\%$ mean Ki-67 staining (21), and $8.15 \pm 1.13\%$ and $16.86 \pm 3.61\%$ mean Ki-67 staining, respectively (9). A significant correlation of Ki-67 staining with tissue type (benign, low- and high-grade cancer) was also observed in this study, but there was no correlation observed between Ki-67 and tissue phospholipid metabolite concentration. The lack of correlation between phospholipid metabolite levels and proliferation is consistent with published studies of prostate and breast cancer (17,47), and suggests that the elevation of the *in vivo* total choline peak observed in prostate cancer patients is attributable to an alteration of phospholipid metabolism and not simply to increased proliferation.

The staging of prostate cancer is important because it contributes both to predicting prognosis and planning treatment. In this study, phospholipid metabolite concentrations and mean Ki-67 staining were higher in cancer samples taken from patients with extracapsular extension (stage T3A) and seminal vesicle invasion (stage T3B) as compared to those with localized disease at surgery (stage T1 and T2). While using the results of a localized sample to predict a patient level out-come such as cancer stage is not optimal, it can add additional predictive ability to MRI. For example, men with a Gleason pattern 4 in any of their biopsies, are assumed to be of higher risk for cancer spread, and are treated more aggressively (7,8). The results of this study suggest that high phospholipid metabolite concentrations and increased Ki-67 staining from a biopsy may increase the ability of MRI to predict disease stage. Two *in vivo* studies have suggested that the addition of MRSI to MRI data can significantly improve prostate cancer staging. In one study of 53 patients with early stage prostate cancer, tumor volume estimates based on MRSI findings were combined with MRI criteria (48) in order to assess the ability of combined MRI/MRSI to predict extracapsular cancer spread. It was found that tumor volume per lobe estimated by MRSI was significantly ($p < 0.01$) higher in patients with ECE than in patients without ECE. Moreover the addition of a MRSI estimate of tumor volume, based on elevated choline plus creatine to citrate ratios (Cho+Cr/Cit), to MRI findings for ECE (49) improved the diagnostic accuracy and decreased the inter-observer variability of MRI in the diagnosis of extracapsular extension of prostate cancer (48). In another study of 383 prostate cancer patients prior to radical prostatectomy, 1.5T MRI/ ^1H MRSI data were added to a biopsy based staging nomogram for predicting organ-confined prostate cancer (OCPC, no ECE or SVI) and provided a significant improvement in predictive accuracy (50). The results of the current exam support the potential role of *in vivo* ^1H MRSI for improved staging prostate cancer since elevated total choline is associated with higher grade, more proliferative, later stage prostate cancer.

This study had several limitations. The primary limitation of the clinical utility of the phospholipid concentration, and Ki-67 findings is that, although the changes with pathologic grade and stage are large, there is significant overlap between individual patient values. This overlap is in part due to the true biologic heterogeneity of prostate cancer(51). We attempted

to overcome the subjectivity of Gleason scoring by averaging duplicate readings of two prostate pathologists with extensive experience, but a more quantitative pathologic approach would be important in future studies(52). Another limitation of this study was the variability of percent cancer within the tissue samples used. This is an unfortunate consequence of the fact that many men undergoing surgery have very small amounts of prostate cancer. We found that correcting the samples for percent cancer was problematic for samples containing small amounts of prostate cancer. Most likely the differences between benign, low and high grade tissues would be even more significant if samples could be obtained with higher percentages of cancer and the phospholipid metabolite concentration were corrected for the percentage of cancer in the sample. The use of tissue level phospholipid metabolite concentration and Ki-67 findings to predict prostate cancer stage was also limited by not utilizing MR targeted tissues to ensure that the index or dominate tumor was sampled. Recent FDA approved MR guided biopsy approaches(53) could ensure the collection of higher percentages of cancer from the dominant lesion in the prostate for tissue level metabolic, pathologic, genomic and proteomic data, thereby improving future studies of metabolic biomarkers. The findings of this study also need to be validated in a larger cohort of surgical patients with clinical outcome data.

CONCLUSION

The results of this study demonstrate that high-grade prostate cancers, i.e. cancers having a dominant Gleason pattern 4, have significantly higher Ki-67 staining and concentrations of PC and GPC. Clinically, cancers with a dominant Gleason pattern 4 are considered to be more aggressive with a high likelihood of metastases, and in this study, near significantly higher Ki-67 and phospholipid concentrations in cancer samples taken from patients with extracapsular extension and seminal vesicle invasion as compared to those with localized disease at surgery. The study also demonstrated that phospholipid metabolism and proliferation in prostate cancer tissue is different from human prostate cancer cells in culture, presumably due to a lack of critical stromal-epithelial interactions in cell cultures. Specifically, PC and Ki-67 was dramatically lower and GPC higher in tissues as compared to cell cultures. The findings of this study will need to be tested in a larger cohort of surgical patients with clinical outcome data. However, the results look promising and support the role of *in vivo* ^1H MRSI to discriminate low- from high-grade prostate cancer based on the magnitude of elevation of the *in vivo* total choline resonance.

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The authors would like to dedicate this work to the memory of Dr. Andrew S. Zektzer, a gifted scientist, colleague and friend.

Abbreviations

HR-MAS	High-resolution magic angle spinning
^{31}P	NMR active isotope of phosphorus
1D	One-dimensional
T_R	repetition time
S/N	Signal-to-noise ratio

TNM Stages	Tumor classification system where T describes the size of the tumor and invasion, N describes the lymph nodes involved and M describes distant metastasis
PSA	prostate specific antigen
TSP	3-(trimethylsilyl)propionic-2,2,3,3-d4 acid
H&E	hematoxylin and eosin
Cho	Choline
PC	Phosphocholine
GPC	Glycerophosphocholine
Eth	Ethanolamine
PE	Phosphoethanolamine
GPE	Glycerophosphoethanolamine

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Figure 1.

A comparison of representative benign prostate tissues (40% glandular, 60% stromal, Ki-67-negative, PC: 0 millimolal, GPC: 0.24 millimolal), low-grade (50% Gleason score 3+3, Ki-67 < 5 %, PC: 0.17 millimolal, PC+GPC: 0.63 millimolal) and high-grade (40% Gleason 4+4, Ki-67: >10% PC: 426 PC+GPC: 5.3) grade prostate surgical tissues; (A) one dimensional ^1H HR-MAS spectra, (B) Corresponding upper diagonal $\text{CH}_2\text{-CH}_2$ side chain region of the two dimensional ^1H HR-MAS TOCSY spectra. (C) Corresponding Ki-67 and H&E stained histology. All scales are in scales are in ppm.

Figure 2.

Quantitative comparison of the changes in phospholipid metabolite concentrations, (A) [PE + GPE], (B) [PC + GPC], and (C) total phospholipid ([PE + GPE + PC + GPC]) between benign, low- and high-grade prostate cancer. It is visually clear in the box plots of the phospholipid metabolite concentrations that while there are significant differences between the mean ratios (green lines) of all three phospholipid metabolite combinations and tissue types, there is overlap of individual values. The best discrimination of benign and low-grade prostate cancer was provided by [PE + GPE], while [PC + GPC] provided the best discrimination of low- and high-grade prostate cancer. GS – Gleason score.

Figure 3.

A plot demonstrating the % of benign, low- and high-grade prostate cancer samples having increasing levels of Ki-67 antigen staining (unstained, < 5%, 5–10%, >10%) with indicated Gleason score. Although there was overlap in Ki-67 antigen staining of individual tissues between the three tissue types, there was significantly relationship between tissue type and Ki-67 staining.

Table 1

Comparison of Choline and Ethanolamine Phospholipid Metabolite Concentrations Between Benign, Low- and High-Grade Prostate Cancer

A. Comparison Cancer and Benign Tissues

Metabolite	Benign (N=14)	Cancers (N=35)	p-Value
[PC]	0.003 ± 0.009	0.049 ± 0.116	0.013
[GPC]	0.260 ± 0.190	1.07 ± 1.44	< 0.005
[Eth]	1.10 ± 0.702	0.599 ± 0.525	0.025
[PE]	0.749 ± 0.701	1.51 ± 0.97	< 0.005
[GPE]	0.277 ± 0.433	0.988 ± 1.06	< 0.005
[PC+GPC]	0.263 ± 0.187	1.12 ± 1.45	< 0.005
[PE+GPE]	1.03 ± 0.893	2.50. ± 1.52	< 0.005
[PC+GPC+PE+GPE]	1.29 ± 0.864	3.62 ± 2.53	< 0.005

B. Low- and High-Grade Cancer, and Benign Comparison

Metabolite (millimolal)	Cancer (3+4) (N=22)	Cancer (4+3) (N=13)	p –Values *, **, ***
[PC]	0.028 ± 0.063	0.084 ± 0.170	0.452, 0.035 , 0.082
[GPC]	0.643 ± 0.475	1.79 ± 2.13	0.331, 0.001 , 0.006
[Eth]	0.661 ± 0.601	0.495 ± 0.356	0.031 , 0.009 , 0.418
[PE]	1.48 ± 1.00	1.55 ± 0.935	0.023 , 0.025 , 0.810
[GPE]	0.872 ± 0.945	1.87 ± 1.27	0.070 , 0.016 , 0.348
[PC+GPC]	0.671 ± 0.461	1.87 ± 2.15	0.3033, < 0.005 , < 0.005
[PE+GPE]	2.35 ± 1.66	2.74 ± 1.24	0.007 , 0.002 , 0.425
[PC+GPC+PE+GPE]	3.03 ± 1.97	4.62 ± 3.09	< 0.005 , 0.021 , 0.037

Free choline (Cho) was not included because it co-resonates with myo-inositol.

P-values in bold are significant

* low grade versus benign

** high grade versus benign

*** high grade versus low grade