Monitoring Tumor Response of Prostate Cancer to Radiation Therapy by Multi-parametric 1H and Hyperpolarized 13C Magnetic Resonance Imaging

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by

Vickie Yi Zhang

DISSERTATION

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by

Vickie Yi Zhang
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Yi (Vickie) Zhang
January 9th, 2014
Abstract

Monitoring tumor Response of Prostate Cancer to Radiation Therapy by Multi-parametric
$^1$H and Hyperpolarized $^{13}$C Magnetic Resonance Imaging

By

Vickie Yi Zhang

Radiation therapy is one of the most common curative therapies for patients with localized prostate cancer, but despite excellent success rates, a significant number of patients suffer post-treatment cancer recurrence. The accurate characterization of early tumor response remains a major challenge for the clinical management of these patients. Multi-parametric MRI/$^1$H MR spectroscopy imaging (MRSI) has been shown to increase the diagnostic performance in evaluating the effectiveness of radiation therapy. $^1$H MRSI can detect altered metabolic profiles in cancerous tissue. In this project, the concentrations of prostate metabolites from snap-frozen biopsies of recurrent cancer after failed radiation therapy were correlated with histopathological findings to identify quantitative biomarkers that predict for residual aggressive versus indolent cancer. The total choline to creatine ratio was significantly higher in recurrent aggressive versus indolent cancer, suggesting that use of a higher threshold tCho/Cr ratio in future in vivo $^1$H MRSI studies could improve the selection and therapeutic planning for patients after failed radiation therapy.
Varying radiation doses may cause a diverse effect on prostate cancer micro-environment and metabolism, which could hold the key to improving treatment protocols for individual patients. The recent development and clinical translation of hyperpolarized $^{13}$C MRI have provided the ability to monitor both changes in the tumor micro-environment and its metabolism using a multi-probe approach, [$^{1}$-$^{13}$C]pyruvate and $^{13}$C urea, combined with $^{1}$H Multi-parametric MRI. In this thesis, hyperpolarized $^{13}$C MRI, $^{1}$H dynamic contrast enhancement, and diffusion weighted imaging were used to identify early radiation dose response in a transgenic prostate cancer model. Hyperpolarized pyruvate to lactate metabolism significantly decreased in a dose dependent fashion by 1 day after radiation therapy, prior to any changes observed using $^{1}$H DCE and diffusion weighted imaging. Hyperpolarized $^{13}$C urea and $^{1}$H DCE both show increase in perfusion/permeability by 4 days post-radiation. In tumor region treated with high dose radiation, ADC values significantly increased post-radiation, suggesting a decrease in cellular density. These dose dependent changes can be used as markers of early tumor response to the impact of increasing doses of radiation therapy. In addition, a spectral-spatial pulse sequence was developed for the 14T to dynamically observe kinetic information in a transgenic prostate cancer model before and after radiation therapy. A novel modeling approach was proposed to parameterize perfusion in the kinetic modeling of pyruvate to lactate conversion for better characterization of pyruvate metabolism. Unlike single time point HP $^{13}$C urea imaging, quantitative pharmacokinetic parameters such as blood flow and extracellular extravascular volume fraction can be extracted from dynamic acquisitions. Blood flow measured by hyperpolarized $^{13}$C urea was highly correlated with $K^{\text{trans}}$ measured by $^{1}$H DCE, suggesting hyperpolarized urea might be able to provide similar information as $^{1}$H DCE.
The results of this thesis show that Multi-parametric MRI, including functional MRI, $^1$H MRSI, and hyperpolarized $^{13}$C, holds great potential for evaluating early tumor response to radiation therapy of prostate cancer. The findings of this thesis will be useful in designing future studies for using combined Multi-parametric $^1$H and hyperpolarized $^{13}$C MRI to improve planning and assessing radiation therapy in individual prostate cancer patients.
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Chapter 1: Introduction

Radiation therapy is one of the most common curative therapies for localized prostate cancer(1). However, despite the excellent success rates, a significant number of patients suffer post-treatment local cancer recurrence(2,3). Additionally, several dose-escalation trials have shown improved local and biochemical disease control for patients with localized prostate cancer(3-7). Hypofractionated radiation therapy regimes that deliver larger radiation doses per fraction has been suggested to be more biologically advantageous for treating prostate cancer, but they could be greatly improved by a non-invasive imaging approach that could titrate the consequences of such a dose escalation on both the tumor micro-environment (perfusion, cellularity) and metabolism.

Multi-parametric MR imaging examination, including diffusion weighted (DWI) imaging, dynamic contrast enhancement imaging (DCE), and proton MR spectroscopy (¹H MRSI), has demonstrated great potential for improving evaluation of response to radiation therapy in prostate cancer patients. Preclinical studies of prostate cancer have shown a radiation dose dependent increase in ADC early after therapy(8,9). Clinical studies have shown that although water ADC values are increased in both regions of recurrent cancer and benign treated tissues after radiation treatment, ADC values were still significantly lower in regions of recurrent prostate cancer (1.0 x 10⁻³ mm²/s) relative to surrounding regions of benign treated tissues (1.6 x 10⁻³ mm²/s)(10,11). Clinical DCE study shows that prostate cancer recurrence after failed radiation therapy is characterized by earlier and faster enhancement and earlier contrast material washout comparing...
to the surrounding treated benign tissue(12). Haider et al(13) found DCE to be more accurate than T2 weighted imaging at detecting cancer recurrence after radiation therapy. 1H MRSI can non-invasively detect abnormalities of metabolism that occur in prostate cancer. Clinical studies have shown the addition of metabolic information provided by 1H MRSI to that obtained by MRI can significantly improve the ability of MRI to local cancer within the prostate, assess cancer aggressiveness, and evaluate response to radiation therapy. Hyperpolarized 13C MRI is a revolutionary new molecular imaging technique with an increase in signal intensity of 10,000-fold that can be used to monitor perfusion, uptake, and metabolism of endogenous molecules(14,15).

The goal of this dissertation project was to improve the assessment of the micro-environmental and metabolic response of prostate cancer to a range of radiation doses, ranging from ablative to non-lethal, using Multi-parametric MR, including DCE, diffusion weighted imaging, 1H MRSI, and hyperpolarized 13C imaging. The first project identifies 1H MR biomarkers that could discriminate recurrent proliferative from indolent/dying prostate cancer following radiation therapy. The next project focuses on determining the early radiation dose response in a transgenic prostate tumor model using Multi-parametric 1H and hyperpolarized 13C MRI. The last project develops a pulse sequence for the 14T scanner to study dynamic changes in perfusion and metabolism in prostate tumors early after radiation therapy. This dissertation is organized in the following manner:

In Chapter 2, I provide the rationale for the studies conducted by reviewing the clinical rationale for radiation therapy of prostate cancer, indicating some of the current limitations in treatment
and therapeutic monitoring. The recent advancements in radiation therapy, including technical developments and new insights on radiation therapy regimens for prostate cancer are also discussed.

In Chapter 3, I present the basic physics of MRI and different MR methods including DCE, DWI, $^1$H MRSI, and hyperpolarized $^{13}$C imaging. An overview of the diagnostic performance of these methods in evaluating prostate cancer response to radiation therapy is provided.

In Chapter 4, I investigate the correlation between metabolite concentrations from snap-frozen, prostate cancer patient biopsies of residual cancer after radiation therapy with histopathological findings, including pathologic grade and Ki-67 immunohistochemistry, to identify quantitative metabolic biomarkers that predict for residual aggressive versus indolent/dying cancer.

In Chapter 5, I probe the early radiation dose dependent response in a transgenic prostate cancer model using both $^1$H MR imaging, including DCE and diffusion weighted imaging, and hyperpolarized $^{13}$C imaging, including $[1-^{13}$C]pyruvate and $^{13}$C urea. I also correlate the Multi-parametric MRI data with histopathology and gene expression analysis to better understand the mechanisms underlying the observed changes in the imaging parameters.

In Chapter 6, I describe a spectral-spatial excitation pulse sequence for the 14T scanner to study the real time flux of $[1-^{13}$C]pyruvate to $[1-^{13}$C]lactate, and obtain a dynamic measure of perfusion using hyperpolarized $^{13}$C urea. Each excitation pulse was followed by a flyback echo planar readout. The total pulse was kept at 6.7 ms to accommodate the short $T_2^*$ effect. Variable
flip angle scheme was individually designed for each resonance to optimize SNR while considering signal decay due to hyperpolarized $T_1$ relaxation decay, prior RF excitations, and metabolic conversion. The sequence was used in conjunction with the same TRAMP radiation model used in Chapter 5 to monitor dynamic changes in perfusion and metabolism with radiation dose. The value of this information was compared with perfusion measured by $^1$H DCE.

Reference:


Chapter 2: Radiation Therapy of Prostate Cancer

Clinical Prostate cancer

The clinical stage of prostate cancer is essential in management of patients(1). The most important clinical question is whether the disease is pathologically organ-confined or non-organ-confined. The goal of staging patients with prostate cancer is to find out how far the cancer has spread to evaluate prognosis and determine treatment options. All patients are first clinically staged, through a combination of information such as serum PSA, digital rectal examination (DRE), prostate biopsy results (including Gleason score), and imaging results. Pathologic stage is a much more accurate assessment of the extent of cancer, which is based on surgery and examination of the removed tissue for histological evaluation of pelvic lymph nodes and the prostate. Essentially, pathologic stage differentiates between organ-confined or non-organ-confined disease. Radiation therapy can be used both as a curative treatment for disease that has not spread outside the gland, or as a palliative treatment for advanced cancer to provide relief from symptoms. As a salvage therapy, radiation therapy is used when cancer recurs in the area of the prostate after surgery or first round radiation therapy.

The Gleason scoring system is used to grade prostate cancer based on its microscopic appearance from prostate biopsy samples(2). Since an individual prostate cancer lesion can have multiple grades of cancer, the highest two grades are reported. The primary pattern represents the dominant tissue pattern in the tumor, and the secondary pattern represents the next most frequent pattern (primary + secondary Gleason pattern). Each pattern is given a grade from 1 to 5, with 1 looking the most like normal prostate tissue and 5 looking the most abnormal(3). As grade
increases, cells invade or infiltrate the surrounding tissue and lose glandular architecture. Pattern 1 corresponds to a well-differentiated carcinoma, pattern 2 and 3 correspond to moderately differentiated carcinoma, and pattern 4 and 5 correspond to poorly differentiated carcinoma. Prostate cancer of Gleason pattern 1 and 2 are rarely seen. Prostate cancers with a primary Gleason 4 pattern are more aggressive and have a worse prognosis after any therapy including radiation therapy(4). A hypoxic tumor microenvironment within the high grade prostate cancer has been postulated to have an important impact in selectively promoting the survival of a more aggressive cell phenotype and inducing radioresistance(5). Additionally, some prostate cancers can grow and spread quickly, but most grow slowly. Prostate tumors, even the high-grade tumors, contain smaller fractions of cycling cells compared to other cancer types.

There are several treatment options for patients with clinically localized prostate cancer, including active surveillance, surgery, radiation therapy, and hormone therapy(1,4). Active surveillance is closely monitoring patient’s condition without giving any treatment unless there are symptoms or changes in test results. During active surveillance, patients are given certain exams and tests, including DRE, PSA test, transrectal ultrasound, and transrectal needle biopsy. When the cancer begins to grow, treatment is given to cure the cancer. Surgery is the most common aggressive therapy for the treatment of prostate cancer if the disease has not spread outside the gland. Radical prostatectomy is the main type of surgery, which consists of removing the entire prostate, surrounding tissue, and seminal vesicles. Another common treatment for prostate cancer is radiation therapy, which uses high-energy rays or particles to kill cancer cells. There are two main types of radiation therapy: external beam radiation and brachytherapy (internal radiation)(6). Hormone therapy, also called androgen deprivation therapy, reduces the
levels of male hormones to prevent them from stimulating prostate cancer cells to grow. Lowering androgen levels often makes prostate cancers shrink or grow more slowly. However, hormone therapy also does not cure prostate cancer and eventually, it stops helping when prostate cancer becomes hormone resistant. Hormone therapy is often used along or prior to radiation therapy to make the treatment more effective.

**Fundamentals of Radiation therapy**

Radical prostatectomy and radiation therapy are the most common curative treatments for patients with disease confined to the prostate and adjacent tissues. Radiation therapy is often the preferred treatment for patients with high-risk factors such as PSA > 30 ng/dl, Gleason score > 7 (either 3+4 or 4+3). Furthermore, patients with pre-existing medical conditions such as obesity, diabetes mellitus, or cardiovascular disease are limited to radiation therapy. The risks of surgery include higher incidences of incontinence and impotence, leading some men to select radiation therapy, based on quality of life considerations(6).

Radiation therapy uses high-energy radiation to kill cancer cells and thereby locally control the lesion. Ionizing radiation can inflict cellular damage directly or indirectly. The Direct Action theory refers to the direct effects of radiation energy on cells. Damages caused by the direct effects of radiation take place within fractions of a microsecond. These direct effects result in tumor tissue ablation. Cell death due to necrosis and apoptosis is a typical pathological feature following ablative radiation therapy. High doses of radiation may increase lysis of tumor cells, which would result in break down of the plasma membrane, the cytoplasmic contents, including
lysosomal enzymes are released into the extracellular fluid(7). Single doses of 10-15 Gy have been shown to inhibit the growth of tumor cells by releasing the pro-apoptotic second-messenger ceramide from sphingomyelin in membranes of endothelial cells. The ceramide release was mediated by acid sphingomyelinase (asmase) and resulted in apoptosis of endothelial cells(8). Preliminary evidence suggested that endothelial apoptosis might radiosensitize tumor cells by interfering with DSB repair(9). High dose radiation of the microvasculature may lead to adhesion of platelets to endothelial cells with formation of thrombi(10).

The Indirect Action theory refers to the formation of DNA lesions produced by the free radicals. Free radicals are formed when free moving electrons from radiation interact with water molecules within the tissues. These free radicals react with molecules within the cell to cause damage(11). These chemical reactions are enhanced by the presence of oxygen. The half-life of the free radicals is short so the damages that they induce are limited to the path of the radiation and would not spread to any great extent through the tissues. It is generally accepted that the most important target for radiation-induced damage is the cellular DNA. While ionizing radiation is absorbed in random locations, there are multiple copies of most cellular molecules, but DNA is present in only two copies. Also, a number of experiments have demonstrated higher radiosensitivity of the nucleus relative to the cytosol following irradiation(12). Cell membrane may be another important target for radiation-induced damage caused by both direct action and free radicals formed from indirect action(13). While a portion of the cancer cell death can occur early after radiation damage, the majority of radiobiological cell death occurs after the cells attempting mitosis one or more times. This is called mitotic catastrophe. Cells can also enter radiation-induced senescence, which is a state of permanent cell cycle arrest.
The amount of energy deposited by ionizing radiation per unit mass is defined as the radiation dose received by a tissue, which in SI units is given in Gray (Gy). The absorption of 1 Gy of radiation will give rise to approximately $10^5$ ionizations per cell. This quantity is associated with the amount of biological damage, which is described by the linear quadratic (LQ) equation:

$$p = e^{-(\alpha D + \beta D^2)}$$

Where $p$ is the survival fraction. $D$ is the total dose. $\alpha$ and $\beta$ are constants, descriptive of the linear and quadratic components of the equation, respectively. The dose-cell survival relationship has been characterized through in vitro studies measuring the proportion of surviving cells, or survival fraction, after irradiation. Figure 2.1 shows the dose-cell survival curve with a low $\alpha/\beta$ ratio (less than 3). The shape of any cell survival curve is determined by the $\alpha/\beta$ ratio. The unit of the $\alpha/\beta$ ratio is Gy. Low $\alpha/\beta$ ratios are associated with slowly dividing tissues, which show late reactions to radiation. The initial slope of the dose-cell survival curve ($\alpha$) represents the intrinsic radiosensitivity of the cell, and is a non-repairable type of damage. It is linearly dependent on dose. The curvature of the dose-cell survival curve ($\beta$) represents a repairable type of injury with time, responsible for the dose-per-fraction and dose-rate variations in radiobiology. It is proportional to the square of the dose.
Two most important biological factors that affect the effects of radiation are oxygenation and the cell cycle. Multiple experiments have shown oxygen enhances the cellular damage caused by the radiation-induced free radicals. Hypoxia reduces the development of damaging free radicals and thus the degree of radiation damage. Cells undergoing mitosis (M phase) and cells in G3 phase are the most sensitive to radiation, while cells at the end of DNA synthesis (late S phase) are the most radioresistant (14,15). In general, cancer cells have high \( \alpha/\beta \) ratio and are proliferating very fast (not true for prostate cancer) and therefore, more susceptible to radiation.

Conventional radiation therapy takes the advantage of the fact that cancer cells have a high \( \alpha/\beta \) ratio by delivering the doses in fractions. In principle, it increases the chance of catching a tumor
cell at a radiosensitive stage of the cell cycle, by killing sensitive cells early and establishing cell cycle synchronization. Fractionation allows radiation to target cancers with high $\alpha/\beta$ ratios while sparing normal tissues with low $\alpha/\beta$ ratios. This process is called re-assortment. However, the biological effectiveness of a given dose of radiation in general is less if it is divided between several fractions. Therefore, to produce the same dose effect, using fractionation, a greater total dose is required.

$$BED = nd(1 + d / [\alpha/\beta])$$

A Biologically Effective Dose (BED) can be calculated for any dose per fraction $d$ (and for a total dose $nd$ where $n$ denotes the number of fractions), if a value for the appropriate tissue $\alpha/\beta$ ratio is assumed.

**Hypofractionation for prostate cancer**

 Unlike most of types of cancer, prostate cancer has a very low $\alpha/\beta$ ratio(16), because it has the longest tumor cell population doubling time of any human cancer, from 15 to more than 70 days. Most other carcinomas have median cell doubling time of 4 – 9 days. Rapidly proliferating types of cancer have high $\alpha/\beta$ ratios of 10 Gy or higher. Many clinical studies have shown the $\alpha/\beta$ ratio for prostate cancer is approximately 1.5 Gy(17). Therefore, while it is logical to treat most cancers with a large number of small doses of radiation therapy to keep damage to normal tissues low, hypofractionation with a larger dose per fraction is a more biologically advantageous treatment regimen for prostate cancer. Since the $\alpha/\beta$ ratio for prostate cancer is actually significantly below that for late rectal complications ($\alpha/\beta = 3$ Gy), the hypofractionated schedules can be designed to keep rectal complications low and constant, and also provide a
greater cancer cell kill. Table 2.1 provides calculated rectal complications and cancer cell kills for different hypofractionation schedules using the BED equation. The results demonstrate that increased tumor cell kill can be achieved while reducing the total dose and keeping rectal complications constant and relatively low.

<table>
<thead>
<tr>
<th>Hypofractionated Schedule</th>
<th>Total Dose (Gy)</th>
<th>Rectal BED Gy3 for α/β = 3 Gy</th>
<th>Late rectal Complications NTD2Gy (α/β = 3Gy)</th>
<th>Calculated NTD2Gy (α/β = 1.5Gy)</th>
<th>Estimated bNED</th>
</tr>
</thead>
<tbody>
<tr>
<td>37F × 2.09</td>
<td>74.0</td>
<td>123.3</td>
<td>74.0 Gy</td>
<td>74.0 Gy</td>
<td>75.5%</td>
</tr>
<tr>
<td>25F × 2.69</td>
<td>65.73</td>
<td>123.3</td>
<td>74.0</td>
<td>78.7</td>
<td>82.8</td>
</tr>
<tr>
<td>20F × 3.06</td>
<td>61.11</td>
<td>123.3</td>
<td>74.0</td>
<td>79.6</td>
<td>84.0</td>
</tr>
<tr>
<td>15F × 3.69</td>
<td>55.33</td>
<td>123.3</td>
<td>74.0</td>
<td>82.0</td>
<td>87.3</td>
</tr>
<tr>
<td>10F × 4.77</td>
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<td>123.3</td>
<td>74.0</td>
<td>85.4</td>
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</tr>
<tr>
<td>5F × 7.3</td>
<td>36.16</td>
<td>123.3</td>
<td>74.0</td>
<td>90.2</td>
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<td>29.10</td>
<td>123.3</td>
<td>74.0</td>
<td>93.1</td>
<td>95.8</td>
</tr>
</tbody>
</table>

Table 2.1. Hypofractioned schedules calculated for external beam radiation therapy for constant late rectal complications assuming α/β = 3 Gy. New total dose = 74*(1+2/3)/(1+d/3) for the constant late BED of 123.3 Gy. NTD2Gy = normalized total dose, the schedule using 2 Gy fractions that would give the same log cell kill. The final two columns also present the consequent prostate tumor effects, as normalized total dose (NTD2Gy in 2 Gy fractions) assuming α/β = 1.5 Gy; and the 5 year percentage bNED (biochemical non-evidence of disease) estimated from prior clinical data from intermediate risk prostate tumors. Adapted from Fowler JF. The radiotherapy of prostate cancer including new aspects of fractionated radiotherapy. Acta Oncol 2005;44(3):265-276.

Figure 2.2 shows the graphical representation of increasingly more effective tumor doses can be delivered while a constant level of late complications is maintained(18). The ratio of the EQD2 (equivalent dose delivered in 2 Gy fractions) doses for tumor (assuming α/β of 1.5) to late normal tissue (assuming α/β = 3) is plotted as a function of increasing fraction size, while limiting total dose to maintain acceptable late complication risks. However, some analyses have suggested that the LQ model under-predicts the total dose effect for larger fraction sizes, such as larger than 6 Gy per fraction, resulting in a less toxic but also less effective treatment(19). Some
factors such as reoxygenation and redistribution are particularly relevant as the total number of fractions in a treatment course decreases.

Figure 2.2. Increasing therapeutic advantage with increasing hypofractionation. The equivalent total doses if delivered in 2 Gy fractions for prostate tumor (\(\alpha/\beta = 1.5\)) and normal tissue late effects (\(\alpha/\beta = 3\)) are shown versus fraction size-number combinations that preserve similar late effect levels. A reduction in total dose is required with increasing hypofractionation to maintain similar predicted late effects. The difference between the solid lines and dotted extensions on the right indicate in non-quantitative fashion a potential, over-prediction of biological effect by the linear quadratic model for very large fraction sizes. Adapted from Ritter M, Forman J, Kupelian P, Lawton C, Petereit D. Hypofractionation for prostate cancer. Cancer J 2009;15(1):1-6.
Advancements in radiation therapy for prostate cancer treatment

Significant technical advancements have been made in the definitive use of various forms of radiation therapy in the management of clinically localized prostate cancer. The conventional radiation therapy, or external beam radiation therapy, employs the standard dose fractionation schemes with fraction sizes of about 2 Gy and total dose of approximately 70-80 Gy. More than 50% of patients with clinically localized prostate cancer experience biochemical failure following conventional radiation therapy.(20,21) 3D conformal radiation therapy utilizes 6-field 3D conformal techniques to shape the radiation dose distribution to the tumor volume in three dimensions. Based on the CT images, special software can precisely map the location of the prostate to assure that specified normal tissue tolerances are accounted for. This technique allows modest dose escalation in the tumor volume and dose reduction in normal tissues. To further control the dose distribution within the tumor volume and surrounding normal tissues, Intensity-modulated radiation therapy (IMRT) uses a computer-driven machine that rotates around the patient as it delivers radiation. The edges of the irradiating field are shaped to conform to the shape of the tumor volume, and the radiation dose distribution within the treated volume is modulated. In addition, the intensity of the beams can also be adjusted to minimize the dose reaching to the surrounding normal tissues. Imaging scanners have been built into the IMRT machines to provide clinicians image guidance to assure the precise overlap of the delivered dose distribution to the tumor volume, as it is position in the patient. Hypofractionation clinical trials using these advanced external beam radiation techniques have achieved an increased dose per fraction of 2-5 Gy with a reduced number of fractions. Assuming tumor $\alpha/\beta$ ratio of 1.5 Gy, the equivalent doses in 2 Gy fractions were calculated to be between 80 to 90 Gy, which is higher than the conventional external beam radiation therapy. These treatments were generally well
tolerated, with low reported toxicity. Similar 5-year biochemical control results were obtained from these treatments comparing to the standard dose fractionation schemes of 2 Gy per fraction(22).

Extreme hypofractionation can be accomplished by brachytherapy. Brachytherapy delivers radiation internally by placing the radioactive seeds within the tumor volume. The small radioactive seeds are about the size of a grain of rice. Prostate brachytherapy is achieved by interstitial placement of radioactive seeds into the prostate through thin, hollow surgical needles. The technique is performed under trans-rectal ultrasound (TRUS), CT, or MRI guidance for accurate placement of the needle applicators(18). Because the radioactive seeds are placed inside of the target volume, very high doses are delivered to the tissues next to the seeds. The radiation intensity drops exponentially with distance due to both tissue attenuation and geometric dose fall-off from the source (1/distance squared). As long as the placement of the seeds is correct, very high doses can be delivered to the tumor and a minimal dose given to the surrounding normal tissues. Accurate and precise placement of the seeds is thus extremely important to ensure the tumors get irradiated, not the normal tissues. There are two types of brachytherapy – Low dose rate (LDR) brachytherapy and high dose rate (HDR) brachytherapy(6,18). For LDR brachytherapy, the radioactive seeds (Pd-103 or I-125) are placed permanently inside the prostate. Pd-103 has a half-life of 17 days while I-125 has a half-life of 60 days. Therefore, the source can stay radioactive for months inside the patients. Clinical trials performed using LDH brachytherapy reported equivalent dose in 2 Gy fractions administered to the patients to be over 140 Gy. Biochemical and clinical tumor control appears to be as effective as after radical prostatectomy or conventional external beam radiation therapy in early prostate cancer(6). For
HDR brachytherapy, the radioactive seeds (Ir-192 or Cs-137) are placed temporarily in the prostate to deliver high dose per fraction radiation treatments. A variety of treatment schedules have been performed in clinical trials(23). Dose escalation trials used HDR brachytherapy as a boost in conjunction with external beam radiation therapy. Increasingly large fractions of HDR treatment, ranging from 5.5-6.5 Gy (dose per fraction) × 3 (number of fractions) to 8.25-11.5 Gy × 2 fractions, combined with 46-50 Gy of external beam radiation therapy. A clinical trial used the schedule of 2 Gy × 25 fractions external beam radiation therapy with two HDR brachytherapy boost doses of 10 Gy, an equivalent dose in 2 Gy fractions of 116 Gy was achieved in the tumors while only 71 Gy to the rectum, because their brachytherapy dose to the rectal wall does not exceed 60% of the tumor-prescribed dose(17,24). Patients selected for the combination treatment are generally those at intermediate-to-high risk who may benefit from dose escalation(25). There also had been interest in developing HDR monotherapy for patients with early-stage prostate cancer. Multiple clinical studies with different schedules have demonstrated the feasibility of this approach, including 6 Gy × 8-9 fractions, 8.5 Gy × 4 fractions, 9 Gy × 4 fractions, 10.5 × 3 fractions, and etc. Early results suggest an excellent biochemical control and no difference in the acute and late toxicity comparing to conventional external beam radiation therapy(26). S. Nilsson et al. at the Karolinska Institute, Stockholm and B. Lennernas et al. at the Sahlgrenska Hospital, Goteborg developed a schedule consists of 15 Gy × 2 fractions two weeks apart for early stage prostate tumors(17). This scheme delivered a tumor equivalent dose in 2 Gy fractions of 141 Gy, but because all of the rectal wall could be kept below 60% of the prescribed tumor dose, the rectal equivalent dose in 2 Gy was only 59 Gy.

A major concern with multifractionated HDR treatments is that catheter might move between treatments, which could cause degradation of dosimetry(27,28). The preservation of dosimetry is
critical to ensure satisfactory biochemical control and low toxicity using HDR brachtherapy. Overall, there is a growing interest in shorter, more hypofractionated HDR approaches. Clinical dose escalation trials have shown efficacy and safety for hypofractionated treatments with HDR boost, however, evidence is just beginning to emerge for HDR monotherapy.

There are multiple radiobiological factors that may contribute to the differential response of tumors to radiation therapy. Among them, the cancer cells’ intrinsic cellular radiosensitivity, repopulation, reoxygenation capacity, and tumor microenvironment including the level of perfusion and hypoxia have been extensively investigated in experimental and clinical studies(29). Clinical dose-escalation trials reported high radiation dose significantly improve biochemical control and clinical disease-free survival for locally advanced prostate cancer patients(30-32). A hypoxic tumor microenvironment within the prostate has been postulated to have an important impact in selectively promoting the survival of a more aggressive cell phenotype and inducing radioresistance(5). Serially monitor cancer biomarkers such as tumor cell metabolism and perfusion during radiation therapy can guide radiation oncologists to optimize dose prescription, selection of hypofractionated treatment schemes, and identify residual recurrent cancer for salvage therapy for individual patient.

Reference


Chapter 3: Fundamentals of MR

Basics of Magnetic Resonance Imaging (MRI)

By definition, nuclear magnetic resonance (NMR) is a process involving the absorption and emission of energy by nuclei. Atoms with an odd number of protons and/or odd number of
neutrons possess a nuclear spin angular momentum, and therefore exhibit the MR phenomenon. Each atomic nucleus can be visualized as spinning charged sphere to gives rise to a magnetic moment ($\mu$). These MR-relevant nuclei are referred as spins. The nuclear magnetic dipole moment can be shown as:

$$\mu = \frac{\gamma h}{4\pi}$$  \[3.1\]

Where $\gamma$ is the gyromagnetic ratio intrinsic to the given nucleus and $h$ is Planck’s quantum constant. Table 3.1 shows the values of $\gamma$ for several isotopes commonly used for NMR and MRI experiments. The majority of current clinical imaging utilizes signals from the nuclei of hydrogen. However, other atoms such as phosphorus ($^{31}$P) and carbon ($^{13}$C) are also of interest.

In the presence of an external magnetic field $B_0$, the magnetic moment vectors either align with $B_0$ (spin-up) or align against the $B_0$ (spin-down). Most nuclei will align with $B_0$ (ie, in a lower energy state). The energy difference between the two spin states is given by the following equation:

$$\Delta E = \gamma h B_0$$  \[3.2\]

Where $h$ is Planck’s constant divided by $2\pi$. The Boltzmann probability distribution describes the proportion of aligned nuclei to anti-aligned nuclei:

$$\frac{N_+}{N_-} = \exp\left(-\frac{\Delta E}{kT}\right)$$  \[3.3\]

Where $N_+$ and $N_-$ represent the number of spins one would expect to measure in the spin-up and spin-down configurations, $k$ is the Boltzmann constant ($1.38 \times 10^{23}$ joules/K), and $T$ is the
absolute temperature in Kelvin. The proportion depends on the energy difference ($\Delta E$) between the two spin states and the thermal energy. As the temperature approach to absolute zero, the vast majority of the spins are expected to be in the lower energy state. However, at body temperature, thermal motions tend to equalize the spin population between two energy states. MR signal is proportional to the population difference between the two energy states. Polarization ($P$), is the ratio of the difference between the two energy states and the number of total spins, which can be described by the following equation:

$$P = \frac{N_+ - N_-}{N_+ + N_-} = \frac{\gamma B_0}{2kT}$$

[3.4]

At body temperature in a field of 1.0 T, this equation predicts that $N_+$ and $N_-$ are nearly equal; only a small difference of spins between the two states ($\sim 10^{-6}$) can be expected to found when measured.

Table 3.1. Gyromagnetic ratio for isotopes commonly used for NMR and MRI experiments. Most atomic nuclei have positive gyromagnetic ratios, but a few nuclei and the electron have negative values ($\gamma < 0$).

<table>
<thead>
<tr>
<th>Nucleus or Particle</th>
<th>Gyromagnetic Ratio ($\gamma$) MHz/T</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^1$H</td>
<td>42.58</td>
</tr>
</tbody>
</table>
In the presence of an external magnetic field $B_0$, a net magnetization vector $(M)$ is induced to point in the z-direction (the direction of the main field $B_0$) of strength $M_0$ and resonances at the Larmor frequency:

$$\omega_0 = \gamma B_0$$ \[3.5\]

Larmor frequency is defined by both the gyromagnetic ratio of the specific nucleus and the main magnetic field $B_0$. To obtain an MR signal, a radiofrequency (RF) magnetic pulse $B_1$ tuned to the Larmor frequency of the spins is applied in the $xy$ (transverse) plane to excite these spins out of equilibrium. $B_1$ field can be applied to direct $M$ from aligning with $B_0$ (z direction) to a plane perpendicular to $B_0$ (the transverse plane). This procedure is called an RF excitation pulse. After the $B_1$ field turns off, $M$ precesses in the $xy$ plane at the Larmor frequency, generating an electromotive force (EMF) signal in the receiver coil. The magnetization vector $M$ relaxes back to the equilibrium (re-aligned with $B_0$) according to Bloch equation, given by

$$\frac{dM}{dt} = M \times \gamma B - \frac{M_z i + M_y j}{T_2} - \frac{(M_z - M_0)k}{T_1}$$ \[3.6\]

Where $i$, $j$, and $k$ are unit vectors in $x$, $y$, and $z$ directions respectively, $M_0$ is the equilibrium magnetization arising from the main field $B_0$, and $B$ includes the various magnetic fields applied.
The time constant characterizing the return of the magnetization vector along the z-axis is called $T_1$, while the time constant characterizing the decay of the vector component in the transverse plane is called $T_2$. $M_0$ is proportional to the density $\rho$ of the atoms. $T_1$, $T_2$, and $\rho$ have distinct values for different tissues and therefore, are important MR parameters to generate contrast. After an excitation, the $M_z$ grows back to its equilibrium according to the following equation:

$$M_z(t) = M_0 (1 - \exp(-t/T_1))$$  \[3.7\]

Longitudinal relaxation is the process by which spins transfer energy from the spin system to the environment allowing the $M_z$ to recover to equilibrium $M_0$. Transverse relaxation is the process by which spins lose their phase coherence due to the energy transfer between neighboring spins. $M_{xy}$ returns to zero according to the following equation:

$$M_{xy}(t) = M_0 \exp(-t/T_2)$$  \[3.8\]

$T_2$ measures the transverse decay of the MR signal that arise from natural interactions at the atomic and molecular levels within the tissue of interest. However, in practice inhomogeneities in the main magnetic field and susceptibility artifacts produced by tissue can also cause transverse signal decay. This additional dephasing, combined with the $T_2$, represents $T_2^*$ relaxation.

As mentioned above, $T_1$, $T_2$, and density $\rho$ are key to generating contrast in MR images. Contrast in MR images can be manipulated by altering acquisition parameters. Repetition time (TR)
between excitations controls $T_1$ weighting, while time to form an echo (TE) controls $T_2$ weighting, as described:

$$
S = \rho (1 - e^{-TR/T_1}) e^{-TE/T_2}
$$

[3.9]

Where $S$ is the acquired MR signal.

In MR imaging, spatial localization is achieved by applying linear gradient magnetic fields in addition to $B_0$. To obtain a 3D image, gradients are applied in the x, y, and z dimensions to sample the inverse image domain, known as k-space. An MR signal is detected from the receiver coil, which is designed to measure changes in flux of the transverse magnetization. Therefore, the received time varying signal comes from the contribution of all transverse magnetizations in a volume. The Fourier transform will convert the measured net transverse magnetization into the image domain:

$$
S_r(t) = \int_x \int_y \int_z M_{xy}(x,y,z) e^{-i2\pi (xG_x + yG_y + zG_z)t} \, dx \, dy \, dz
$$

[3.10]

Where $S_r$ is the received MR signal and $G$ is the magnitude of the magnetic gradient in the x, y, or z direction. This equation can be re-written as signal measured in k-space:

$$
S_r(k_x,k_y,k_z) = \int_x \int_y \int_z M_{xy}(x,y,z) e^{-i2\pi (xk_x + yk_y + zk_z)t} \, dx \, dy \, dz
$$

[3.11]
Where $j$ can be either x, y, or z. The two most common ways to acquire k-space samples are phase encoding and frequency encoding. As we can see from the equation above, $k_j$ can be acquired by varying the gradient or time. Phase encoding acquires the localization information by varying the gradient strength by a discrete amount for a constant time duration. While during frequency encoding, a constant gradient is played over time and discrete samples are acquired at varying time intervals.

**Basics of Magnetic Resonance Spectroscopy (MRS)**

Chemical composition in the tissue of the body can be identified based on the chemical shift information from compounds of physiologic interest. Chemical shift is a small shift of the resonant frequency due to shielding created by the orbital motion of the surrounding electrons in response to the main $B_0$ field. As a result, the resonance frequency from equation 3.5 becomes:

$$\omega = \gamma B_0 (1 - \sigma)$$

Where $\omega$ is the actual resonance frequency and $\sigma$ is the shielding constant that is dependent on the chemical environment. The change in frequency is proportional to the magnetic field $B_0$. The chemical shift is defined with respect to a reference frequency $\omega_r$. If the resonant frequency of our sample is $\omega$, then the chemical shift $\delta$, in parts per million, is:

$$\delta = \frac{\omega - \omega_r}{\omega_r} \times 10^6$$
Chemical shift is reported on a parts per million (ppm) scale and does not depend on field strength. Chemical shift provides the basis of MR spectroscopy, which has been used to probe molecular structure, analyze chemical exchange rates, and follow metabolic processes. MR spectroscopy obtains spectra based on the relative concentrations of cellular chemicals/metabolites. With proton MR spectroscopy imaging (\(^1\)H MRSI), specific resonances for the metabolites; citrate, choline, creatine, and various polyamines from contiguous small volumes throughout the gland are observed(1). As seen in Figure 3.1, prostate cancer (right) is metabolically different from healthy peripheral zone (left) based on significant decreases in citrate and polyamines and an increase in choline.

Figure 3.1. 3D *in vivo* \(^1\)H MRSI spectra from prostate cancer patients. Left: tumor confirmed by histopathologic section indicating the presence of Gleason 3+4 prostate cancer. Cancer is demonstrated through elevated choline-containing compounds (Cho) and reduced citrate. Right:
Healthy tissue shows high levels of citrate and polyamines (PA) and low levels of choline-containing compounds. Adapted from Swanson MG, et al. Proton HR-MAS spectroscopy and quantitative pathologic analysis of MRI/\(^1\)H MRSI targeted postsurgical prostate tissues. Magn Reson Med 2003;50(5):944-954.

Current MRI and MR spectroscopy techniques for assessing prostate cancer and its response to radiation therapy

\(T_2\) Weighted MRI

MRI is an excellent prostate imaging modality to delineate prostatic anatomy and pathology due to its high spatial resolution, superior tissue contrast, and multiplanar capability. Current receiver coil technology includes pelvic phased-array coils with an endorectal coil, which adds approximately an order of magnitude to the available SNR(2). In general, \(T_1\) weighted images are not so helpful since prostate gland appears with homogenously intermediate signal intensity. However, \(T_2\) weighted images are much more sensitive at depicting the prostate zone anatomy. The contour of the prostate gland is clearly delineated by \(T_2\) weighted images. Prostate cancer has typically low signal intensity in \(T_2\) weighted images, comparing to peripheral zone, which has high signal intensity. The reason for lower signal intensity in the cancer regions compared to normal peripheral zone tissue is a loss of normal ductal morphology and associated long \(T_2\) water. Central zone is usually heterogeneous due to presence of benign prostatic hyperplasia, therefore, it is more difficult to detect cancer in the central gland. The signal intensity of prostate cancer can vary due to the grade of the disease. High-grade prostate tumors have higher signal intensity than lower grade cancers in \(T_2\) weighted images. The interpretation of prostate cancer
on T₂ weighted imaging can be affected by false-positive findings such as prostatitis, post-biopsy hemorrhage, and fibrosis(3,4). T₂ weighted MRI is also limited in evaluation prostate cancer after radiation therapy by the post-treatment loss of zonal anatomy and diffuse low signal, which hinders tumor detection(5-8).

Significant advances in MR imaging methods has allowed the simultaneous measurement of micro-environmental and metabolic changes in prostate cancer in a single Multi-parametric exam to improve cancer diagnosis, individualized therapy planning, and evaluation of response to treatment. Options considered include diffusion-weighted imaging (DWI), dynamic contrast enhancement (DCE) imaging, and MR metabolic imaging (¹H MRSI).

**Diffusion Weighted MRI**

DWI measures the rate of Brownian motion of water molecules in tissues by adding a pair of strong gradient pulses to the pulse sequence. The first pulse dephases the spins, and the second pulse rephases the spins if no net movement occurs. If net movement of spins does occur, signal would be attenuated depending on the magnitude of the molecular movement and diffusion weighting. Apparent diffusion coefficient (ADC) maps of water are generated from diffusion-weighted images. Low ADC represents restricted water molecular motion. In normal prostate the ADC is variable depending on the anatomic zone of the prostate. The healthy peripheral zone consists primarily of glandular lumen embedded within a stromal matrix. The macromolecular water content and free water content are low in stroma and high in luminal space in these normal structures. These characteristics result in a relatively unrestricted water diffusion and high water ADC values in the healthy peripheral zone. Healthy central and transition zones consist not only
of the same tissue components as peripheral zone but with increasing amounts of smooth muscle as well varying amounts of benign prostatic hyperplasia (BPH) which can be composed of predominately glandular tissues. Therefore, healthy central and transition zones can appear more heterogeneous than the surrounding peripheral zone. During prostate cancer formation, the normal ductal morphology is lost and is replaced by more densely packed malignant epithelia cells. Several histopathologic studies have demonstrated an inverse relationship between cellular density of a prostate tumor and (9,10). ADC values also have been shown to correlate with the Gleason score of prostate cancers(11-13).

Preclinical studies of prostate cancer have shown a radiation dose dependent increase in ADC early after therapy. This increase in ADC is thought to be primarily due to a reduction in cancer cell density caused by radiation induced cell death(14,15). Clinical studies have shown that although water ADC values are increased in both regions of recurrent cancer and benign treated tissues after radiation treatment, ADC values were still significantly lower in regions of recurrent prostate cancer \( (1.0 \times 10^{-3} \text{ mm}^2/s) \) relative to surrounding regions of benign treated tissues \( (1.6 \times 10^{-3} \text{ mm}^2/s) \) (16,17). Moreover, a study by Westphalen et. al. demonstrated that incorporation of DWI to T2 weighted and/or 1H MRSI significantly improves the assessment of patients with suspected recurrence after radiotherapy and a combined approach with all three modalities may have the best diagnostic performance (17).

_Dynamic Contrast Enhanced MRI_

DCE is another physiological MRI technique based on the alterations in vascularity of prostate cancer by means of angiogenesis. Tumor hypoxia induces formation of new blood vessels in
prostate cancer(16). Tumor angiogenesis influences microvascular blood flow, surface area, and permeability. The newly formed blood vessels initiated by tumor angiogenesis tend to be disorganized with weak walls and increased permeability. The goal of DCE imaging is to capture the passage of contrast material, such as gadolinium (Gd) DTPA, using $T_1$ weighted sequences. Since prostate cancer is a highly vascularized tumor with increase blood vessel permeability, cancer appears to have increased uptake and fast washout of contrast material in DCE images in comparison with benign prostatic tissues(17).

The contrast material is not directly observed in DCE MRI, rather, we observe it effect indirectly though its impact on water. Gd influences the surrounding water by catalyzing, or shortening the proton relaxation, which can be thought as transient chemical bonding between water protons and the paramagnetic ion. The relaxivity of the contrast material measures the degree of relaxation enhancement. Contrast material concentration can be assumed to be linearly related to relaxation rate ($1/T_1$):

$$\frac{1}{T_1} = \frac{1}{T_{10}} + r[Gd]$$

[3.15]

Where $r_1$ is the $T_1$ relaxivity constant of the contrast material and $T_{10}$ is the spin-lattice relaxation times in the absence of contrast material. Assuming signal intensity after Gd injection is proportional to $1/T_1$:

$$\frac{S_{Gd} - S_0}{S_0} = rT_{10}[Gd]$$

[3.16]
Where $S_{\text{Gd}}$ is the signal intensity after Gd injection and $S_0$ is signal intensity before Gd injection. Therefore, the relative increase of signal intensity following administration of contrast agent is related to both the $T_1$ relaxivity of the contrast material and the pre-contrast $T_1$ of the tissue. Semi-quantitative analysis incorporates time signal intensity parameters such as onsite time (time from injection to the first increase in tissue signal enhancement), uptake slope, time to peak enhancement, magnitude of peak enhancement, area under the time curve, and washout slope. These model-free quantification parameters provide reliable and reproducible information that have a bearing on the kinetics of contrast agent. However, these parameters do not provide any specific information on tissue properties as they reflect a combination of blood flow, blood volume, endothelial permeability, and the extravascular extracellular space volume.

Pharmacokinetic tracer kinetic compartmental models have been proposed to extract physiologically meaningful parameters from the DCE images. The most commonly used models is a four compartment Toft’s model, which includes extracellular extravascular space, vascular plasma space, intracellular space and a catch-all for all the other microscopic tissue components, such as membranes, fibrous tissues, etc, as shown in Figure 3.2. The contrast material, Gd, does not enter the cells. This model calculates physiological parameters such as $K^{\text{trans}}$ (related to the perfusion and permeability of the vessels), $K_{\text{ep}}$ (reverse flux rate constant between extracellular and vascular space), and extravascular extracellular space $v_e$. Concentration of contrast material in the tissue can be modeled as following:

$$C_{\text{tissue}}(t) = v_p C_p(t) + K^{\text{trans}} \times \exp(-k_{\text{ep}} t) \otimes C_p(t)$$ [3.17]
Where $C_{tissue}$ is the concentration of contrast material in the tissue, $v_p$ is the fraction of vascular plasma space, and $C_p$ is the concentration of contrast material in $v_p$.

Figure. 3.2. Diagram shows quantitative parameters and method for calculating pharmacokinetic parameters using four-compartment model devised by Tofts et al (18). These compartments are usually expressed as fractions of tissue volume. Where $v_e$ is fractional extracellular space, $v_p$ is fraction occupied by plasma, and $v_i$ is fraction occupied by intracellular space. Low-molecular-weight contrast agent, such as Gd DTPA (yellow dots) distributes exclusively in intravascular blood plasma and in extravascular extracellular space. Pharmacokinetic parameters listed are $K_{trans}$ and $K_{ep}$. Transfer constant, $K_{trans}$, describes diffusion of intravascular contrast medium into extracellular space. When distribution in body and renal elimination cause contrast medium concentration in plasma to drop below that in extracellular space, contrast medium from extracellular space diffuses back into plasma, which is described by rate constant, $K_{ep}$. $K_{trans}$
[min\(^{-1}\)] = permeability surface area product per unit volume of tissue, \(K_{ep} [\text{min}^{-1}] = \) efflux rate constant, \(v_p = \) fractional plasma volume, \(v_e = \frac{K_{\text{trans}}}{K_{ep}} = \) extracellular extravascular volume fraction. Adapted from Verma S, et al. AJR Am J Roentgenol 2012;198(6):1277-1288.

Prostate cancer lesions show earlier and faster enhancement and earlier contrast material washout compared with healthy prostate tissues. Semi-quantitative analyses have shown to be useful in differentiating malignant from benign prostatic tissue(19-22). Noworolski et al(19) found regions of cancer in the peripheral zone cancer have a higher peak enhancement, faster enhancement rate, and faster washout slope than normal peripheral zone tissues. Stromal BPH has the fastest enhancement rate of all tissues and tended to have the greatest enhancement. The differences between cancer and predominantly stromal BPH indicate that DCE may also aid in diagnosing cancer in the central gland. Physiologic parameters such as \(K_{\text{trans}}\) and \(k_{ep}\) have been shown to elevate in prostate cancer and used to detect cancer in the prostate, often in a Multi-parametric approach in combination with other sequences such as \(T_2\) weighted MRI, DWI, and MR spectroscopy imaging(23-25). DCE MRI alone has reported to have only 43% sensitivity, however, the sensitivity increased to 83% when combined with \(T_2\) weighted MRI and DWI(26). Another study reported that combined use of ADC maps and \(K_{\text{trans}}\) values extracted from DCE improved tumor detection in the central gland(27). Hyperemia, an increase in blood flow, has been shown to occur soon after radiation therapy in prostate cancer patients based on an increased permeability surface area product(28). Another clinical study using functional CT also demonstrated acute hyperemic response in the prostate glands of prostate cancer patients following radiation therapy as early as 1-2 weeks following completion of treatment and this remained so for 6-12 weeks(29). There was no evidence of tumor recurrence in both studies. In
addition, preclinical study of a xenograft prostate tumor mouse model showed a significantly increased $K_{\text{trans}}$ in irradiated region of the tumor 4 days after treatment, indicating the acute hyperemic phenomenon. A DCE clinical study shows that for patients with average 4 years post-radiation therapy, radiation therapy induced fibrosis in the prostate causes the prostate to enhance less and washout slower than normal prostatic tissue\(^{(30)}\). Prostate cancer recurrence after failed radiation therapy has been characterized by earlier and faster enhancement and earlier contrast material washout comparing to surrounding treated benign tissue\(^{(31)}\). Haider et al\(^{(30)}\) found DCE to be more accurate than $T_2$ weighted imaging at detecting cancer recurrence after radiation therapy.

The above calculations on Gd tissue concentration (equation 3.15 and 3.16) assume that the water exchange is fast between intracellular space and extracellular space, therefore, the tissue has a single, well defined, $T_1$. However, studies have shown that transcytolemmal water exchange departs from the fast exchange regime in many tissues even with using very low doses of contrast material\(^{(32,33)}\). As the contrast material cannot enter the cells, water must pass out of the cell to interact with the contrast material and influence signal changes in the extracellular space. The largest population of water is typically found inside cells. An implication of the slower water exchange is that $1/T_1$ in equation 3.15 is no longer linearly related to the concentration of the contrast material. As a water molecular moving from inside of the cell, through the cellular membrane and into the extracellular space, a $T_1$ measurement will contribute a relaxation behavior that represents an average of the intrinsic $T_1$ of the cell and the intrinsic $T_1$ of the interstitial space weighted by the time it spent in each of those spaces during the measurement. The shutter-speed pharmacokinetic model was developed to allow finite inter-
compartmental water exchange kinetics (34-36). Intracellular water lifetime was mapped for the prostate and was shown to significantly decrease in tumor compared with both peripheral zone and central gland (37). A preliminary study comparing shutter-speed analyses of prostate DCE MRI data with pathologic analyses of biopsy core specimens showed high specificity in distinguishing malignant from benign prostate tissue (38). Prostate glandular duct spaces are particularly abundant in the peripheral zone. In normal prostate, these spaces may be inaccessible to the contrast material (39,40), while in advanced cancer contrast material can enter the duct. A novel pharmacokinetic model incorporating a Gd-inaccessible luminal water parameter has been proposed (40). This model has demonstrated promise in better understanding prostate tissue histopathology and to potentially aid in the pathologic grading of prostate cancer and in predicting response to therapy.

*High-resolution magic angle spinning (HR-MAS)*

In addition to anatomic and functional imaging, valuable metabolic information can also be obtained from *ex vivo* MR spectroscopic analysis of image guided prostate biopsies. High-resolution magic angle spinning (HR-MAS) NMR spectroscopy can be applied to intact biopsy or surgical tissue samples to obtain a wealth of metabolic information *ex vivo*. The same tissue samples can subsequently undergo histopathologic and immunohistochemical analysis. Metabolic information determined by HR-MAS spectroscopy can then be correlated with quantitative histopathologic findings from the same tissues to determine the metabolic profiles associated with benign, glandular and stromal tissues, the presence, extent, and pathologic grade of prostate cancer, and any other immunohistochemical findings. Prior published HR-MAS studies have shown that distinct spectral patterns can be observed for different benign tissue
types and prostate cancer when there is at least 20% cancer present in the sample(41). An *ex vivo* HR-MAS study of surgically removed prostate tissues shows that proliferative (Ki67 staining ≥ 5%), high grade prostate cancers also have significantly higher concentrations of the choline-containing phospholipid metabolites phosphocholine (PC) and glycerophosphocholine (GPC) than do low grade prostate cancers and benign prostate tissues(42). This *ex vivo* finding is consistent with other *ex vivo*(41,43-47) and *in vivo*(48-51) $^1$H spectroscopy studies, which have correlated the degree of elevation of the individual phospholipid metabolites and the composite *in vivo* choline resonance with cancer aggressiveness (Gleason grade). PC and GPC serve as a precursor and a degradation metabolites of phospholipid metabolism, respectively. Increase in choline-containing compounds in prostate cancer is due to a combination of cellular proliferation and an altered phospholipid metabolism. A recent HR-MAS study has also identified lactate and alanine as biomarkers of prostate cancer presence(52), suggesting a higher overall glycolytic activity in prostate cancer compared to healthy tissues. In addition to significantly lower levels of choline-containing compounds, healthy glandular tissue can also be discriminated from prostate cancer based on significantly higher levels of citrate and polyamines. Predominantly stromal tissue lacks both citrate and polyamines, but it can still be discriminated from cancer based on the significantly lower levels of choline-containing compounds. Citrate levels in prostatic cancer are grade dependent, with citrate levels been low in well-differentiated, low grade prostate cancer and absent in poorly differentiated high grade prostate cancer(53,54). Healthy prostatic epithelial cells synthesize and secrete very high levels of citrate, due to high levels of zinc, which inhibit the enzyme aconitase and prevent the oxidation of citrate in the citric acid cycle(55). Citrate is a major component in prostatic secretions and it is believed to be involved in sperm activation and motility. In prostate cancer, zinc levels drop sharply, citrate turnover increases,
and prostate epithelial cells lose their capability for net citrate production and secretion. The transformation of healthy secretory prostate cells to malignant citrate oxidizing cells dramatically increases energy production capability of the cells, which is essential to the process of malignancy and metastasis(56).

Proton MR spectroscopy imaging (\(^1\)H MRSI)

\(^1\)H MRSI can non-invasively detect changes in metabolism (changes in choline-containing metabolites, polyamines, and citrate) that occur in prostate cancer. Clinical studies have shown compelling evidence that the addition of metabolic information provided by \(^1\)H MRSI to that obtained by MRI can significantly improve the ability of MRI to localize cancer within the prostate, assess cancer aggressiveness(57), and evaluate response to radiation therapy(58). Key metabolic resonances for in vivo \(^1\)H MRSI include citrate, polyamines, creatine, and choline. In vivo choline peak is a composite peak made up of PC, GPC, ethanolamine, phosphoethanolamine (PE), and glycerol-phosphethanolamine (GPE). The combined MRI/\(^1\)H MRSI demonstrated the ability to discriminate residual/recurrent prostate cancer from benign tissues and atrophic/necrotic tissue after radiation therapy. These studies have relied on elevated choline to creatine as a metabolic marker for residual prostate cancer presence, since the prostate metabolites polyamines and citrate, which are present in prostate tissue prior to therapy, are reduced to undetectable levels early after radiation therapy in both post-radiated benign and malignant tissues(1,59). Two published MRI/\(^1\)H MRSI studies of prostate cancer patients after radiation therapy have demonstrated that three or more consecutive spectroscopic voxels having total choline/creatine > 1.5 resulted in the ability to predict the presence of cancer after radiation therapy with an accuracy of \(\approx 80\%\)(5,60). Moreover, the addition of MR spectroscopic imaging
to T$_2$ weighted MRI (area under the receiver operating characteristic curve or AUC = 0.79) was shown to significantly improve the diagnostic accuracy of T$_2$ weighted MRI alone (AUC = 0.67) in the detection of locally recurrent prostate cancer after definitive external beam radiation therapy(60). Additionally, the lack of metabolic evidence of locally recurrent cancer after radiation therapy also predicts effective local treatment(59). Westphalen et al. found the combination of $^1$H MRSI, diffusion weighted MRI, and T$_2$ weighted MRI significantly improves the assessment of patients with suspected local recurrence after radiation therapy(58). The Multi-parametric MRI approach may have the best diagnostic performance in evaluation response to radiation therapy of prostate cancer.

$^{13}$C Hyperpolarization using Dynamic Nuclear Polarization

Hyperpolarized MR is a revolutionary new molecular imaging technique that can provide more than 10,000-fold signal enhancement for $^{13}$C labeled compounds by temporarily redistributing the populations of nuclear spins between the two energy states in a magnetic field. This technique allows for rapid in vivo imaging of alterations in metabolic and physiologic processes that could not be accomplished using traditional MR methods. Dynamic nuclear polarization or DNP is based on the transfer of polarization from the electron spins of paramagnetic centers to the neighboring nuclear spins by microwave irradiation at 3 to 5T and $\approx 1^\circ$ K(61). As shown in equation 3.4, the polarization of NMR active nuclei is:

$$P = \frac{N_+ - N_-}{N_+ + N_-} = \frac{\gamma h B_0}{2 k T}$$

[3.4]
Based on this equation, the polarization can be increased by increasing the magnetic field strength \((B_0)\), by decreasing the temperature \((T)\), or by using a particle with a larger gyromagnetic ratio \((\gamma)\). Nuclear polarization of \(^1\text{H}\) and \(^{13}\text{C}\) in a 3.0 T magnet field at 37°C are 9.34e-6 and 2.34e-6, respectively, or just 0.000934% and 0.000234%. DNP increases the polarization by increasing \(B_0\), decreasing \(T\), and increasing the effective \(\gamma\) by transferring polarization from electrons to the \(^{13}\text{C}\) nuclei. Interestingly, as shown in Table 3.1, the \(\gamma\) of an electron is 2,540 times larger than the \(\gamma\) of a \(^{13}\text{C}\) nucleus. As shown in Figure 3.3, electrons in a 3.35 T magnetic field are nearly 100% polarized at 1.4 K.

![Figure 3.3](image)

Figure 3.3. Thermal polarization for \(^{13}\text{C}\) nuclei, proton nuclei, and electrons when they are exposed to a 3.35 T external magnetic field. The polarizations were computed using equation 3.4.

During the polarization transfer process, the \(^{13}\text{C}\) molecules are doped with a substance containing free radicals in a glassy frozen solution. The transfer of polarization is achieved from the electrons to the neighboring nuclear spins through dipolar interactions by irradiating with
microwaves at frequency equal to the resonance frequency of electron. For the technique to be efficient, the radicals have to be homogenously distributed in the frozen solution containing the molecules of interest. The optimal temperature for an efficient polarization transfer is about 1° K(62). The DNP process can increase the $^{13}$C nuclear polarization in the solid state to 20-30%. After the polarization transfer process is completed, the sample is quickly dissolved and transferred to the MRI or NMR scanner for the spectroscopic experiment. Figure 3.4 shows a HyperSense polarizer and the schematic depiction of DNP process in a $^{13}$C sample. Once taken out of the polarizer, the hyperpolarized state decreases to its equilibrium value with a time constant according to $T_1$. 

Figure 3.4. The picture of a HyperSense polarizer and the schematic depiction of DNP mixing process in a $^{13}$C sample.
**Imaging of hyperpolarized $^{13}$C agents**

[1-$^{13}$C]Pyruvate has been the most widely studied substrate, due to its central role in cellular metabolism, the ease with which it can be hyperpolarized, its relatively long $T_1$ relaxation time, and its very rapid transport across the cell membrane and subsequent metabolism. Pyruvate is a key molecule involved in glycolysis. The production of lactate from pyruvate mediated by lactate dehydrogenase (LDH) is up-regulated in many tumor types (63). Flux of hyperpolarized $^{13}$C label between pyruvate and lactate has been used as a marker of prostate cancer presence and progression, with the levels of hyperpolarized lactate increasing with increasing tumor grade in the TRAMP model (64), as shown in Figure 3.5.
Figure 3.5. (A) Representative hematoxylin and eosin–stained pathologic sections (magnification, ×40) and hyperpolarized $^{13}$C spectra from a normal mouse prostate, an early stage and late transgenic mouse prostate tumor (TRAMP), and a lymph node metastases. Below the histologic sections are representative hyperpolarized $^{13}$C spectra acquired after injection of hyperpolarized [1-$^{13}$C]pyruvate and normalized to correct for differences in polarization. The normalized spectra exhibited a visually clear increase of hyperpolarized lactate and hyperpolarized lactate-to-pyruvate ratio with progression from the normal to early and late-stage tumors and metastases. (B) Axial T2 weighted $^1$H image depicting the primary tumor and lymph node metastasis from a TRAMP mouse with a late-stage primary tumor and the overlay of hyperpolarized [1-$^{13}$C]lactate image after the injection of 350 $\mu$l of hyperpolarized [1-$^{13}$C]pyruvate. Hyperpolarized [1-$^{13}$C] lactate increased in going from normal to prostate cancer and with disease progression. (C) A box plot quantitatively summarizing the peak area-to-noise ratios of the [1-$^{13}$C]lactate-to-noise ratio for the four histologically defined groups. The lactate peak area SNR values were statistically different (P < .05) for all four groups, except that early stage tumors were not significantly different from lymph node metastases. In addition, there was minimal overlap between individual [1-$^{13}$C]lactate-to-noise ratios between normal prostates and early and late-stage tumors. Adapted from Albers MJ, et al. Hyperpolarized 13C lactate, pyruvate, and alanine: noninvasive biomarkers for prostate cancer detection and grading. Cancer Res 2008;68(20):8607-8615.

Prior preclinical studies have also demonstrated the potential for using hyperpolarized [1-$^{13}$C]pyruvate to monitor disease progression and for making an early assessment of response to
therapy(64-71). Reduction of the flux between \([1^{-13}C]lactate\) and \([1^{-13}C]pyruvate\) observed in breast cancer model after radiation therapy appeared to be associated with cell death, changes in cellular membrane transport of pyruvate and lactate and lactate dehydrogenase expressions(71). Another hyperpolarized biomarker, HP \([^{13}C]urea\), is metabolically inactive and has shown great potential for perfusion imaging(72). Unlike Gd compounds, as an endogenous compound, HP \([^{13}C]urea\) is nontoxic. Other advantages of HP \([^{13}C]urea\) over Gd DCE include direct proportionality of signal to tracer concentration, absence of background signal, and the ability to co-polarize with HP \([1^{-13}C]pyruvate\) for combined, simultaneous perfusion and metabolic imaging(73).

The ability to probe changes in metabolism and perfusion early after therapy may allow clinicians to determine whether a cancer is responding to the treatment. If the tumor is not responding, a more effective treatment regimen could be initiated promptly(62). A recent clinical study has shown the safety and feasibility of hyperpolarized \([1^{-13}C]pyruvate\) as an agent for noninvasively characterizing alterations in tumor metabolism in patients with prostate cancer(74). Tumors were detected in all patients with biopsy-proven cancer. Also, cancer in regions of prostate that were previously considered to be tumor-free upon inspection with other conventional anatomic imaging methods was detected. This study demonstrated the potential of using hyperpolarized \(^{13}C\) MRI for both initial diagnosis and monitoring therapy in patients.

**14T hyperpolarized \(^{13}C\) 3D GRASE imaging sequence**

The unique combination of a 14T micro-imaging spectrometer and DNP polarizer provides the sensitivity necessary to obtain high spatial and temporal resolution \(^1H\) and hyperpolarized \(^{13}C\)
MR data from the murine prostate (≈ 4 mm in diameter). A novel, single-shot, frequency and time specific 3D imaging sequence was developed by Sukumar S. et al(75) to measure hyperpolarized $^{13}$C substrates in vivo at 14T. This pulse sequence utilizes chemical shift selective pulses to overcome the challenge of large spectral bandwidth at high field.

Figure 3.6. 3D GRASE imaging sequence developed for 14T HP $^{13}$C MR studies. $n$- number of first phase encoding; $m$- number of second phase encoding; $f$- frequency of the metabolite of interest. The sequence can be repeated for multiple frequencies.

The pulse sequence as shown in figure 3.6, was based on the spin-echo EPI technique and, as in the GRASE (gradient and spin-echo) imaging method, uses multiple 180° refocusing pulses during the echo train to minimized $T_2^*$ effects. The 90° and 180° pulses used in the sequence are chemical shift selective pulses designed to excite only the resonances of interest. Standard phase encoding can be added to the sequence to provide high resolution (2.5 × 3.3 × 3.3 mm$^3$ resolution) hyperpolarized $^{13}$C MR images in very short scan times (153 ms) per image. This very short acquisition time allows for the acquisition of multiple frequencies, probes and metabolites, after a single injection of co-hyperpolarized pyruvate and urea. For the 14T MR murine studies performed in this thesis, $^{13}$C image data acquisition started 44 s after injection.
This acquisition time was based on the slab-selective dynamic study from a TRAMP tumor at shown in Figure 3.7. A detailed overview on the challenges involved in acquiring hyperpolarized $^{13}$C data at high field are provided in Chapter 4.

![Figure 3.7. Dynamic hyperpolarized signal curves from a TRAMP tumor at 14T.](image)

Reference


apparent diffusion coefficient with histologic grade after radical prostatectomy. AJR Am J Roentgenol 2011;196(2):374-381.


Chapter 4: The Role of Metabolic Imaging in Radiation Therapy of Prostate Cancer

Abstract

The goal of this study was to correlate prostatic metabolite concentrations from snap-frozen patient biopsies of recurrent cancer after failed radiation therapy with histopathological findings, including Ki-67 immunohistochemistry and pathologic grade, in order to identify quantitative metabolic biomarkers that predict for residual aggressive versus indolent cancer. A total of 124 snap-frozen transrectal ultrasound (TRUS) – guided biopsies were acquired from 47 men with untreated prostate cancer and from 39 men with a rising PSA and recurrent prostate cancer following radiation therapy. Biopsy tissues with Ki-67 labeling index ≤ 5% were classified as indolent cancer, while biopsy tissues with Ki-67 labeling index > 5% were classified as aggressive cancer. The majority (15 out of 17) of cancers classified as aggressive had a primary Gleason 4 pattern (Gleason score ≥ 4+3). The concentrations of choline – containing phospholipid metabolites (PC, GPC and free Cho), and lactate were significantly elevated in recurrent cancer relative to surrounding benign tissues. There was also a significant increase in [PC] and reduction in [GPC] concentration between untreated and irradiated prostate cancer biopsies. The concentration of the choline – containing phospholipid metabolites was significantly higher in recurrent aggressive (≈ 2 fold) than in recurrent indolent cancer biopsies, and the receiver operating characteristic (ROC) curve analysis of total choline to creatine ratio (tCho/Cr) demonstrated an accuracy of 95% (confidence interval = 0.88 to 1.00) for predicting aggressive recurrent disease. The tCho/Cr was significantly higher for identifying recurrent
aggressive versus indolent cancer (tCho/Cr = 2.4 ± 0.4 versus 1.5 ± 0.2) suggesting that use of a higher threshold tCho/Cr ratio in future in vivo $^1$H MRSI studies could improve the selection and therapeutic planning of patients that would benefit most from salvage focal therapy after failed radiation therapy.

Introduction

An estimated 241,740 men will be diagnosed with prostate cancer in the United States in 2012 (1) and many of these men will be treated with external beam radiation therapy (2). Approximately half of these patients are expected to develop biochemical failure, i.e. a rising serum prostate-specific antigen (PSA) after a post-treatment nadir has been reached (3), which triggers investigation for the presence of recurrent local and/or metastatic disease. The diagnosis of locally recurrent prostate cancer after radiation therapy is largely dependent on histopathology obtained from transrectal ultrasound (TRUS) – guided prostate biopsies. TRUS localizes the prostate, however often does not visualize the malignant focus well because 37% to 50% of cancers may be isoechoic or only slightly hypoechoic (4). Accordingly, TRUS-guided biopsy has demonstrated false negative rates of up to 30% (5). Post-radiation prostate biopsies are also prone to problems in pathologic interpretation due to radiation effects confounding assessments of the presence, aggressiveness and viability of the residual tumor (6-8). The diagnosis of recurrent cancer after radiation therapy is further hindered by the long time to reach a PSA nadir after treatment, a process that can take up to 24 months (9,10).

Endorectal magnetic resonance (MR) imaging allows for an assessment of the entire prostate. However, T2-weighted MR imaging of the irradiated prostate is limited by the post-treatment
loss of zonal anatomy and diffuse low signal, which hinders tumor detection (11-14) (Figure 1A). Proton MR spectroscopic imaging ($^1$H MRSI) diagnoses of prostate cancer is based on the identification of neoplastic metabolism (15,16) and studies have demonstrated the ability of combined MRI/$^1$H MRSI to discriminate residual or recurrent prostate cancer from residual benign tissue and atrophic/necrotic tissue after radiation therapy (17,18) (Figure 1C, D). These studies have relied on elevated choline to creatine as a metabolic marker for residual prostate cancer presence since the prostate metabolites polyamines and citrate, which are present in prostate tissue prior to therapy, are reduced to undetectable levels early after radiation therapy in both post-radiated benign and malignant tissues (17,18). Two published MRI/$^1$H MRSI studies of prostate cancer patients after radiation therapy have demonstrated that three or more consecutive spectroscopic voxels having total choline/creatine >1.5 resulted in the ability to predict the presence of cancer after radiation therapy with an accuracy of ≈ 80% (11,19) (Figure 1C, D). Moreover, the addition of MR spectroscopic imaging to T$_2$-weighted MR imaging (area under the receiver operating characteristic curve or AUC = 0.79) was shown to significantly improve the diagnostic accuracy of T$_2$-weighted MR imaging alone (AUC = 0.67) in the detection of locally recurrent prostate cancer after definitive external beam radiation therapy (19). Alternatively, the lack of metabolic evidence of locally recurrent cancer after radiation therapy predicts for effective local treatment. This in the face of a rising serum PSA triggers concern for the presence of metastatic disease, thereby changing therapeutic intervention from a focal to a systemic approach.
Figure 4.1. (A) Representative $T_2$-weighted axial image of a 63 year old patient with a PSA of 2.4 ng/ml, 2.5 years after external beam radiation therapy. (B) Corresponding apparent diffusion coefficient (ADC) image and (C and D) 0.16 cc $^1$H MRSI spectral array. The red arrows on the ADC image indicate a region of clear cut ADC reduction in the right midgland of the prostate ($\leq 1.0 \times 10^{-3}$ mm$^2$/sec), which was not clear on the corresponding $T_2$-weighted image (A). Spectra overlapping the region of reduced ADC (C and D) demonstrated and absence of citrate and polyamines and a very elevated choline to creatine ratio. A subsequent MR targeted TRUS guided biopsy demonstrated a large volume of recurrent Gleason 4+4 cancer in the same location as the ADC and metabolic abnormalities.
It has also been shown that exposure of tumors to radiotherapy consistently lead to measurable increases in water diffusion in cases of favorable treatment response, however regions of cancer still remained lower than surrounding benign and atrophic tissues (Figure 4.1B) (20,21). After radiation therapy, the mean apparent diffusion coefficient (ADC) values of the biopsy-proven cancer areas (0.98 ± 0.23 x 10^{-3} mm²/sec) were shown to be significantly lower than those of benign tissue (1.60 ± 0.21 x 10^{-3} mm²/sec (20). A significantly greater area under the ROC curve was determined for combined T2 MRI and diffusion weighted imaging, or DWI (AUC = 0.88, P < 0.01) as compared to T2 MRI alone (AUC = 0.61) (20). In another study, incorporation of ¹H MRSI to T2-weighted (AUC = 0.84) and/or diffusion-weighted MRI (AUC = 0.86) significantly improved the assessment of patients with suspected recurrence after radiotherapy and a combined approach with all three modalities (AUC = 0.87) may have the best diagnostic performance (21). Similar results were obtained by Haider et al. investigating the diagnostic value of dynamic contrast-enhanced (DCE) MR imaging after radiation therapy (22). Specifically, on a sextant basis, peak enhancement on DCE MRI had significantly better sensitivity (72% vs. 38%), positive predictive value (46% vs. 24%) and negative predictive value (95% versus 88%) than T2-weighted MRI for detecting residual disease after radiation therapy.

The non-invasive detection of residual/recurrent cancer at an early time following treatment using Multi-parametric MRI would have a significant clinical value by allowing earlier intervention with a number of possible salvage therapy approaches (23-27). However, because of long prostate cancer doubling times and the fact that radiation therapy causes post-mitotic cell death, histologic tumor clearance can be delayed, with 30% of patients with positive early biopsy findings demonstrating tumor resolution by 30 months after therapy (28). For the same reason,
early $^1$H MRSI findings positive for cancer often resolve themselves over time, with the mean time for metabolic resolution of prostate cancer being on the order of 24 to 40 months after radiation therapy depending on radiation dose (11,17,29). A recent study investigated early (within first eight weeks) changes of ADC coefficients and T$_2$ relaxation time and determined six weeks to be the optimum time point to detect radiation induced changes but the prediction of clinically progressive recurrent disease was not determined in this study (30). The selection of appropriate salvage therapy candidates early after radiation therapy requires the identification of aggressive/progressive versus indolent or dying prostate cancer.

The nuclear antigen, Ki-67, is present in proliferating cancer cells, and the presence of high Ki-67 staining has been associated with aggressive, high pathologic grade prostate cancer (31-33). Furthermore, >5% Ki-67 staining in post-radiation biopsy tissues has been associated with subsequent local failure (8,34,35) and the high likelihood of metastatic disease (32,33). In a ex vivo high resolution magic angle spinning (HR-MAS) spectroscopy study of surgically removed prostate tissues prior to therapy, Keshari et al. showed that proliferative, high-grade prostate cancers also have significantly higher concentrations of the choline-containing phospholipid metabolites, phosphocholine (PC) and glycerophosphocholine (GPC), than do low-grade prostate cancers and benign prostate tissues (36). This ex vivo finding is consistent with other ex vivo (37-42) and in vivo (43-46) $^1$H spectroscopy studies, which have correlated the degree of elevation of the individual phospholipid metabolites and the composite in vivo choline resonance with cancer aggressiveness (Gleason grade). Recent studies have also identified other prostate metabolites, such as lactate and glutamate, as biomarkers of prostate cancer presence and aggressiveness (39,47,48). Based on these findings it is hypothesized that the identification of
quantitative metabolic biomarkers of proliferative/aggressive locally recurrent cancer could improve the selection of patients that would benefit from salvage focal therapy and potentially improve the targeting of the focal therapy (49).

Accordingly, the goal of this study was to correlate metabolite concentrations from snap-frozen, patient biopsies of residual cancer after radiation therapy with histopathological findings, including pathologic grade and Ki-67 immunohistochemistry, to identify quantitative metabolic biomarkers that predict for residual aggressive versus indolent/dying cancer. These quantitative metabolic biomarkers will be used in future in vivo Multi-parametric MR studies to investigate the improvement they provide for the selection and therapeutic planning of patients that would benefit most from salvage focal therapy after failed radiation therapy.

Methods

Subjects: Our institutional research board reviewed and approved this study, it was compliant with United States Government Health Insurance Portability and Accountability Act (HIPAA) requirements, and informed consent was obtained from all patients.

A total of 124 TRUS-guided biopsy specimens acquired from 47 men with untreated prostate cancer and from 39 men with recurrent prostate cancer following radiation therapy were used for this study. The mean time interval from patient receiving radiation therapy administered to the prostate to TRUS-guided biopsy of the prostate was 6.4 years (range, 2 to 15 years). Detailed patient characteristics, including the types of radiation therapy received, are described in Table 4.1 and their biopsy characteristics are provided in Table 4.2.
Under TRUS-guidance, 16 to 20 core biopsy specimens were obtained as part of usual clinical care; two additional specimens were acquired for research purposes. These two specimens were immediately snap-frozen on dry ice in cryovials, and stored in -80°C until further analysis. Tessem MB et al. have detailed this process in a previous publication (48).

<table>
<thead>
<tr>
<th>Table 4.1: Patient Characteristics</th>
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<tbody>
<tr>
<td>Pretreatment patients (n=47)</td>
</tr>
<tr>
<td>Age (years mean ± SD)</td>
</tr>
<tr>
<td>PSA (ng/ml mean ± SD)</td>
</tr>
<tr>
<td>Type of Radiation therapy</td>
</tr>
<tr>
<td>External beam</td>
</tr>
<tr>
<td>Permanent prostate seed implantation (PPI) brachytherapy</td>
</tr>
<tr>
<td>Combination of external beam and PPI brachytherapy</td>
</tr>
<tr>
<td>High-dose-rate (HDR) brachytherapy</td>
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<tr>
<td>Combination of external beam and HDR brachytherapy</td>
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<tr>
<td>Proton beam</td>
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<tr>
<td>Combination of external beam and proton beam</td>
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\textit{^1H HR-MAS Spectroscopy Acquisition}

Acquired biopsy tissues (5.4±1.2 mg) were prepared as previously described (48), and placed into custom designed 20 µl or 35 µl leak proof zirconium rotors containing 3.0 µl deuterium oxide and 0.75 wt% sodium-3-trimethylsilylpropionate-2,2,3,3-d$_4$ acid (D$_2$O+TSP). Quantitative 1D \textsuperscript{1}H HR-MAS ‘presat’ data were acquired at 11.7T (500 MHz for \textsuperscript{1}H), 1°C, and 2,250 Hz spin rate using a Varian INOVA spectrometer, equipped with a 4 mm gHX nanoprobe (Varian Inc, Palo Alto). Fully relaxed water pre-saturated spectra were acquired using 40,000 points, 20,000-Hz spectral window, 2-s pre-saturation delay, 2-s acquisition time (total 4-s repetition time, or TR), four steady-state pulses, and 124 transients.

For quantification of prostate metabolites, an electronic reference was added using the electronic reference to access \textit{in vivo} concentrations (ERETIC) method (50). The phase and amplitude of the ERETIC peak were chosen to match other peaks in the spectrum, and the signal was transmitted during acquisition using 0 dB of power, a full width at half height of 3.5 Hz, and an offset frequency equivalent to -0.5 ppm. The ERETIC signal was calibrated monthly using standard solution of D$_2$O + TSP.

2D total correlation spectroscopy (TOCSY) was acquired after the 1D ‘presat’ HR-MAS data acquisition. The TOCSY spectra were acquired using a rotor synchronized adiabatic (WURST-8) mixing scheme with 1-s pre-saturation delay, 0.2-s acquisition time, 40-ms mixing time, 24 transients/increment, 20,000 × 6000 Hz spectral width, 4096 × 64 complex points, and a time of ~1 hour (36,40).
*1H HR-MAS Spectroscopy Data Analysis and Quantification*

Acquired HR-MAS biopsy data were processed offline using ACD/Labs 1D and 2D NMR processor version 9 (ACD/Labs, Toronto, Canada). 1D spectra were first prepared by linearly predicting the first two points of the FID. Each spectrum was phase–corrected, frequency–referenced to the CH₃ peak of creatine, and the residual water peak was removed using the Java-based graphical user interface for the Magnetic Resonance User Interface (jMRUI) package (51). 1D data were then quantified using the high resolution quantum estimation (HR-QUEST) method (52). To determine goodness of fit, HR-QUEST estimated the Cramér–Rao Lower Bounds (CRLB) percentages of the measured metabolites for each prostate biopsy spectrum. Metabolites with CRLB >15% were excluded from analysis. By modeling the broad lipid and macromolecule resonance overlapping lactate at 1.33 ppm, HR-QUEST was able to resolve the lactate amplitude.

In 3 out of 53 biopsies, lactate measurements were considered unusable due to improper fit of the lipid and macromolecule peak at 1.33 ppm. Concentrations were calculated relative to the amplitude of the ERETIC signal according to Eq. [1]:

\[
\frac{A_m}{A_e} \times [H_e] \times \frac{nH_e}{nH_m} \times \frac{1}{\text{Tissue mass}} = [M] 
\]

[1]

Where \( A_m \) = the metabolite amplitude, \( A_e \) = the amplitude of ERETIC signal, \( [H_e] \) = the standard moles of protons associated with the ERETIC signal in mmol, \( nH_e \) = the number of protons of ERETIC (\( N = 1 \)), \( nH_m \) = the number of protons corresponding to the main metabolite resonance.
used in HR-QUEST fitting, tissue mass = mass of the prostate biopsy (kg), and [M] = the metabolite concentration in mmolal.

2D TOCSY data were processed and quantified as described previously (36,40). PC and GPC concentrations were estimated using the side chain CH$_2$-CH$_2$ cross-peak volumes relative to the diagonal of TSP. PC, GPC and TSP cross-peaks were volume integrated and corrected for the magnetic transfer efficiency (K$_{MT}$). Concentrations were then calculated relative to the TSP cross-peak volume, TSP 1D integrated area and ERETIC 1D integrated area according to Eq. [2]:

\[
\frac{V_{\text{met}}}{V_{\text{TSP}}} \times K_{MT} \times \frac{\text{Area}_{\text{TSP, 1D}}}{\text{Area}_{e}} \times [H_e] \times \frac{nH_e}{nH_m} \times \frac{1}{\text{Tissue mass}} = [M]
\]

Where $V_{\text{met}}$ = integrated volume of either PC or GPC cross-peak, $V_{\text{TSP}}$ = integrated volume of the TSP diagonal peak, $\text{Area}_{\text{TSP, 1D}}$ = integrated area of the TSP peak from the 1D spectrum, $\text{Area}_{e}$ = integrated area of the ERETIC peak from the 1D spectrum.

Histopathologic Analysis

Following $^1$H HR-MAS acquisition, biopsy tissues were frozen in Tissue-Tek® optimal cutting temperature (OCT) tissue – embedding medium (Fisher, Pittsburgh, PA, USA), and sectioned at -22°C using a Leica CM1850 cryostat (Leica Microsystems, Wetzlar, Germany). Tissues were sectioned at 5 to 7 µm intervals, placed on individual histology slides, and stained with hematoxylin and eosin (H&E) using a standard protocol. Additional sections were cut for high-molecular-weight keratin staining to confirm the presence of cancer, and Ki-67
immunohistochemical staining (53). For Ki-67, the slides were incubated for 60 min with a monoclonal mouse Ki-67 antibody, clone MIB-1 (M7240, Dako, Copenhagen, Denmark), diluted 1:100 at room temperature. Secondary antibody was peroxidase-labeled horseradish peroxidase polymer (Dako), incubated for 60 min. The antigen localization was achieved by the 3,3’-diaminobenzidine chromogen (Dako). Nuclei were considered to be Ki-67 positive if any nuclear staining was present, regardless of staining intensity.

Two pathologists (12 and 16 years of experience) reviewed the slides and estimated the percentage of benign glandular epithelium, stroma, prostatic intraepithelial neoplasia (PIN), benign prostatic hyperplasia (BPH), chronic inflammation (prostatitis), prostate cancer (Gleason grade), and % of Ki-67 positively stained tissue in each core. Benign prostatic biopsy tissues that consisted of ≥ 25% glandular tissue were defined as predominately glandular tissue. 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. The malignant biopsy tissues were further separated according to their pathologic grade and Ki-67 labeling index. Biopsy tissues with Ki-67 labeling index ≤ 5% were classified as indolent cancer (8,34,35), while biopsy tissues with Ki-67 labeling index > 5% were classified as aggressive cancer (Table 4.2).

Statistical Analysis

Statistical analyses were performed using JMP Software (SAS Institute Inc., 2008, version 8.0). Metabolite concentrations (mmol/kg) were compared between benign and residual cancer tissues
as well as among benign, indolent and aggressive residual cancer using a linear mixed-effects model (54). The model used metabolite concentrations as the dependent variable, disease status (Ki-67 labeling index and grade) as fixed effect and individual patients as random effect. Thus, effects from repeated samples of patients were removed. Non-parametric Wilcoxon Rank Sum Test or Kruskal-Wallis Test was used for multiple comparisons. For all statistical analyses, a probability value of less than 0.05 was considered significant. The performance of using total choline (PC + GPC + free choline) to creatine ratio to diagnose aggressive residual disease was described using receiver operating characteristic (ROC) curve. Biopsy tissues with Ki-67 staining greater than 5% were considered to be aggressive cancer (8).

**Results**
The average serum PSA level of patients with prostate cancer after therapy was significantly lower than prior to therapy, even for patients with similar amounts of recurrent cancer after therapy (Table 4.1). Figures 4.2 and 4.3 demonstrate representative $^1$H HR-MAS spectra, H&E and Ki-67-stained histologic sections from benign, indolent and aggressive prostate cancer biopsies from patients prior to and after radiation therapy. Of the 71 untreated prostate biopsy specimens, histopathology demonstrated that 58 were benign tissues (38 predominately glandular, 20 predominately stromal), 5 were indolent cancers, and 8 were aggressive cancers. Of the 53 post-radiation prostate biopsies, 32 were benign tissues, 7 indolent/dying cancers, and 12 were aggressive (Table 4.2). The percentage of the biopsy cores that were positive for cancer ranged from 5 to 60% (14.0 ± 8.8%). No significant difference was observed between the average percentage of biopsy core positive for untreated and treated cancer samples or between the recurrent indolent and aggressive cancer samples.
Ki-67 labeling index was very low and not different between untreated predominately glandular and stromal benign samples (Table 4.2). For both untreated and post-radiation biopsy tissues, Ki-67 labeling index for aggressive cancer (11.0 ± 1.7% and 16.0 ± 3.3%, respectively) was significantly higher compared to benign (0.23 ± 0.14% and 0.10 ± 0.08%, respectively) and indolent cancer biopsies (1.40 ± 0.33% and 1.00 ± 0.42%, respectively). Interestingly, benign tissues had a significant reduction and recurrent aggressive cancer had higher Ki-67 labeling after radiation therapy. Of particular importance to the current study, was a significant correlation between Ki-67 labeling index and [PC + GPC + free Cho] in both untreated (Spearman ρ = 0.57; p < 0.001) and irradiated (Spearman ρ = 0.62; p < 0.001) cancer biopsy
specimens, and the majority of the (15 out of 17) cancers classified as aggressive by the Ki-67 staining had a primary Gleason 4 pattern (Gleason score $\geq 4+3$), and the other two had had $\geq 3+4$ (Table 2).

Figure 4.2. A comparison of representative 1D HR-MAS spectra, and corresponding H&E and Ki-67-stained sections of snap-frozen prostate biopsy tissues taken from regions of (A) benign stromal tissue (5% glandular, 95% stromal, 0% Ki-67), (B) benign glandular tissue (38%
glandular, 62% stromal, 0% Ki-67), (C) indolent cancer (10% Gleason 3+3, 30% glandular, 60% stromal, 1% Ki-67) and (D) aggressive prostate cancer (27% Gleason 4+3, 15% glandular, 38% stromal, 13% Ki-67). (Left) One-dimensional HR-MAS spectra. Corresponding hemoxylin/eosin (H&E)-stained histologic (middle) and Ki-67-stained sections (right). The major metabolites resonances are shown. PC, phosphocholine; GPC, glycerophosphocholine; Cho, free choline; Cr, creatine; PA, polyamines; MyoI, myo-inositol; Glu, glutamate; Ala, alanine; Lac, lactate;

<table>
<thead>
<tr>
<th>Metabolite (mmolal ± SE)</th>
<th>Benign (n=58)</th>
<th>Cancer (n=13)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>[PC + GPC + free Cho]</td>
<td>0.61 ± 0.05</td>
<td>1.57 ± 0.19</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>[PE]</td>
<td>0.78 ± 0.09</td>
<td>1.15 ± 0.17</td>
<td>0.03</td>
</tr>
<tr>
<td>[lactate]†</td>
<td>0.78 ± 0.16</td>
<td>1.60 ± 0.31</td>
<td>0.01</td>
</tr>
<tr>
<td>[alanine]</td>
<td>0.27 ± 0.03</td>
<td>0.54 ± 0.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>[Glutamate]</td>
<td>1.57 ± 0.17</td>
<td>2.62 ± 0.26</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>[Citrate]†</td>
<td>6.11 ± 0.75</td>
<td>4.48 ± 0.74</td>
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<tr>
<td>[Polyamines]†</td>
<td>0.74 ± 0.20</td>
<td>0.35 ± 0.15</td>
<td>0.0036</td>
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<th>Metabolite (mmolal± SE)</th>
<th>Benign (n=58)</th>
<th>Indolent Cancer (n=8)</th>
<th>Aggressive Cancer (n=5)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>[PC + GPC + free Cho]</td>
<td>0.61 ± 0.05</td>
<td>1.22 ± 0.19</td>
<td>2.06 ± 0.23</td>
<td>0.003, &lt;0.001, 0.02</td>
</tr>
</tbody>
</table>

* n = 54 for lactate in benign and n = 10 for lactate in cancer biopsy samples.
† Only predominately glandular biopsies (N=38). [Citrate] and [polyamines] were significantly higher in predominantly glandular relative to predominantly stromal benign biopsies (p ≤ 0.001).

---

Table 4.3. Significant Metabolic Differences Between Benign and Malignant Untreated Prostate Biopsies

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Representative fully relaxed $^1$H HR-MAS spectra of untreated predominately stromal (A), predominately glandular (B) benign prostate samples, and indolent (C), and aggressive cancer (D) samples are shown in Figure 4.2. Similar to previous HR-MAS studies of surgically removed prostate tissues, predominately glandular biopsy benign tissues (Figure 4.2B) demonstrated high levels of citrate (doublet of doublets at 2.55 ppm) and polyamines (broad multiplet at 3.13 ppm), which were progressively reduced in both indolent and high-grade prostate cancer spectra (36,40-42). Predominantly benign stromal tissues also demonstrated a significant reduction ($p \leq 0.001$) in citrate and polyamines relative to benign glandular tissues (Figure 4.2, Table 4.3). In cancer there was also a clear increase in the free Cho (3.21 ppm), PC (3.23 ppm) and GPC (3.24 ppm) resonances in prostate cancer relative to benign glandular and stromal prostate tissues. Quantitatively, these observations proved true, with the concentration of citrate and polyamines being significantly lower and phospholipid metabolites significantly higher in prostate cancer relative to benign prostate tissues (Table 4.3). Moreover there was a significant increase in the concentration of phospholipid metabolites, [PC + GPC + free Cho], between benign and indolent cancer biopsies and between indolent and aggressive cancers (Figure 4.4A and Table 4.3). However, there was overlap of individual [PC + GPC + free Cho] values in indolent cancer with both benign and aggressive cancer biopsy tissues (Figure 4.4A). Other lower signal to noise prostate metabolites also demonstrated significant differences in their concentrations between untreated cancer and benign samples. These included significant increases in glutamate (multiplet at 2.35 ppm), alanine (doublet at 1.48 ppm) and lactate (doublet at 1.33 ppm) (Figure 4.4C) in untreated cancer relative to benign biopsies (Table 4.3).
Figure 4.3. A comparison of representative 1-D HR-MAS spectra, and corresponding H&E and Ki-67-stained sections of snap-frozen post-radiation prostate biopsy tissues taken from regions of (A) residual benign tissue (100% stromal, 0% Ki-67), (B) indolent/dying recurrent cancer (45% Gleason 3+4, 5% glandular, 50% stromal, 1% Ki-67) and (C) aggressive cancer (42% Gleason 4+3, 5% glandular, 53% stromal, 10% Ki-67). (Left) One-dimensional HR-MAS spectra. Corresponding hemoatoxylin/eosin (H&E)-stained histologic (middle) and Ki-67-stained sections (right).
Table 4.4. Metabolite Concentrations in Treated Biopsies

<table>
<thead>
<tr>
<th>Metabolite (mmolal ± SE)</th>
<th>Benign (n=34)</th>
<th>Cancer (n=19)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>[PC + GPC + free Cho]</td>
<td>0.44 ± 0.03</td>
<td>0.91 ± 0.07</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>[Lactate]</td>
<td>0.70 ± 0.05</td>
<td>1.04 ± 0.06</td>
<td>0.001</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Metabolite (mmolal)</th>
<th>Benign (n=34)</th>
<th>Indolent Cancer (n=7)</th>
<th>Aggressive Cancer (n=12)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>[PC + GPC + free Cho]</td>
<td>0.44 ± 0.03</td>
<td>0.66 ± 0.07</td>
<td>1.05 ± 0.07</td>
<td>0.37a, &lt;0.001b, 0.04c</td>
</tr>
</tbody>
</table>

*a* Indolent versus Benign.  
*b* Aggressive versus Benign.  
*c* Indolent versus Aggressive.

Representative fully relaxed $^1$H HR-MAS spectra of radiation-treated benign prostate samples (A), and indolent (B), and aggressive cancer (C) samples are shown in Figure 4.3. Effective radiation therapy induces glandular atrophy resulting in irradiated benign prostatic tissues being predominately stromal tissue (Figure 4.3A) similar to untreated benign stromal tissues (Figure 4.2A). Since citrate and polyamine production are associated with glandular prostate tissues not stroma (55), there was a loss of citrate and polyamines in irradiated benign tissues (Figure 4.3A). A number of post radiation benign and malignant tissues did retain some glandular architecture and had detectable levels of polyamines and citrate in the associated $^1$H HR-MAS spectra. In both irradiated benign and malignant tissues, there was a significant reduction in [PC + GPC + free Cho], but the concentration of phospholipid metabolites in recurrent malignant tissues remained significantly higher than in irradiated benign tissues (Table 4.4). Similar to pre-treatment (Figure 4.4A), there was an increase in [PC + GPC + free Cho] between benign,
indolent and aggressive cancer biopsies (Figure 4.4B and Table 4.4). Of particular importance to the current study, [PC + GPC + free Cho] was significantly higher in aggressive recurrent cancer than in benign tissues and indolent/dying tumors (Figure 4.4B and Table 4.4). [Lactate] was also significantly higher in post-radiation residual prostate cancer relative to benign tissue, but did not discriminate aggressive and indolent recurrent cancer (Figure 4.4D and Table 4.4). The amplitudes of glutamate and alanine were typically reduced to noise level of $^1$H HR-MAS spectra after radiation therapy and therefore were not useful as biomarkers of cancer presence or aggressiveness.

Figure 4.4. (A and B) Quantitative comparison of phosphocholine (PC), glycerophosphocholine (GPC) and free choline concentrations in untreated and post-radiation prostate biopsy tissues. In untreated biopsy tissues (left), [PC+GPC] was found to be statistically significant between all
three tissue types (benign, indolent and aggressive cancer). In post-radiation biopsy tissues (right), [PC+GPC] was found to be statistically significant between benign and recurrent aggressive cancer, and between recurrent indolent and aggressive cancer). (C and D)

Quantitative comparison of lactate concentrations in untreated and post-radiation prostate biopsy tissues. [Lactate] was found to be statistically significantly different between benign and malignant biopsy tissue in both untreated and post-radiation biopsy tissues, but did not discriminate between indolent and aggressive cancer.

Figure 4.5. A comparison of representative 2-D HR-MAS total correlation spectra of snap-frozen prostate biopsy tissues taken from regions of untreated aggressive prostate cancer (27% Gleason 4+3, 15% glandular, 38% stromal, 13% Ki-67) (A) and post-radiation aggressive prostate cancer (42% Gleason 4+3, 5% glandular, 53% stromal, 10% Ki-67) (B). The major
metabolites resonances are shown. PE, phosphoethanolamine; GPE, glycerophosphoethanolamine; Eth, ethanolamine;

Figure 4.5 demonstrates the upper diagonal CH$_2$-CH$_2$ region of the 2D TOCSY spectra from the representative untreated and irradiated aggressive cancer biopsies shown in Figures 4.2D and 4.3C. On visual inspection, GPC cross-peaks were higher than PC in untreated cancer versus post-radiation cancer biopsy tissues. Quantitatively, these observations proved true with [GPC] and [PC] being the most dominant phospholipid metabolites in untreated and treated aggressive cancers, respectively. Specifically, in untreated aggressive cancer tissues, [GPC] was $1.10 \pm 0.46$ mmolal, almost 2-fold higher than [PC] ($0.60 \pm 0.20$ mmolal). On the contrary, in post-radiation aggressive cancer tissues, [PC] was $0.96 \pm 0.21$ mmolal, more than 7-fold higher than [GPC] ($0.13 \pm 0.06$ mmolal). On average, the post radiation biopsies studied had a significantly ($p \leq 0.001$) higher Ki-67 labeling index and were of higher pathologic grade (Table 4.2).
Figure 4.6. The performance of using total choline (PC+GPC+Cho) to creatine ratio to diagnose aggressive residual disease was described using receiver operating characteristic (ROC) curve. The area under the ROC curve was 95% (confidence interval = 0.88 to 1.00) indicating that the magnitude of the in vivo total choline to creatine ratio would be an accurate predictor of aggressive recurrent prostate cancer after radiation therapy.

Figure 4.6 is a ROC curve demonstrating the performance of using the total choline to creatine ratio, [PC + GPC + free Cho]/[Cr], to predict aggressive recurrent prostate cancer after radiation therapy. Creatine concentration did not significantly change between benign (1.30 ± 0.10 mmolal), indolent (1.31 ± 0.22 mmolal) and aggressive cancer (1.30 +/- 0.22 mmolal) tissues after radiation therapy. Therefore the total choline-to-creatine ratio can be used in in vivo $^1$H MRSI studies where concentrations are difficult to measure. The mean tCho/Cr concentration
ratio significantly (p = 0.02) increased in indolent (0.50 ± 0.09) cancer versus benign tissues (0.38 ± 0.04) and in aggressive cancer (0.82 ± 0.17) versus indolent cancer (p ≤ 0.005). Since the choline and creatine head-groups have 9 and 3 equivalent protons and similar T₁ and T₂ relaxation times (56), respectively, the in vivo tCho/Cr peak ratio will be approximately 3 times larger than the concentration ratio (1.1 ± 0.1, 1.5 ± 0.2 and 2.4 ± 0.4 for benign, indolent, and aggressive recurrent cancer, respectively). The area under the ROC curve for predicting aggressive cancer using [PC + GPC + free Cho]/[Cr] ratio was 0.95 (confidence interval = 0.88 to 1.00).

Discussion

A growing number of prostate cancer patients receiving Multi-parametric¹H MR exams are referred for suspected local cancer recurrence after radiation therapy. Recurrent cancer is typically suspected in these patients due to a detectable or rising PSA (3), however as demonstrated in this study, even patients with aggressive recurrent cancer often have much lower PSA concentrations than those with similar cancer burdens prior to therapy (3.40 ± 0.91 versus 19 ± 13 ng/mL for aggressive recurrent cancer and aggressive untreated cancer patients). This is most likely due to the reduction in contribution of serum PSA arising from benign prostate tissues effectively treated during radiation therapy, since a significant amount of serum PSA arises from benign prostate tissues.

The identification of recurrent prostate cancer after radiation by either a PSA nadir or pathology of subsequent prostate biopsies is further confounded by the relatively slow rate of radiation-induced cellular death (28), and radiation-induced pathologic changes (6-8), resulting in the
inability to accurately predict clinical progressive recurrent cancer within the first two years after radiation therapy. However, prior published studies have associated high cellular staining of the nuclear antigen Ki-67 with high pathologic grade prostate cancer (31-33) and with subsequent local failure of radiation therapy of prostate cancer (8,34,35). Using greater than 5% Ki-67 staining of prostate cancer as a measure of recurrent aggressive cancer (8,34,35), this study demonstrated that the concentration of phospholipid metabolites, [PC + GPC + free Cho], was significantly higher in these recurrent tumors as compared to both benign tissues and/or indolent/dying tumors after radiation therapy. Moreover, the majority of cancers classified as aggressive by Ki-67 staining had a primary Gleason 4 cancer (Gleason score ≥ 4+3), a pathologic pattern considered to be clinically aggressive/progressive disease (57,58). The ability to discriminate recurrent aggressive cancer following radiation therapy has clinical significance, as the detection of residual cancer at an earlier time after treatment could allow earlier intervention with additional salvage therapy.

The 2D TOCSY HR-MAS data also demonstrated that glycerophosphocholine was the dominant choline-containing phospholipid metabolite in aggressive untreated human prostate cancer tissue (PC/GPC = 0.6), which is in agreement with prior studies of surgical human prostate specimens (36,40). Whereas phosphocholine was found to be the dominant phospholipid metabolite with GPC being dramatically reduced (PC/GPC = 7.6) in recurrent aggressive prostate cancer after radiation therapy. In previous studies of culture-immortalized (37) and primary human prostate cancer cells (39), [PC] was also the dominant phospholipid. PC and GPC serve as a precursor and a degradation product of phospholipid metabolism, respectively. Enhanced [PC]/[GPC] ratio has been suggested to indicate tumor progression from lower to higher malignancy grades in
breast cancer cell lines and brain tumors (38,59-63). PC has also been proposed to be mitogenic by acting as a mediator of growth factor-induced cell proliferation (38,61,63-65). The [PC]/[GPC] ratio difference observed in this study between untreated and post-radiation residual prostate cancer, especially in high-proliferative cancer specimens, might be a result of the survival of a more aggressive cell phenotype with higher radio-resistance following radiation therapy. Several clinical studies have shown that conventional-dose radiation therapy does not eradicate prostate cancer in a significant proportion of cases, leading to eventual clinical recurrence (66,67).

The concentration of lactate was also found to be significantly higher in post-radiation residual prostate cancer relative to benign tissue, but [lactate] did not reach statistical significance in discriminating aggressive and indolent recurrent cancer. The significant increase in lactate concentrations found in both untreated and post-radiation residual prostate cancer is in agreement with previous $^1$H HR-MAS study of prostate tissues (48) and other human cancers (68-71). In vivo prostate lactate levels have not been typically monitored by $^1$H MRSI studies of patients due to complications with overlapping lipid. However, with improved volume selection, outer voxel suppression techniques, and higher-field (3T) scanners, it is becoming feasible to use spectral-spatial RF editing sequences to detect lactate (72). Additionally, new hyperpolarized $^{13}$C MR techniques could potentially take advantage of elevated lactate in proliferating residual cancer after radiation therapy. Elevated hyperpolarized [1-$^{13}$C] lactate levels have been shown to correlate with prostate cancer grade (47), and with failed androgen deprivation therapy in the transgenic adenocarcinoma of mouse prostate (TRAMP) model (73). Hyperpolarized [1-$^{13}$C] lactate levels have also been shown to be a sensitive index of radiation-induced biochemical
changes (74,75). Therefore, the changes in lactate observed in the present study could be used to improve clinical diagnosis and monitoring of prostate cancer progression after radiation therapy using hyperpolarized $^{13}$C MR techniques.

Other prostate metabolites, including citrate, polyamines, alanine, and glutamate, which demonstrated significant differences in their concentrations between untreated cancer and benign biopsy tissues in this study and in a number of prior in vivo $^1$H MRSI (16,44-46,76) and ex vivo $^1$H HR-MAS spectroscopy studies (41,42,48,77), were typically reduced to noise level in $^1$H HR-MAS spectra of post radiation biopsies. Since radiation therapy induces glandular atrophy, effectively irradiated benign prostatic tissues were observed to be pathologically similar to untreated benign stromal tissues, with an associated loss of the prostatic secretory metabolites polyamines and citrate in the $^1$H HR-MAS spectra. The time-dependent loss of citrate and polyamines in both benign and malignant tissues after radiation therapy has been observed in several serial $^1$H MRSI studies of prostate cancer patients (17,18). Additionally, prostate tissues receiving insufficient doses of radiation in these in vivo studies often had $^1$H MRSI spectra containing observable citrate and polyamine metabolite resonances and the associated prostate tissue presumably retained some glandular architecture (17,18) similar to the glandular pathology that was observed in a number of post-radiation biopsies that also demonstrated $^1$H HR-MAS spectra detectable citrate and polyamine concentrations. There was also an approximate 50% reduction in the concentration of all prostate metabolites reliably detected in both pre-treated and irradiated biopsy tissues, but the concentration of the choline-containing phospholipid metabolites remained in the in vivo $^1$H MRSI detectable millimolar concentration range and were approximately 3 fold higher than irradiated benign tissues and approximately 2 fold higher than
recurrent indolent cancer biopsies. Indeed, successful radiation treatment has been characterized by *in vivo* \(^1\)H MRSI as a lack of metabolic activity or “metabolic atrophy” (17,18). Whereas, recurrent prostate cancer can be detected by *in vivo* \(^1\)H MRSI based on an elevated *in vivo* total choline resonance (11,19).

Since absolute concentrations of individual choline-containing phospholipid metabolites are very difficult to measure using *in vivo* \(^1\)H MRSI, the total choline to creatine ratio has been used to identify recurrent prostate cancer after radiation (11,19). In this study, the concentration of creatine did not significantly change between benign, indolent and aggressive cancer biopsy tissues after radiation therapy, indicating that the changes observed in the total choline to creatine ratio after radiation predominately reflect changes in the concentration of choline-containing phospholipid metabolites. There was significant changes in the mean tCho/Cr ratio between benign indolent and aggressive cancer tissues after radiation therapy, and the area under the ROC curve analysis of tCho/Cr demonstrated an accuracy of 95% (confidence interval = 0.88 to 1.00) for predicting aggressive recurrent disease. These findings are consistent with prior MRI/\(^1\)H MRSI studies of prostate cancer patients after radiation therapy demonstrating that tCho/Cr provided the most accurate predictor of the presence of recurrent cancer after radiation therapy (11,19). The mean tCho/Cr ratio threshold for the detection of any (indolent or aggressive) recurrent cancer (≥ 1.5 ± 0.2) after radiation in this study was not different from the ratio that was determined from a ROC analysis of prior *in vivo* data (tCho/Cr threshold ratio of ≥ 1.5). The tCho/Cr ratio was significantly higher for identifying aggressive cancer (tCho/Cr = 2.4 ± 0.4) suggesting that a higher threshold tCho/Cr ratio could improve the selection and
therapeutic planning of patients that would benefit most from salvage focal therapy after failed radiation therapy.

This study has several limitations. Although biopsy tissues provided a more accurate snapshot of \textit{in vivo} metabolism, there were a number of contaminants compared to surgical samples. These contaminants arose from periprostatic lipid contamination and the topical anesthetic (Hurricane®; Beulich) that was applied prior to the TRUS procedure. This resulted in 5 (~6%) prostate biopsy samples being unusable for determining lactate concentrations. The lack of significance of lactate concentration to predict recurrent aggressive cancer could be due to this smaller sample size since there was trend towards significance. Additionally, only two additional research biopsies could be obtained per patient and the sample sizes were much smaller than what can be obtained from surgical specimens. Nevertheless, the use of snap-frozen biopsy tissues was critical for this study in order to minimize the impact of anaerobic glycolysis, which occurs in surgical specimens. We attempted to overcome the subjectivity of determining cancer percentage, Ki-67 labeling index and cancer grade for biopsies by averaging duplicate readings of two prostate pathologists with extensive experience, but a more quantitative pathologic approach would be important in future studies (78). Another limitation of this study was the variability of the cancer percentage within the biopsy tissues used. This is an unfortunate consequence of the fact that many TRUS – guided biopsy tissues obtained have very small amounts of prostate cancer. We found that correcting the samples for percentage cancer was problematic for specimens containing small amounts of prostate cancer. However, to minimize the impact of the % cancer on the statistical comparison of tissue types before and after radiation therapy, we made sure there was no significant difference was observed between the average
percentage of biopsy core positive for untreated and treated cancer samples or between the recurrent indolent and aggressive cancer samples. Most likely, the differences between benign, indolent and aggressive cancer would be even more significant if tissues could be obtained with higher percentages of cancer. Future studies could use direct MR-guided biopsies, recently approved by the Food and Drug Administration (79) to provide improved the collection of higher percentages of cancer from the dominant lesion in the prostate.

**Conclusion**

This study demonstrated that the concentrations of phospholipid metabolites (PC, GPC and free Cho), and lactate were significantly elevated in recurrent cancer as compared to surrounding benign tissues. There was also a significant increase in phosphocholine concentration and reduction in glycerophosphocholine concentration between untreated and irradiated prostate cancer biopsies that were associated with significantly higher Ki-67 staining indices and pathologic grade. Other prostate metabolites, including citrate, polyamines, alanine, and glutamate which demonstrated significant differences in their concentrations between untreated cancer and benign biopsy tissues were typically reduced to the noise level of $^1$H HR-MAS spectra for post radiation biopsies. The concentration of the choline-containing phospholipid metabolites and the associated total choline to creatine ratio in recurrent aggressive cancer was significantly higher than irradiated benign tissues ($\approx$3 fold) and recurrent indolent cancer biopsies ($\approx$ 2 fold). These results support the role of *in vivo* $^1$H MRSI to discriminate between aggressive/progressive and indolent dying prostate cancer following radiation therapy based on the magnitude of elevation of the *in vivo* total choline to creatine ratio. This hypothesis will need
to be tested through future in vivo Multi-parametric MR studies of patients with rising PSA after failed radiation therapy.

References


75. Zhang VY, Bok R, Sukumar S, Cunha A, Hsu IC, Pouliot J, Vigneron D, Kurhanewicz J. Detecting Early Tumor Response of Prostate Cancer to Radiation Therapy Using Multi-Parametric 14T \textsuperscript{1}H and Hyperpolarized \textsuperscript{13}C MR Imaging. 20th Scientific Meeting and Exhibition of ISMRM, Melbourne, Australia 5-11 May, 2012, program# 4319.


Chapter 5: Detecting Early Radiation Dose-Dependent Changes of Prostate Cancer using Multi-parametric 14T $^1$H and Hyperpolarized $^{13}$C MR Imaging

Introduction

External beam radiation therapy is one of the most common therapeutic modalities for treating clinically localized prostate cancer(1), but despite excellent success rates, a significant number of patients suffer post-treatment cancer recurrence(2,3). Several dose-escalation trials have shown improved local and biochemical disease control in localized prostate cancer(3-7). The low alpha-beta ratio for prostate cancer suggests that a hypofractionated regimen with larger doses per fraction is biologically advantageous(8,9). Significant technological advancements such as image–guided intensity modulated radiation therapy and brachytherapy have improved the delivery of larger and more biologically effective doses to prostate cancer(4,6,7). These targeted radiation therapy techniques require high accuracy and precision in the delivery of large doses to the tumors while sparing the surrounding healthy tissues. The ability to accurately assess early tumor response to radiation in a dose dependent fashion is crucial for clinicians to optimize treatment regimen personalized to individual patients. Additionally, assessment of early dose response of the tumor verifies whether irradiation to the target volume in prostate cancer has been delivered as planned. The ability to monitor localized dose depositions in the tumors allows clinicians to modify the dose-planning system to amend the fractionation therapy in the subsequent dose delivery.
Fluoro-2-deoxyglucose (FdG) positron emission tomography (PET) studies have shown reduced glucose uptake can identify early treatment response (10-13). However, FdG-PET uptake has played a limited role in prostate cancer mainly because of the relatively low differential uptake of FdG in most primary prostate tumors and because the bladder produces a very strong FdG signal near the prostate. Proton MR spectroscopic imaging ($^1$H MRSI) diagnoses of prostate cancer are based on the identification of neoplastic metabolism (14,15) and studies have demonstrated the ability of combined MRI/$^1$H MRSI to discriminate residual or recurrent prostate cancer from residual benign tissue and atrophic/necrotic tissue after radiation therapy (16,17). A study from snap-frozen, patient biopsies after radiation therapy has shown phospholipid metabolites discriminate between aggressive/progressive and indolent/dying prostate cancer following radiation therapy (18). $^1$H MRSI may therefore be a sensitive tool for monitoring early radiation-induced changes in tumors.

Early changes in tumor microenvironment following radiation therapy have been investigated using diffusion weighted imaging (DWI) and dynamic contrast enhancement (DCE) MRI. Apparent diffusion coefficient (ADC) calculated from DWI measures the water diffusion within biological tissues, which is related to tissue cellularities and the integrity of cell membranes. Clinical studies (19-21) have shown that ADC increased significantly following radiation therapy in prostate cancer patients, indicating a decrease in cellular density. ADC has also been shown in increase in a dose dependent fashion in preclinical dose escalation studies using murine and rat glioma models (22,23). DCE MRI investigates the tumor microvasculature by measuring tissue perfusion, microvessel permeability, and extravascular, extracellular space. DCE MRI has shown
early enhancement following radiation therapy in prior studies of patients with prostate cancer(24,25) and xenograft TRAMP tumors(25).

Hyperpolarized (HP) MR is a revolutionary new molecular imaging technique that can provide more than 10,000-fold signal enhancement for $^{13}$C labeled compounds, allowing for rapid imaging of alterations in metabolic and physiologic processes non-invasively(26-28). Prior studies have demonstrated the potential for using hyperpolarized [1-$^{13}$C]pyruvate to monitor disease progression and for making an early assessment of response to therapy(29-36). The production of lactate from pyruvate mediated by lactate dehydrogenase (LDH)(29,37,38) is part of glycolysis, which is up-regulated in many tumor types(34,39-41). Reduction of the flux between [1-$^{13}$C]lactate and [1-$^{13}$C]pyruvate observed in breast cancer model after radiation therapy appeared to be associated with cell death and changes in cellular membrane transport of monocarboylic acid and lactate dehydrogenase expressions(36). Cell death, senescence, and growth arrest are typical pathological features after radiation therapy(42-45). Following radiation therapy, tumor cell death is correlated with dose levels described by the linear quadratic survival curve(46). Another hyperpolarized biomarker, HP [$^{13}$C]urea, is metabolically inactive and has shown great potential for perfusion imaging(47). Unlike gadolinium (Gd) –containing (paramagnetic) compounds, as an endogenous compound, HP [$^{13}$C]urea is nontoxic. Other advantages of HP [$^{13}$C]urea over Gd DCE include direct proportionality of signal to tracer concentration, absence of background signal, and the ability to co-polarize with HP [1-$^{13}$C]pyruvate for combined perfusion and metabolic imaging(48).
In this study, we investigated the early radiation dose dependent changes of the transgenic adenocarcinoma of mouse prostate (TRAMP) tumor using both the clinical $^1$H MR imaging approach, including DCE and DWI, and a clinical translatable hyperpolarized $^{13}$C technique, including [1-$^{13}$C]pyruvate and [$^{13}$C]urea. We also correlated the multi-parametric MRI data with histopathological findings and gene expression analysis to identify physiological and metabolic changes in tumors to the impact of increasing radiation doses. These early, dose dependent tumor responses might improve radiation treatment planning and the optimization of radiation therapy regimen to individual patients.

**Methods**

*Animal preparations and treatment*

All animal studies were studied according to a protocol approved by the UCSF Institutional Animal Care and Utilization Committee. When the tumor of a TRAMP mouse reached approximately 2 cc in volume, the mouse was either sacrificed for histopathological and gene expression analyses as a control or treated with radiation. This relatively large tumor volume was selected for this study in order to be able to place the surface radioactive seed close to the tumor, have a sufficient range of radiation doses across the tumor and to minimize radiation doses to surrounding healthy tissues. A total of 24 mice were included (13 treated and 11 untreated) in this study. Multi-parametric MRI was acquired from the 13 treated TRAMP mice at pre-radiation and 1 day, 4 days, and 7 days after radiation. Histopathological and gene expression analyses were performed on both untreated and treated TRAMP tumors. During radiation therapy, the mice were anesthetized with a mixture of 1-1.5% isoflurane and O$_2$ at 1 l/min. Ionizing radiation was delivered to the tumors using the Nucletron microSelectron system (Ir-192). A Leipzig
HDR applicator made of tungsten alloy was used to deliver radiation to the tumor. Ten Gy in single fraction was prescribed to a 3 mm depth. This provides a steep dose gradient within the tumor with 14 Gy delivered on the ventral side of the tumor and 4 Gy on the dorsal side of the tumor, as shown in Figure 4.1. The tumor region receiving 9-14 Gy was defined as region treated with high dose radiation, while tumor region receiving 4-9 Gy was defined as region treated with low dose radiation. Following radiation treatment, axial CT images (matrix = 512×512×106 and spatial resolution = 0.10547 × 0.10547×1 mm³) were acquired (Somatom; Siemens Medical Solutions, Malvern, PA). Based on the CT images, a radiotherapy dose distribution map was generate from the radiation treatment plan (OncentraBrachy; NucleotronInc., Veenendaal, The Netherlands). A rigid registration algorithm based on Mattes Mutual information registration metric was used for co-registration of CT and MR images (3D Slicer version 3.6.3, available online: http://www.slicer.org)(49,50).

Figure 5.1: Radiation dose gradient overlaid on top of a T₂-weighted axial MR image of a tumor.

A Leipzig HDR applicator was position on the ventral side of the tumor and delivered a steep dose gradient within the tumor. The ventral side of the tumor received the highest dose of 14 Gy, while the dorsal side of the tumor received lower dose of 4 Gy. Tumor region receiving 9-14 Gy (ventral half of the tumor) was defined as region treated with high dose radiation and tumor
region receiving 4-9 Gy (dorsal half of the tumor) was defined as region treated with low dose radiation.

\(^1\)H and hyperpolarized \(^{13}\)C MRI in vivo studies

**Hardware and agent**

All MR experiments were done using a Varian 14.1T imaging spectrometer with a 98 mm bore vertical magnet and controlled by a Direct-drive console (Agilent Technologies, Santa Clara, CA, USA). The system is equipped with a 55 mm 100 G/cm gradients and insert volume RF coils for \(^1\)H and \(^{13}\)C supplied by the manufacturer.

A HyperSense™ DNP polarizer (Oxford Instruments, Abingdon, UK) was used to polarize the \(^{13}\)C agents as described previously\(^{(48)}\). 24 µl neat \([^{1-^{13}}\text{C}]\text{pyruvic acid with 16.5 mM trityl radical (GE Healthcare)}\) and 1.5 mM Dotarem® (Guerbet) and 55 µl \([^{13}\text{C}]\text{urea (6.4 M in glycerol)}\) with 17.5 mM trityl radical OX63 (Oxford Instruments) and 0.2 mM Dotarem® were co-polarized. Optimum polarization was achieved by adding the urea and pyruvic acid solutions to a sample cup separately and freezing them rapidly in a liquid nitrogen bath to form two separate glass layers. This was followed by dissolution in 4.5 ml of buffer containing 40 mM Tris, 80 mM NaOH, and 0.3 mM Na\(_2\)EDTA. The resulting dissolution mixture contained 80 mM \([^{1-^{13}}\text{C}]\text{pyruvate and 74 mM }^{[^{13}\text{C}]\text{urea}}\)(48).

**MRI experiments**

MRI data were acquired at several time points (within 1 day before radiation, and 1, 4, and 7 days after radiation). At the time of the MR experiment, the mice were anesthetized using a
mixture of 1-1.5% isoflurane and 100% O₂ at 1 l/min, kept warm with a circulating water heating pad, and positioned vertically in the magnet using a custom animal positioning apparatus. Care was taken during setup so that the mouse position in the magnet was similar between studies of the same mouse. The respiration was continuously monitored using an animal monitoring system (SA Instruments, Stony Brook, NY, USA). For ⁴¹H MRI studies, data acquisition was gated with respirations to reduce imaging artifacts. A 400 µl volume of the mixture with prepolarized [¹³C]pyruvic acid and [¹³C]urea, followed by a 150 µl saline flush, was injected through the catheter secured in the tail vein or jugular vein of the mouse over a period of about 15 s. The pH of the dissolution mixture was verified to be within the range of 7-7.5 prior to the injection into the mice. For the hyperpolarized dynamic study, a ¹³C spectrum was acquired every 3 s from a 10 mm thick slab centered on the primary tumor in a TRAMP mouse. The spectra were recorded using a 5° excitation pulse, 20k points, and a 20 kHz bandwidth. Hyperpolarized dynamic study was performed on 5 untreated TRAMP mice.

Single shot, frequency and time specific ¹³C 3D imaging was performed on the mice using an echo-planar (GRASE) sequence (51) with multiple 180° refocusing pulses during the echo train to minimize T₂* effects. The 90° and 180° pulses used in the sequence were chemical shift selective pulses designed to excite only the resonances of interest. The ¹³C image data acquisition was initiated 45 s after the injection. Resonances of [¹-¹³C]lactate, [¹-¹³C]alanine, [¹-¹³C]pyruvate, [¹³C]urea, HP001 (bis-1,1-(hydroxymethyl)-¹³C-cyclopropane-d₈; reference phantom) were excited and sampled sequentially. Phase encode steps were kept at 12×12×16 resulted in 40×40×40 mm³ FOV, 2.5×3.3×3.3 mm³ resolution and scan time of 153 ms per image. The ¹³C data were zero-filled to 32×32×32 and the magnitude images were reconstructed.
to a 1.25×1.25×1.25 mm³ resolution. T₂-weighted ¹H anatomical images were acquired using a spin-echo pulse sequence (Axial: FOV = 40×40 mm, 256×256 matrix, 1.25 mm slice thickness, total of 24 slices from the same slice locations of the ¹³C images; Coronal: FOV = 256×256 matrix, 1.25 mm slice thickness, total of 24 slices) for localization and overlay of the ¹³C images. ROIs around the tumor were drawn on axial T₂-weighted ¹H anatomical images using 3D Slicer, tumor volume was calculated as summed ROI multiplied by the slice thickness. Hyperpolarized urea signals were normalized to the urea signals from the kidneys of the same animal from the same study.

Prior to the hyperpolarized ¹³C study, diffusion weighted ¹H MR imaging was performed using a spin-echo sequence with the diffusion-sensitizing gradient applied along the y-axis, with the following parameters: TE/TR = 20/1200 ms, matrix = 256×128 zero-filled to 256×256, FOV = 40×40 mm², slice thickness = 1 mm with 0.25 mm gap, 18 slices, gradient duration = 2 ms, delay between gradients = 13 ms, and b values of 18.60, 181.74, 324.49, 508.03 s/mm²). ADC value was generated for each image voxel online using Varian VNMRJ 3.1A software (Agilent Technologies, Santa Clara, CA)

DCE ¹H MR imaging was performed following the hyperpolarized ¹³C study. A T₁-weighted gradient echo sequence was acquired a period of 5 min with the following parameters: TE/TR =1.11/39 ms, flip angle = 40°, matrix = 128×128, FOV = 40×40 mm², spatial resolution = 0.3125×0.3125 mm², slice thickness=1 mm with 0.25 mm gap, 10 slices, and temperature resolution=6 s. A bolus dose of 100 µL of Gd-DTPA (1/6 x 500 mmol in normal saline with heparin (2 unit/ml); Magnetvist; Bayer Healthcare) followed by 150 µL of saline flush was
injected into the tail vein or the jugular vein of the mouse over 15 s after the first 5 measurements. The baseline $T_1$ values were calculated from the images acquired with multiple flip angles (2, 5, 15, 25, 35, and 40). A series of 40 dummy scans was incorporated to ensure steady state was reached prior to acquisition of the relevant data. The signal intensity of each voxel was converted into the contrast agent concentration by solving the nonlinear relationship between signal intensity and contrast agent concentration (52). Sum of concentration observed over 5 min (area under the time curve, or AUC), uptake slope, and washout slope were calculated for each voxel.

Ex vivo assays

**Histopathologic analysis**

TRAMP tumor tissues were harvested at pre-radiation, 4 days, or 7 days post-radiation. Pimonidazole was injected into the mice 45 – 60 min prior to dissection. The histopathology was correlated with the anatomic images by displaying the axial, coronal, and sagittal images of the TRAMP mice on a laptop computer during the dissection using 3D Slicer. Additionally, digital photographs were taken during the dissection and subsequently reviewed. For each tumor, excised tissues were taken from tumor region treated with high dose radiation (9-14 Gy, the ventral region of the tumor) and low dose radiation (4-9 Gy, the dorsal region of the tumor). Next, each excised region was further dissected with one portion snap-frozen (and stored at -80 °C) and the other portion immediately fixed in 10% buffered formalin and embedded in paraffin blocks. Tissue blocks were cut into 5 μm thick sections on a Leica microtome. Sections were dried onto glass slides and stained for hematoxylin and eosin (H&E) using standard protocols. Additional sections were cut and stained with mouse monoclonal antibodies using standard
protocols: anti-Ki-67 (DAKO), anti-caspase-3/ASP175 (Cell Signaling), and anti-pimonidazole IgG1 (Hypoxprobe-1 MAb1 kit). The stained slides were submitted to an oncologist for histopathological and immunohistochemical evaluations.

**Quantitative real-time PCR analysis**

Total RNA was extracted from the snap-frozen tissues and purified with RNeasy procedure kit (Qiagen). The quality of RNA was confirmed using an RNA 6000 Nano Chip kit (Agilent, Santa Clara, CA). Reverse transcription was performed using the iScript cDNA Synthesis kit (BioRad Laboratories). PCR was conducted in triplicate for the lactate dehydrogenase-α (LDHA), lactate dehydrogenase-β (LDHB), the monocarboxylate transporters 1, 2, and 4 (MCT1, 2, 4), Hif1-α, VEGF, glucose transporter 1 (Glut1), and hexokinase 2 (HK2) on the ABI 7900HT (LApplied Biosystems). L19, a nonregulated ribosomal housekeeping gene, was used as the internal control. The relative fold difference was calculated for each primer/probe combination.

**LDH activity assay**

Total LDH activity was measured spectrophotometrically by quantifying the linear decrease in NADH absorbance at varying pyruvate concentrations at 339 nm using an Infinite M200 spectrophotometer (Tecan Group Ltd.). The reaction rate was normalized to total protein concentration (Quick Start Bradford Protein Assay, Bio-Rad). About 2.36 mg of snap-frozen tissue was used for each study. The maximum velocity ($V_{\text{max}}$) and the Michaelis-Menten constant ($K_{\text{m}}$) were estimated using the Lineweaver-Burke plot.
Statistical analysis

Statistical analyses were performed using JMP Software (SAS Institute Inc., 2008, version 8.0). The Kruskal-Wallis test was performed to evaluate the difference among pre-radiation (or untreated), tumor region treated with high dose radiation, and tumor region treated with low dose radiation. Wilcoxon signed-rank test was then used to evaluate pair-wise comparisons. All results were expressed as mean ± SE.

Results

The time course of the delivery of hyperpolarized $^{13}$C urea, pyruvate, and its metabolic conversion to lactate and alanine in a 10 mm thick slab from an untreated TRAMP mouse with 2.1 cc tumor is shown in Figure 5.2. The hyperpolarized urea and pyruvate reached their maximum levels ~15 s after injection. Hyperpolarized lactate was observed almost immediately following the arrival of the labeled pyruvate and increased at a slightly slower rate from the labeled pyruvate. Hyperpolarized urea and pyruvate signals rapidly decreased from their maximum due to $T_1$ relaxation, use of the hyperpolarized signal for data acquisition, and conversion of labeled pyruvate to lactate. Labeled lactate, pyruvate, and urea decreased at a slower rate at ~45 s after injection. Hyperpolarized [1-$^{13}$C] alanine and pyruvate hydrate were observed, however, at a substantially lower signal level. For this study, the time course data was used to define the timing of the 3D GRASE imaging experiments.
Figure 5.2: The time course for the hyperpolarized $^{13}$C urea, [1-$^{13}$C] pyruvate, and its metabolic products following the injection of 350 µl of hyperpolarized urea and pyruvate. The MR spectra were acquired on the 14.1T scanner every 3 s from a TRAMP mouse with a 2.1 cc primary tumor using a 5° flip angle and a 10 mm thick slice. The peak area plot was corrected for the amount of magnetization used for previous acquisition by dividing each peak area by $\cos^0(5^\circ)$. $n =$ number of flip angles. Based on this time course, the subsequent 3D GRASE data was recorded at 45 s, a time when the rates of change for hyperpolarized lactate, pyruvate and urea signals were minimal.

The 13 radiation treated TRAMP mice (with tumor volume = 2.1 ± 0.44 cc) were histologically classified as high-grade tumors(29) as the extracted viable tissues were over 95% poorly differentiated as shown in Table 1. The 11 untreated TRAMP mice used for histopathological
and gene expression analysis comparisons were also classified as high-grade tumors as the extracted tissues were also over 95% poorly differentiated. There was no significant difference in the percentage of poorly differentiated viable tissues between treated and untreated TRAMP tumors, therefore they can be directly compared in histopathology and gene expression analyses.

<table>
<thead>
<tr>
<th>Histology</th>
<th>Untreated</th>
<th>High dose</th>
<th>Low dose</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poorly differentiated</td>
<td>96.3% ± 2.2%</td>
<td>97.0% ± 0.9%</td>
<td>97.2% ± 0.7%</td>
<td></td>
</tr>
<tr>
<td>(% In viable tissue)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Necrotic/fibrotic tissue</td>
<td>16.3% ± 4.3%</td>
<td>40.6% ± 7.4%</td>
<td>20.3% ± 3.6%</td>
<td>.04$^a$, .006$^c$</td>
</tr>
<tr>
<td>(% In total tissue)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( % Stained)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki-67</td>
<td>81.4% ± 3.8%</td>
<td>49.5% ± 8.6%</td>
<td>69.0% ± 7.7%</td>
<td>.004$^a$, .02$^b$, .006$^c$</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>2.8% ± 1.5%</td>
<td>5.8% ± 2.1%</td>
<td>4.2% ± 1.2%</td>
<td></td>
</tr>
<tr>
<td>PIM</td>
<td>28.6% ± 10.2%</td>
<td>20.4% ± 5.1%</td>
<td>15.3% ± 2.9%</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Control versus high dose. $^b$Control versus low dose. $^c$High dose versus low dose.

Table 5.1. Comparison of histology and immunohistochemistry in untreated tumors, tumor region treated with high dose radiation, and tumor region treated with low dose radiation.
Hyperpolarized pyruvate to lactate metabolism significantly decreased in a dose dependent fashion by 1 day after radiation therapy, prior to any changes observed using $^1$H DCE and diffusion weighted imaging.

Representative multi-parametric MRI images including hyperpolarized lactate-to-pyruvate ratio (only within the tumor) (A), hyperpolarized urea (B), uptake slope maps from DCE (C), and ADC maps (D) calculated from diffusion weighted imaging of a TRAMP tumor at pre-radiation, 1 day, 4 days, and 7 days post-radiation are shown in Figure 5.3. As shown in Figure 5.3E, tumor volume stayed relatively constant 1 to 4 days and decreased significantly to 75.0% pre-radiation by 7 days post-radiation. The spatially interpolated hyperpolarized lactate-to-pyruvate ratio image from this mouse at pre-radiation in Figure 5.3A appears to be relatively uniform within the tumor. By 1 day post-radiation, the lactate-to-pyruvate ratio in tumor region treated with high dose radiation (ventral side of the tumor) decreased dramatically while the lactate-to-pyruvate ratio in the tumor region treated with low dose radiation (dorsal side of the tumor) did not change. This dose dependent difference in the lactate-to-pyruvate ratio was still present at 4 days post-radiation. By 7 days post-radiation, the lactate-to-pyruvate ratio in tumor regions treated with both high and low dose radiation decreased to about half of the pre-radiation levels.

Quantitatively, these observations proved true, with the lactate-to-pyruvate ratio significantly decreased in a dose dependent fashion 1 day post-radiation, prior to any changes observed using $^1$H DCE and diffusion weighted imaging. As shown in Figure 5.4A, by 1 day post-radiation, the lactate-to-pyruvate ratio decreased significantly to 80.0 ± 7.8% and 93.1 ± 5.7% pre-radiation ($P$s < .05, < .01 comparing to pre-radiation) in tumor regions treated with high dose and low dose, respectively. This dose dependent decrease in the lactate-to-pyruvate ratio was still present at 4 days post-radiation where the lactate-to-pyruvate ratio significantly decreased in both tumor
regions treated with high dose and low dose (67.6 ± 6.8% and 83.2 ± 6.8% pre-radiation, $P$s < .001, < .01 comparing to pre-radiation, respectively). Finally, by 7 days post-radiation, the lactate-to-pyruvate ratio was decreased similarly in both the high and low dose regions to 48.9 ± 3.6% and 59.5 ± 5.2% pre-radiation ($P$s < .001, < .001 comparing to pre-radiation). The percentage changes in lactate-to-pyruvate ratio post-radiation in tumor regions treated with high dose vs. low dose were significantly different from pre-treatment for 1 day, 4 days, and 7 days ($P$s < .05, < .01, < .05 comparing high dose region vs. low dose region, respectively) after radiation therapy, whereas tumor volume did not change significantly until 1 week after treatment.
Figure 5.3: (A-E) Representative multi-parametric MRI data acquired at different time points (pre-radiation, and at 1, 4, and 6 days post-radiation). (A, B) Hyperpolarized $^{13}$C lactate-to-pyruvate ratio and HP $^{13}$C urea normalized by urea signal in the kidneys overlaid on T$_2$-weighted MRI; (C) AUC values calculated from $^1$H DCE; (D) Uptake slope values calculated from $^1$H
DCE; (E) ADC values calculated from diffusion weighted imaging. (F) Tumor volumes as measured by T2-weighted MRI at different time points (pre-radiation, and at 1, 4, and 6 days post-radiation). The statistical significance compared with pre-radiation: **$P < .01$, not marked = not significant.

*Hyperpolarized $^{13}$C urea and $^1$H DCE both show increased perfusion/permeability by 4 days post-radiation*

We used hyperpolarized $^{13}$C urea and $^1$H DCE to investigate changes in vascular perfusion and permeability following radiation therapy. Figure 5.3(B and C), 5.4B, and 5.5 show normalized hyperpolarized $^{13}$C urea, and initial slope maps calculated from DCE both significantly increased by 4 days post-radiation in both tumor regions treated with high dose and low dose. As shown in Figure 5.4B, at day 4 post-radiation, urea signals significantly increased to $151 \pm 16.9\%$ and $140 \pm 16.2\%$ pre-radiation ($Ps < .01$, $< .05$, comparing to pre-radiation) in tumor regions treated with high dose and low dose, respectively. Hyperpolarized $^{13}$C urea signals dropped slightly by 7 days post-radiation but were still significantly higher than pre-radiation values ($147 \pm 16.6\%$ and $126 \pm 13.8\%$, $Ps < .01$, $< .05$, as compared to pre-radiation) in tumor regions treated with high dose and low dose, respectively.
Figure 5.4. Quantitative analysis of hyperpolarized $^{13}$C metabolic imaging at different time points comparing to pre-treatment for tumor region treated with high dose (9-14 Gy) and low dose (4-9 Gy). (A: left) Ratio of lactate-to-pyruvate measure in the TRAMP tumors. (B: right) Urea signals in the tumors normalized by urea signals in the kidneys. The statistical significance compared with pre-radiation: $*P < .05$, $**P < .01$, $***P < .001$, not marked = not significant.

Figure 5.5 Quantitative analysis of DCE $^1$H MR imaging at different time points comparing to pre-treatment for tumor region treated with high dose (9-14 Gy) and low dose (4-9 Gy) radiation. (A: left) Changes in DCE area under the curve comparing to pre-treatment. (B: right) Changes in DCE Gd uptake slope comparing to pre-treatment. The statistical significance compared with pre-radiation: $*P < .05$, $**P < .01$, $***P < .001$, not marked = not significant.

Figure 5.5B shows following radiation therapy, uptake slope maps calculated from $^1$H DCE increased from pre-radiation in the same fashion as hyperpolarized urea. By day 4 post-radiation, uptake slope values significantly increased to $142 \pm 14.0\%$ and $142 \pm 12.2\%$ pre-radiation ($Ps < .01$, $< .01$, as compared to pre-radiation) in tumor regions treated with high dose and low dose, respectively. Uptake slope values also dropped slightly by 7 days post-radiation but were still
significantly higher as compared to pre-radiation with 130.0 ± 11.4% and 114.2 ± 12.8% pre-radiation ($Ps < .01, < .05$, comparing to pre-radiation) in tumor regions treated with high dose and low dose, respectively.

Figure 5.5A shows the AUC values calculated from DCE were significantly elevated from pre-radiation only at 4 days post-radiation. At 4 days post-radiation, AUC values significantly increased to 132 ± 18.2% and 126 ± 13.2% pre-radiation ($Ps < .05, < .05$, comparing to pre-radiation) in tumor regions treated with high dose and low dose, respectively. By 7 days post-radiation, AUC values dropped in both high and low dose treatment regions and were no longer significantly different from pre-radiation. The differences in percentage change post-radiation between initial slope and AUC, both calculated from DCE, are possibly due to the way they are calculated. Initial uptake measures the Gd uptake within the tissue at the beginning of the DCE acquisition, while AUC is a measurement of Gd concentration within the tissue over 5 min. The observation that the enhancements of AUC values are less than uptake slope values post-radiation is likely due to increased vascular permeability following radiation injury, which would result in Gd leaking out of the tissue earlier during DCE acquisition.

Interestingly, at pre-radiation, hyperpolarized urea measured from ventral portion of the tumor (which received high dose radiation during treatment) was significantly lower ($P < .05$) than the dorsal portion of the tumor (which would be irradiated with low dose radiation during treatment), but this difference was not significant in DCE measurements. An explanation for this could be that the ventral portion of the tumor is further away from the main artery resulting in poorer perfusion. Higher cellular density (reflected by ADC measurements, Figure 5.6) could also
reduce perfusion. The reason that hyperpolarized urea was able to pick up the heterogeneity in perfusion is likely due to the fact that urea is smaller in molecular mass comparing to Gd-DTPA.

Figure 5.6. Quantitative analysis of ADC values calculated from diffusion weighted $^1$H MR imaging at different time points comparing to pre-treatment for tumor region treated with high dose (9-14 Gy) and low dose (4-9 Gy) radiation. The statistical significance compared with pre-radiation: **$P < .05$, *$P < .01$, ***$P < .001$, not marked = not significant.

**ADC values significantly increased post- high dose radiation**

Figure 5.3D and 5.6 show that ADC values significantly increased by 4 days post-radiation in regions of tumor treated with high dose radiation (116 ± 7.7%, $P < .05$ comparing to pre-radiation). By 7 days post-radiation, ADC values continued to increase in these regions (146 ± 23.1%, $P < .05$ comparing to pre-radiation). There was no significant difference in ADC in regions of tumor treated with low dose radiation. The increase in ADC values suggests a reduction in cellular density resulting from a significant portion of the cells receiving a lethal dose of radiation. At pre-radiation, ADC values measured from ventral portion of the tumor
(irradiated with high dose radiation) were significantly lower \((P < .01)\) than the dorsal portion of the tumor (irradiated with low dose radiation), suggesting the ventral portion of the tumors had higher cellular density prior to treatment.

*At pathology, the necrotic/fibrotic tissue percentage significantly increased in regions of high dose radiation*

TRAMP tumors were harvested at baseline, and 4 days or 7 days post-radiation for H&E, Ki-67, Caspase-3, and pimonidazole (PIM) staining and gene expression analysis. No significant difference was observed between tissues harvested at 4 days and 7 days post-radiation. Representative H&E and Ki-67 images from pre-radiation tumor tissue, and tumor regions treated with high- and low-dose radiation are shown in Figure 5.7. In regions of high dose radiation, the histological sections showed hypo-cellularity with necrosis and fibrosis, nuclear shrinkage, and vacuolation changes. In contrast, the untreated tumor exhibited high cellularity, nuclear atypia, and no vacuolation on histology. As demonstrated in Table 5.1, Ki-67 staining significantly decreased \((P < .01, < .05, \text{ in tumor regions treated with high low dose radiation as compared to baseline, respectively})\) in a dose dependent fashion. The percentage of necrotic/fibrotic tissue increased significantly in high dose radiation regions, while no difference was observed in region treated with low dose radiation. This dose dependent change in necrosis/fibrosis post- high dose radiation correlated with the observed ADC findings, indicating significant amount of cell death.
mRNA expression analysis shows LDHA and MCT4 decreased in a dose dependent fashion post-radiation.

To better understand the mechanisms underlying the $^1$H and hyperpolarized $^{13}$C MRI changes associated with increasing radiation dose, we assayed the following key enzymes: activity level of LDH, and expression of LDHA, LDHB, MCT1, 2, 4, Hif1-α, VEGF, Glut1, and HK2. Table 5.2 shows the enzymes whose activity and/or expression changed significantly with radiation treatment, LDH activity was found to be significantly ($p < 0.01$) decreased post-radiation in a dose dependent fashion. As shown in Figure 5.8, LDH activity was reduced by ~75% in tumor region treated with high dose radiation. LDHA encodes the predominantly M isoform of LDH, which catalyzes the conversion between pyruvate and lactate(53). Just like the LDH activity, the mRNA expression of LDHA was also significantly reduced in a dose dependent fashion, however to a somewhat lesser extent ($P < .01$, < .01 for tumor region treated with high and low
dose radiation comparing to untreated, respectively). LDHA expression reduced by ~60% in tumor region treated with high dose radiation. The expression of MCT4, which mediates the efflux of the lactate out of the cells (53), was significantly decreased in tumor region treated with high dose radiation compared to untreated tissues ($P = .01$). Even though hypoxia did not change significantly as measured by PIM staining, the mRNA expression of HIF-1α increased significantly in tumor region treated with high dose radiation ($P = .05$). No significant change was observed in mRNA expression of other genes analyzed.
<table>
<thead>
<tr>
<th>Relative expression (%)</th>
<th>Untreated</th>
<th>High dose</th>
<th>Low dose</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDHA</td>
<td>44.8% ± 3.0%</td>
<td>14.1% ± 1.0%</td>
<td>19.9% ± 2.9%</td>
<td>.002$a$, .008$b$, .04$c$</td>
</tr>
<tr>
<td>MCT4</td>
<td>0.04% ± 0.01%</td>
<td>0.006% ± 0.002%</td>
<td>0.018% ± 0.008%</td>
<td>.01$a$</td>
</tr>
<tr>
<td>Hif1-α</td>
<td>1.4% ± 0.1%</td>
<td>3.4% ± 0.5%</td>
<td>1.8% ± 0.01%</td>
<td>0.05$a$</td>
</tr>
</tbody>
</table>

**Enzyme activity**

| LDH$^*$               | 1.7 ± 0.3 | 0.43 ± 0.04 | 0.62 ± 0.07 | .003$a$, .007$b$, .05$c$ |

Relative expression values are normalized to L19 housekeeping gene.

$a$ Control *versus* high dose.  $b$ Control *versus* low dose.  $c$ High dose *versus* low dose.

$^*$LDH activity as measured by $V_{max}$ (nmol NAD/mg protein/min).

Table 5.2. Comparison of enzyme activity and mRNA expressions in untreated tumors, tumor region treated with high dose radiation, and tumor region treated with low dose radiation.
Figure 5.8 Percentage change relative to untreated tissues in mRNA expression in tumor regions treated with high dose (9-14 Gy) and low dose (4-9 Gy) radiation. The statistical significance compared high dose region and low dose region: *$P < .05$, not marked = not significant.

**Discussion**

Clinical studies have demonstrated radiation dose escalation with hypofractionated radiation therapy regimens significantly improve disease-free survival for high-risk prostate cancer patients(5,54). Accurate assessment of early tumor response to treatment is crucial in determining whether to continue current therapy or switch to an alternative therapy. We investigated the physiologic and metabolic changes in TRAMP tumors following radiation therapy using both clinical $^1$H MR imaging approach, including DCE and diffusion weighted imaging, and a clinical translatable hyperpolarized $^{13}$C technique, and interrogated the histopathological and biochemical basis of the multi-parametric MRI data and their relationship to tumor response to high and low doses of radiation. In this study we show that the hyperpolarized lactate-to-pyruvate ratio decreased significantly by one day after radiation therapy, preceding any changes observed using conventional clinical MR methodologies such as DCE and diffusion weighted imaging. Also, The change in pyruvate metabolism following
therapy was radiation dose dependent. There was a greater reduction in lactate-to-pyruvate ratio in tumor region treated with high dose radiation comparing to tumor region treated with low dose radiation at both 1 day and 4 days post-radiation. Additionally, we found perfusion and/or permeability, measured by hyperpolarized $^{13}$C urea and $^1$H DCE, increased significantly post-radiation. ADC values also increased significantly in tumor region treated with high dose radiation. These radiation dose dependent changes observed by the multi-parametric $^1$H and hyperpolarized $^{13}$C imaging approach can be used as markers of early tumor response to the impact of increasing doses of radiation therapy.

The dose dependent decrease in hyperpolarized lactate-to-pyruvate ratio following radiation therapy is due to a combination of factors including cell death, decrease in pyruvate-to-lactate flux, and changes in tumor microenvironment such as blood vessel perfusion and permeability and hypoxia. The significant decrease in tumor volume by 7 days after radiation indicates tumor shrinkage due to cell death. Cell death due to mitotic catastrophe, apoptosis, and senescence, a form of proliferative cell death, is a typical pathological feature following radiation therapy(42-45). Necrosis also can be initiated by high dose, ablative irradiation(55,56). The impact of radiation on cell death can be described by the linear quadratic survival curve where fraction of cell survival is negatively correlated with radiation dose(46). This study also demonstrated a dose dependent cell death following radiation. Both ADC values and the percentage of necrotic and fibrotic tissue within tumor region treated with high dose radiation significantly increased following radiation. Dose dependent decrease in Ki-67 staining following radiation therapy indicates slowing down of tumor cell growth, especially in the region treated with high dose radiation.
In addition to cell death, significant decrease in cellular pyruvate-to-lactate flux, as shown in the reduction of enzyme activity of LDH and mRNA expression of LDHA, also contributes to the changes observed in the hyperpolarized lactate-to-pyruvate ratio in tumors after radiation therapy. Not only did the LDH enzyme activity and mRNA expression of LDHA decrease significantly, but the decreases were also dose dependent. Because the measured LDH enzyme activity is normalized to total protein content, the cause of the decrease in LDH enzyme activity post-radiation is due a combination of cell death and a reduction in cellular LDH activity. Upon radiation injury, the intracellular protein content is released from severely damaged cells and deposited into the interstitial space. An increase in cell death would both reduce viable LDH and increase total protein content within the tumors. Indeed, reduction in LDH has been shown to be a marker of cell death in cell culture experiments (57,58) and acute kidney injury (59). Even though ~75% reduction in LDH activity was observed post-radiation, ~50% reduction was observed in hyperpolarized lactate-to-pyruvate ratio by the last day of the MRI study. Factors that could contribute to this discrepancy include the inflammatory processes initiated by radiation injury and other microenvironment changes such as tumor perfusion. The decrease in mRNA expression of LDHA indicates that the cellular conversion of pyruvate to lactate also declined. Furthermore, mRNA expression of MCT4 also decreased significantly in tumor region treated with high dose radiation. MCT4 is predominantly responsible for the export of lactate out of the cells (53). Rapid lactate efflux serves to maintain high levels of glycolysis in cancer cells and concurrently acidifies the extracellular environment (60). Increase in MCT4 has been associated with promoting invasion and metastasis of renal carcinogenesis (61). Rapid lactate efflux is essential for maintaining a neutral intracellular pH, and a high rate of glycolysis and
lactate production. Lower MCT4 expression is likely to indicate slower rate of lactate export and a decrease in cancer aggressiveness.

Interestingly, while lower PIM staining, a marker of hypoxia, was observed following radiation therapy, although the difference was not significant, the mRNA expression of HIF-1α increased significantly in tumor region treated with high dose radiation. The inflammatory microenvironment has been shown to activate HIF-1α levels to affect macrophage biology under normoxia (62). Upon radiation injury, an inflammatory process starts by forming reactive oxygen species (ROS) and/or nitrogen species (NO) to activate immune cells. ROS and NO stabilize HIF-1α protein and increase HIF-1 activity (63-65). Some reports show that radiation induces HIF-1 activity in tumor cells and promotes the survival of neighboring endothelial cells (66). The elevated expression of HIF-1α in tumor region treated with high dose radiation comparing to untreated tumors and tumor region treated with low dose is likely due to the severity of tissue damage.

The vasculature of a tumor plays an important role in its response to radiation therapy (67). Proton DCE using Gd-DTPA and hyperpolarized $^{13}$C urea show significant increase in AUC and uptake slope measured by $^1$H DCE, and urea signal by 4 days post-radiation in both high dose and low dose irradiated portions of the tumor. No significant difference was found between percentage change from baseline for hyperpolarized urea and DCE uptake slope or AUC between the low and high doses. The increase in vascular perfusion/permeability measured by hyperpolarized urea and DCE is not dose dependent and likely to be systemic within the entire tumor. Hyperpolarized urea was acquired at 45 s after injection. Presumably urea at this early
time point was still getting taken up into the tumor tissue, therefore, hyperpolarized urea is likely to provide comparable information as uptake slope from DCE. We found the percentage increase in DCE uptake slope is slightly less than the increase observed in hyperpolarized $^{13}$C urea signal following radiation therapy. The molecular weight of urea (60 g/mol) is much lower than the Gd-DTPA (938 g/mol), therefore may result in faster diffusion from the intravascular to the extravascular space. Hyperpolarized $^{13}$C urea has been shown previously to provide perfusion information in TRAMP tumors(47). In this study, the increase in hyperpolarized $^{13}$C urea following radiation therapy is comparable to the increase shown in $^1$H DCE, demonstrating the feasibility of using hyperpolarized $^{13}$C urea to characterize changes in vascular perfusion and/or permeability after therapy. Dynamic hyperpolarized $^{13}$C urea studies were performed in Chapter 6 and compared with DCE MRI in order to more fully understand if hyperpolarized $^{13}$C urea provides the same or different information than DCE MRI.

The enhancement in $^1$H DCE in tumors at the early stage following irradiation is consistent with prior studies of preclinical animal(25) and patient studies(24) The changes observed in hyperpolarized $^{13}$C urea signal and slope and AUC of $^1$H DCE after radiation therapy is likely due to the reduction in the number of cells surrounding the capillaries, which leads to a decrease in the intratumoral pressure and permits reopening of the microcirculation(68). This decrease in cellular density following radiation is consisted with the observed reduction in tumor ADC. In addition, increased vasculature permeability can also contribute to the $^1$H and hyperpolarized $^{13}$C MRI findings. After radiation therapy, the permeability of capillaries has been shown to increase due to disruption of the lining of the endothelial cells(69). This increase in perfusion and/or permeability following radiation therapy would result in the observed higher hyperpolarized $^{13}$C
signal in the tumor and potentially could increase pyruvate delivery thereby explaining the discrepancy in the observed 75% reduction in LDH activity as compared to 50% reduction in hyperpolarized lactate-to-pyruvate ratio after radiation.

In this study we showed that the multi-parametric \(^1\)H and hyperpolarized \(^{13}\)C MRI approach can detect dose dependent changes in metabolism, vascular perfusion/permeability, and cellular density in tumors early after radiation therapy. Specifically, hyperpolarized lactate-to-pyruvate ratio decreased in a dose dependent fashion by 1 day after radiation therapy, prior to any changes observed using conventional \(^1\)H DCE and diffusion weighted imaging. \(^1\)H DCE and hyperpolarized \(^{13}\)C urea both increased by 4 days post-radiation demonstrating an increase in vascular perfusion/permeability. ADC values also increased significantly in tumor region treated with high dose radiation by 4 days post-radiation, indicating cell death. These dose dependent changes can be used as markers of early tumor response to the impact of increasing doses of radiation therapy. However, to interrogate whether these markers can be used to predict therapeutic efficacy, a different treatment setup will be needed in which lethal or therapeutic radiation dose intensities are delivered to the entire tumor volume with longer term imaging and survival studies performed.

Reference


Chapter 6: Spectral-Spatial Excitation Sequence for Dynamic Imaging of Hyperpolarized 13C Chemical Shift Imaging at 14T

Introduction

This chapter describes the development and implementation of a dynamic spectral-spatial excitation pulse sequence that is capable of acquiring dynamic hyperpolarized $^{13}$C MR data from mice on a 14T micro-imaging spectrometer. This sequence would enable dynamic imaging of $[1-^{13}\text{C}]$pyruvate-lactate exchange and obtain a dynamic measure of perfusion using hyperpolarized $^{13}$C urea in cancer. As described below, the ability to acquire dynamic hyperpolarized $^{13}$C data at 14T required overcoming several technical challenges. Additionally, the optimum utilization of hyperpolarized signal, required adjusting frequency specific flip angle schemes for the observation of hyperpolarized $[1-^{13}\text{C}]$pyruvate, $[1-^{13}\text{C}]$lactate, and $^{13}$C urea. The optimized dynamic sequence was then used in conjunction with the same TRAMP prostate cancer radiation model used in Chapter 5 to monitor dynamic changes in perfusion and metabolism and determine the value of this information relative to a single point measurement of hyperpolarized $[1-^{13}\text{C}]$pyruvate metabolism and changes in perfusion as measured by $^1\text{H}$ DCE using gadolinium (Gd) DPTA.

As described in previous chapters, hyperpolarized $^{13}$C offers over a 10,000-fold signal enhancement for $^{13}$C labeled compounds. This technique also allows for rapid in vivo imaging of tumor metabolism and perfusion simultaneously with no $^{13}$C background signals. Chapter 3 presented the current status of multi-parametric MRI and its utility for planning and monitoring radiation therapy. The conclusion of this chapter was that there remained a significant clinical
need for more sensitive and specific imaging in order to better plan and monitor radiation therapy and hyperpolarized $^{13}$C MR may be able to address this clinical need. In Chapter 5, we demonstrated that early radiation dose dependent changes in the micro-environment and metabolism of TRAMP tumors can be measured using a combination of $^1$H MRI, including anatomic T$_2$, DCE and diffusion weighted imaging, and hyperpolarized $^{13}$C imaging, of [1-$^{13}$C]pyruvate and $^{13}$C urea. In this study, the hyperpolarized $^{13}$C data were acquired from the animals at a single time point of 45 sec after injection of the hyperpolarized agents using a 3D GRASE sequence (details in Chapter 3). While this sequence provided excellent signal-to-noise ratio (SNR) and high spatial resolution, the interpretation of the single-time point hyperpolarized MR data acquired is complicated by several factors. The observed $^{13}$C signals from the hyperpolarized probes and their metabolites in this single time-point method can be affected not only by cellular metabolism but also by vascularity/perfusion and agent delivery, and transport in and out of cells, which may change over time(1). Additionally, perfusion, transport and metabolism have been show to change dramatically early after radiation therapy, so selection of the optimum time point for the 3D $^{13}$C MRSI acquisition my change with treatment and time(2,3). A dynamic imaging approach negates the need for pre-selecting an optimum time for data acquisition and also allows for the modeling of metabolic fluxes and physiologic parameters associated with tissue perfusion and permeability.

A technical limitation of hyperpolarized $^{13}$C imaging is the non-renewable nature of the hyperpolarized state and relatively short T$_1$ relaxation time, or the half-life of the hyperpolarized state, that results in the hyperpolarized signal quickly decaying back to its thermal polarization state. In addition, repeated RF excitation pulses applied during data acquisition cause an
irreversible loss of hyperpolarized signal. Therefore, acquisition methods need to minimize the acquisition time, minimize the number of excitation pulses, and maximize the retention of polarized signal. A preferred RF pulse sequence would be able to provide a balance between SNR, temporal and spatial resolution, systemic delivery of the hyperpolarized compound, and the rate of conversion to its downstream metabolic products in the tumor (1). Hence for metabolic imaging of hyperpolarized $^{13}$C, the desired information lies in the spectral domain, with the relative amplitudes of the different chemical shift species, and the spatial and temporal domains. Multiband excitation approaches and spectral-spatial excitation approaches (4,5) have been proposed to efficiently use the hyperpolarized magnetization. Through prior knowledge about the number and relative frequency separation of the resonances of interest, tailored pulses can be made to excite pyruvate with a small flip angle to preserve its magnetization, while its metabolic products such as lactate and alanine are excited with larger flip angles to improve their SNR.

While our high field micro-imaging spectrometer (operating at 14.1T) offers superior SNR and high-resolution multi-parametric $^1$H MRI images, it poses a number of challenges for hyperpolarized $^{13}$C studies. At higher field strengths, hyperpolarized $T_1$ relaxation times for $^{13}$C labeled carbonyl based probes decreases, therefore, particular care must be taken in optimizing excitation parameters to preserve magnetization of the hyperpolarized compounds. Susceptibility effects and magnetic field inhomogeneities are enhanced at high field, which lead to shorter $T_2^*$. Motion and flow artifacts are also increased at high field strengths. The 3D GRASE sequence addresses the short $T_2^*$ issue by incorporating multiple 180° pulses to minimize signal loss during excitation and readout (see Chapter 3). However, a problem specific to our high field micro-imaging systems and is that the $^{13}$C RF coil and the systems 100
Gauss/cm gradient coils do not homogeneously cover the entire mouse. On our 14T system—only 3.5 cm a long the long axis of the mouse (z direction) is effectively covered. When a tumor bearing TRAMP mouse is placed with its tumor in the center of coil, its heart is often located at the edge or outside of the homogeneous field of the $^{13}\text{C}$ RF coil gradients. Therefore, any non-spatially selective 180° refocusing pulses, even adiabatic pulses, could potentially saturate the hyperpolarized signal inside the heart or for that matter any hyperpolarized probes flowing from the heart to the respective tissues of interest. While this issue is acceptable for single time point acquisitions, it is highly detrimental for dynamic studies. Another major concern is the increased spectral dispersion, which can contribute to spatial errors in the slice and readout dimensions for 3D CSI sequences. For echoplanar spectroscopic imaging (EPSI) sequences, the dwell time in the spectral dimension must be kept short to prevent aliasing, which requires large gradients to be switched rapidly during readout. For spectral-spatial sequences, sidebands must be taken into consideration when dealing with multiple resonances of interest. The design of the spectral-spatial pulses can be challenging because the sidebands might overlap with other peaks within the spectrum.

**Theory**

The experiments presented used a spectral-spatial sequence designed for the 14T system to acquire hyperpolarized [1-$^{13}\text{C}$] pyruvate, its metabolic product [1-$^{13}\text{C}$] lactate, and [$^{13}\text{C}$] urea in vivo. The pulse was designed for good SNR of lactate while preserving the hyperpolarized magnetization stored in the pyruvate substrate. The acquisition of urea perfusion was also optimized for SNR. A variable gradient scaling is implemented to reduce non-linear artifacts due
to cumulative radiofrequency (RF) pulses. Each excitation pulse was followed by a flyback echo planar readout.

**RF excitation pulse**

Spectral-spatial RF pulses simultaneously excite magnetization both in frequency and space. A spectral-spatial pulse can be used to excite a slice of magnetization from one chemical species (e.g., hyperpolarized [1-13C] lactate) while leaving the magnetization from another (e.g., hyperpolarized [1-13C] pyruvate) unaffected. A typical spectral-spatial pulse consists of multiple RF subpulses that are played under a broad RF envelope. The spectral and spatial selectivity is achieved with a combination of shaped subpulses and a concurrent oscillating bipolar slice-selection gradient waveform. The spatial profile is determined by the shape of each subpulse and the spectral profile is determined by the envelope of the train of subpulses.

Several critical parameters have to be taken into consideration when designing the spectral-spatial RF pulse. The pulse was designed using the method described in (4,6). The design is very flexible, allowing for adjustments of the spectral bands and spatial profile, and the resulting pulses are efficient in terms of power and duration. The time interval between two subpulses is the inverse of the sampling frequency required. While the full width at half-maximum (FWHM) of the spectral pass-band is governed by the inverse of the total length of the pulse. The selectivity of the spectral profile is determined by the time bandwidth of the spectral envelope (total pulse time × pass-band width). Therefore, total length of the pulse determines the width of the transition between frequencies that are excited versus those that are not perturbed by the RF pulse.
Figure 6.1 shows the spectral-spatial RF excitation pulse designed for the hyperpolarized $^{13}$C experiments at the 14T. The total pulse was kept short at 6.7 ms to accommodate the short $T_2^*$. This pulse has a spatial time bandwidth of 2.0 and ripples of 0.1% with a minimal slice thickness of 8 mm for a gradient amplitude of 27.11 G/cm. A flyback spectral-spatial design was used to avoid “ghosting” artifact due to eddy current. 80% of the sublobe ramps allowed for applying Variable-Rate Selective Excitation, or VERSE(7) to the RF. This was done to increase efficiency and decrease slice thickness. The RF has a 0.0518 G peak amplitude. The spectral response was designed using linear phase filter, while the spatial response was designed using least squares filter. The frequency separation is 3624 Hz with FWHM of the pass-band at 500 Hz. This pulse is able to individually excite $[1^{13}C]$ pyruvate, its metabolic product $[1^{13}C]$ lactate, and $[^{13}C]$ urea without perturbing other resonances, including $[1^{13}C]$ alanine and $[1^{13}C]$ pyruvate hydrate. $[1^{13}C]$ lactate and urea peaks are farthest apart with a frequency separation of 2945 Hz at 14T. Shimming becomes critical as field inhomogeneity can cause frequency shifts and line broadening.
Figure 6.1. Simulated spectral-spatial excitation pulse with a flyback gradient and an 8 mm slice thickness. (a) RF pulse – real (black, solid) and imaginary (red, dashed) components – and accompanying gradient. (b) Spectral profile simulated for an $8^\circ$ flip of pyruvate (pyr). The spectral locations are labeled for resonance of interest: ala = alanine, lac = lactate, pyr-hyd = pyruvate hydrate. (c) Spectral and spatial profile.

Actual spectral and spatial profiles of the excitation pulse were measured using a 5 M, 3.5 mm ID cylindrical urea phantom on the 14.1T spectrometer with the dual $^1$H and carbon coil. Figure 6.2 shows the results. The measured FWHM of the slice profile is 8 mm. The frequency separation is 3600 Hz with total excitation bandwidth (including transition bands) of 650 Hz. The measured pass-band is 300 Hz. $90^\circ$ flip angle calibration was also determined experimentally using the same urea phantom and the dual-tune coil by varying the power, while
keeping the pulse time duration the same. Figure 6.3 shows the flip angle versus signal intensity. The spectral-spatial excitation pulse was designed using the small flip angle approximation. Note how the relationship starts to slightly deviate from linearity with large flip angles.
Figure 6.2. Measure spatial and spectral profiles using a 5 M, 3.5 mm ID cylindrical urea phantom inside the 14.1T spectrometer with dual-tune coil. (a) Measured slice profile with FWHM of 8 mm. (b) Measured frequency separation of 3600 Hz. (c) Measured spectral profile with FWHM of 500 Hz, pass-band of 300 Hz, and total excitation width of 650 Hz.
Figure 6.3. 90° flip angle calibration with flip angle versus signal intensity using the 5 M, 3.5 mm ID cylindrical urea phantom inside the 14.1T spectrometer with dual-tune coil.

*Variable flip angle schemes*

Efficient usage of the nonrenewable hyperpolarized signals requires optimization of the variable flip angle schemes. Factors that need to be taken into consideration while designing a variable flip angle scheme are hyperpolarized $T_1$ relaxation decay, signal decay due to prior RF excitations, and metabolic conversion. An optimized variable flip angle scheme increases the overall SNR and improves the characterization of tissue perfusion and metabolic profiles in $\theta_4[n]$. 
dynamic hyperpolarized MRI. Spectral-spatial excitation approach allows metabolite- specific imaging and independent control of metabolite flip angles. The variable flip angle schemes presented here were designed using the T$_1$-effective method describe in (8). The flip angles calculated for resonances X (i.e. pyruvate, lactate, and urea) were determine using the following model:

$$\theta_i[n] = \cos^{-1} \sqrt{\frac{E_{1X}^2 - E_{2(N-n+i)}^{2(N-n+i)}}{1 - E_{1X}^{2(N-n+i)}}}$$ \hspace{1cm} [6.1]$$

$$E_{1,P} = \exp \left( -TR \left( \frac{1}{T_{1P}} + K_{PL} \right) \right)$$ \hspace{1cm} [6.2]$$

$$E_{1,L} = \exp \left( -TR \left( \frac{1}{T_{1L}} - K_{PL} \right) \right)$$ \hspace{1cm} [6.3]$$

$$E_{1,U} = \exp \left( -\frac{TR}{T_{1U}} \right)$$ \hspace{1cm} [6.4]$$

Where TR is the repetition time and K$_{PL}$ is the metabolic conversion rate from pyruvate to lactate. To increase the temporal window, T$_1$ relaxation times for pyruvate (T$_{1P}$) and lactate (T$_{1L}$) were assumed to be infinite in the model. T$_{1U}$ relaxation time for urea was 23.5 s, which was experimentally determined from 5 tumor-bearing TRAMP mice (tumor volume ranged from 1 – 3 cc). K$_{PL}$ was also experimentally determined from slice-selective dynamic studies of 5 tumor-bear TRAMP mice and modeled using the following two-site exchange model:
\[ \frac{d}{dt} \begin{bmatrix} P(t) \\ L(t) \end{bmatrix} = \begin{bmatrix} -\frac{1}{T_{1P}} - K_{PL} & 0 \\ K_{PL} & -\frac{1}{T_{1L}} \end{bmatrix} \begin{bmatrix} P(t) \\ L(t) \end{bmatrix} \]

\[ [6.5] \]

\( K_{PL} \) was determined to be 0.0188 s\(^{-1}\), which was experimentally determined from 5 tumor-bearing TRAMP mice (tumor volume ranged from 1 – 3 cc), which is similar to previously published values in TRAMP mice(8). This value was then used in equation 6.2 and 6.3 to determine optimal variable flip angle schemes.

For this project, lactate and pyruvate were acquired at every 3 s for the first 21 s and then acquired at every 5 s from 25 s to 50 s after injection. Urea was acquired every 3 s for the first 21 s and then acquired at every 5 s from 25 s to 65 s after injection. Acquisition started at the beginning of the injection of the hyperpolarized \(^{13}\)C compounds and acquired more frequently (every 3 s) during the first half of the dynamic acquisition to better characterize the initial uptake slope and to catch the maximum peak of the signal enhancement. The repetition time increased from 3 s to 5 s for the latter half of the dynamic acquisition to more effectively use the magnetization, increase the temporal window, and better characterize perfusion and metabolism of the hyperpolarized probes. Figure 6.4 shows the variable flip angle schemes for pyruvate, lactate, and urea. The total acquisition time of urea was longer than of lactate and pyruvate.
because urea signal could last longer. Since urea signal decay is only affected by $T_1$, not metabolism.

Figure 6.4. Variable flip angle schemes for each metabolite – lactate, pyruvate, and urea.

Acquisition started at 3 s after injection and lasted for 50 s for acquisition of lactate and pyruvate and 65 s for the acquisition of urea.

Slice profile effects

Ideally, the slice profile for all of the RF excitations played out during a dynamic study with variable flip angle scheme should scale linearly with flip angle, as long as the small flip angle approximation holds:

$$S = M_0 \sin(\theta)$$  \hspace{1cm} [6.6]

However, the transition regions of the slice profile do not experience this linear relationship due to non-linear effects of cumulative RF pulses. It has been shown that in 2D slice selective hyperpolarized $^{13}$C imaging, using variable flip angle schemes that any realistically shaped RF pulse leads to large excess signal from the slice edges (transition bands) in later RF responses(9). During a variable flip angle scheme, increasing the flip angle would result in profile broadening
over time, creating wings in later RF responses. Figure 6.5 shows the geometry used for the slice profile measurements. In this model, the observed slice profile after the nth RF pulse $P_n(z)$, equal to the transverse magnetization, $M_{xy}(z)$, can be expressed as the product of the excitation profile of the RF pulse $E_n(z)$ and the underlying spatial distribution of longitudinal magnetization $M_n(z)$:

$$P_n(z) = E_n(z) \cdot M_n(z)$$

[6.7]

$P_n(z)$ is the slice profile observed experimentally and $E_n(z)$ is the actual excitation profile of the RF pulse.

Figure 6.5. From bottom to top: The distribution of magnetization within the phantom $M_n(z)$, the slice excitation profile $E_n(z)$, and the observed slice profile $P_n(z)$ within the FOV, (a) for the first RF pulse ($n = 1$) and (b) for a later RF pulse ($n > 1$). Before the first RF excitation, $M_1(z)$ is homogeneous over the whole phantom, and the observed profile $P_1(z)$ mirrors the excitation profile $E_1(z)$. For subsequent RF pulses, magnetization is gradually depleted by RF pulses,
leading to a reduced $M_n(z)$ at the slice location. As a consequence, the observed profile $P_n(z) = E_n(z) \cdot M_n(z)$ is distorted. Adapted from Deppe MH, et al. Slice profile effects in 2D slice-selective MRI of hyperpolarized nuclei. J Magn Reson 2010;202(2):180-189.

As there is no recovery of $T_1$ in hyperpolarized MRI, the observed slice profile $P_n(z)$ is distorted due to the depletion of the magnetization at the center of the slice caused by previous RF pulses. A method based on a purely spatial modulation by scaling of the slice gradient amplitude has been shown to correct this artifact(9). The gradient scaling solution proposed aims to keep the total slice signal constant by choosing a lower initial gradient amplitude, which would result in an accurate usage of $M_z$. Using this method, the following variable gradient scaling factors were implemented:

![Figure 6.6. Variable gradient scaling factor over time for each metabolite – lactate, pyruvate, and urea.](image)

Methods

*In vivo imaging*

All animal studies were done according to a protocol approved by the UCSF Institutional Animal Care and Utilization Committee. A total of 5 late stage tumor bearing TRAMP mice (2.03 ±
0.25cc) was treated with radiation therapy using method described in Chapter 5. Multi-parametric $^1$H MRI (T2 and DCE) and multi-probe ([$^{13}$C pyruvate and $^{13}$C urea) hyperpolarized $^{13}$C MRI was acquired on these mice pre-radiation, 1 day, and 4 days after radiation therapy.

All MR experiments were done using a Varian 14T imaging spectrometer. A HyperSense™ DNP polarizer (Oxford Instruments, Abingdon, UK) was used to polarize the $^{13}$C agents as described previously.(10). 24 µl neat [1-$^{13}$C]pyruvic acid with 16.5 mM trityl radical (GE Healthcare) and 1.5 mM Dotarem® (Guerbet) and 55 µl $[^{13}$C]urea (6.4 M in glycerol) with 17.5 mM trityl radical OX63 (Oxford Instruments) and 0.2 mM Dotarem® were co-polarized. This was followed by dissolution in 4.5 ml of buffer containing 40 mM Trist, 80 mM NaOH, and 0.3 mM Na$_2$EDTA. The resulting dissolution mixture contained 80 mM [1-$^{13}$C]pyruvic acid and 74 mM $^{[13}$C]urea. 500 µl (with 150 µl remained in the tail vein catheter) of this solution was injected into the tail vein of the TRAMP mouse over 12 s. the spectral-spatial pulse shown in Figure 6.1 was used to dynamic acquire [1-$^{13}$C]pyruvate, its metabolic product lactate, and $^{[13}$C]urea in vivo. Variable flip angle schemes and gradient scaling factors shown in Figure 6.4 and 6.6 were implemented as described above. The spectral-spatial sequence was used to acquire an 8 mm slice and a 40×40 mm$^2$ FOV with 3.3×3.3 mm$^2$ in-plane resolution. TR = 18 ms and a temporal resolution of 3 s for the first 21 s and 5 s for the 25 s to 50 s were used to acquire [1-$^{13}$C]pyruvate and lactate. Temporal resolution of 3 s for the first 21 s and 5 s for the 25 s to 65 s were used to acquire [$^{13}$C]urea. A total of 5 tumor-bearing TRAMP mice were treated with radiation therapy and imaged serially with hyperpolarized $^{13}$C probes in this study. Dynamic contrast enhancement (DCE) MR imaging was performed on 4 out of the 5 mice following the
hyperpolarized study, methods as described in Chapter 5 (diffusion weighted imaging was not conducted).

Typical methods multi-compartmental modeling of dynamic data are model-dependent and prone to misinterpretation. Therefore, a model-free approach is used to analyze dynamic pyruvate-to-lactate exchange. Ratio of total lactate signal to total pyruvate signal was calculated for each voxel using the following equation:

$$\frac{\text{Lactate}}{\text{Pyruvate}} = \frac{\sum_{i=1}^{N_t} L_i}{\sum_{i=1}^{N_t} P_i} \quad [6.8]$$

Where $N_t$ is the number of time points, $L_i$ and $P_i$ are lactate and pyruvate signals at each time point. It has been demonstrated that the area under the time curve ratio is proportional to the forward rate constant of pyruvate to lactate(11,12):

$$\frac{\text{Lactate}}{\text{Pyruvate}} = \frac{\sum_{i=1}^{N_t} L_i}{\sum_{i=1}^{N_t} P_i} = \frac{k_{PL}}{k_{LP} + r_L} \quad [6.9]$$

$k_{PL}$ and $k_{LP}$ are the effective conversion rate constants for the chemical exchange reaction between pyruvate and lactate. $r_L$ is the effective relaxation rate of hyperpolarized $^{13}$C signals of
lactate. Unlike the modeling approach, area under the time curve is independent of both the input function and of any other metabolic pathways arising from the injected metabolite.

Perfusion parameterization

DCE MRI

Quantitative parameters of $K_{\text{trans}}$ and $v_e$ were calculated for each voxel using a nonlinear least-squares estimation algorithm. $K_{\text{trans}}$ represents the volume transfer constant from the intravascular system to the extravascular extracellular space, while $V_e$ represents extracellular extravascular volume fraction. The following equation was used to model $K_{\text{trans}}$ and $v_e$:

$$C_{\text{tissue}}(t) = v_p C_p(t) + K_{\text{trans}} \times \exp(-k_{ep} t) \otimes C_p(t)$$

$$v_e = \frac{K_{\text{trans}}}{K_{ep}}$$

Where $C_{\text{tissue}}$ is the concentration of contrast material in the tissue, $v_p$ is the fraction of vascular plasma space, and $C_p$ is the concentration of contrast material in $v_p$.

Hyperpolarized $^{13}$C urea imaging

In this study, HP $^{13}$C urea also has been assessed as a potential marker of perfusion.

Hyperpolarized urea is acquired over a time interval of 65 s after injection. Presumably urea at this early time point was still getting taken up into the tumor tissue. The molecular weight of
urea (60 g/mol) is much lower than the Gd-DTPA (938 g/mol), therefore may result in faster
diffusion from the intravascular to the extravascular space.

To quantify urea perfusion, we modified the modeling equation from Von Morze, et al(13):

\[
C_{\text{tissue}}(t) = v_p C_p(t) + F \times \exp(-\frac{F}{V_e} t) \otimes C_p(t)
\]  \[6.12\]

Where \(C_{\text{tissue}}\) is the hyperpolarized \(^{13}\text{C}\) urea signal in the tissue, \(F\) is perfusion of urea from the arterial input function into the tissue. \(C_p\) is the hyperpolarized urea signal from arterial input function. The flip angles applied to the arterial input function were the same as to the rest of the tissue and assuming \(T_1\) decay of hyperpolarized \(^{13}\text{C}\) urea in the arterial input function is the same as in the tissue, these two effects can be ignored. Hyperpolarized urea signal from arterial input function is calculated as signal normalized to the percentage of the voxel within the arterial input function. This percentage is determined from Gd \(^1\text{H}\) DCE data.

**Results**

Figure 6.7 shows a representative dynamic hyperpolarized \(^{13}\text{C}\) lactate, pyruvate, and urea data acquired within a TRAMP tumor over 50 s for lactate and pyruvate and 65 s for urea. The SNR of lactate was at the peak at the 8\(^{th}\) time frame (25 s after injection) and then dropped sharply due to repetitive pulsing with 55\(^\circ\) flip angles. Figure 6.8 shows the dynamic profiles of lactate, pyruvate, and urea from a voxel overlapping the artery were very different from the profiles taken from a voxel within the prostate tumor. Within the voxel of arterial input function, or AIF, both pyruvate and urea peaked at 15 s after injection. This is consistent with the AIF observed in \(^1\text{H}\) DCE in TRAMP mice, where the AIF peaks between 15 to 20 s after injection. The lactate
signal was low within the AIF. In comparison, substantial lactate signal was detected in the tumor voxel. This observation demonstrates most of the lactate signal is generated from within the tumor, while tissues outside of the tumor show low lactate signal. This is consistent with previously published human clinical study(14). The variable flip angle schemes used for pyruvate and urea result in optimized SNR at later time points, which allows for a longer observation of the metabolite distribution and dynamics.
Figure 6.7. Dynamic [1-\(^{13}\)C] pyruvate, lactate, and \(^{13}\)C urea data. Left: Axial images of hyperpolarized [1-\(^{13}\)C] pyruvate, lactate, and \(^{13}\)C urea from 3 s to 21 s in a tumor bearing TRAMP mouse (3 s between frames), overlaid on T\(_2\)-weighted images. Right: Axial images of hyperpolarized [1-\(^{13}\)C] pyruvate, lactate, and \(^{13}\)C urea from 25 s to 65 s (50 s for pyruvate and
lactate) in the same tumor bearing TRAMP mouse (5 s between frames). The [1-\textsuperscript{13}C] pyruvate, lactate, and \textsuperscript{13}C urea images have been zero-filled and resized for display.

Figure 6.8. Dynamic hyperpolarized [1-\textsuperscript{13}C]pyruvate, [1-\textsuperscript{13}C]lactate, and \textsuperscript{13}C urea signal curves as a function of time. Bottom left: The signal from voxel overlapping the artery shows the arterial input function of pyruvate and urea. Pyruvate signal is scaled down by a factor of 4. The time evolution of pyruvate, lactate, and urea is not only affected by the animal
physiology/metabolism but also flip angles applied and hyperpolarized T<sub>1</sub> decay. Bottom right: dynamic signal curves from a representative tumor voxel.

*Evaluating early response of prostate tumor after radiation therapy*

Figure 6.9 shows representative hyperpolarized lactate-to-pyruvate ratio, perfusion calculated from hyperpolarized $^{13}$C urea and $K_{\text{trans}}$ calculated from Gd $^1$H DCE images overlaid on the corresponding T<sub>2</sub>-weighted images of a TRAMP tumor acquired pre-radiation, 1 day, and 4 days post-radiation. Tumor volume had not changed significantly at 4 day post-radiation. The area of the dynamic curve for lactate-to-pyruvate ratio decreased by 1 day post-radiation, and continued to decrease by 4 days post-radiation. Both tumor perfusion measured by hyperpolarized $^{13}$C urea and and Gd $^1$H DCE increased by 4 days post-radiation. Quantitatively (Figure 6.10), area under the curve of lactate-to-pyruvate ratio significantly (AUC mean ± sd, $p \leq 0.05$) decreased by 1 day post-radiation and continued to significantly (AUC mean ± sd, 0.014 ) decrease by 4 days post-radiation, which is consistent with the single time-point 3D $^{13}$C MRSI results (mean lactate-to-pyruvate ratio ± sd at 1 and 4 days) shown in Chapter 5.

TRAMP tumor perfusion and permeability were measured using both hyperpolarized $^{13}$C urea and Gd $^1$H DCE, as shown in Figure 6.11. Perfusion measured by hyperpolarized $^{13}$C urea increased significantly by 51.7% 4 days post-radiation ($p$-value = 0.021). $K_{\text{trans}}$ measured by Gd $^1$H DCE increased by an average of 14.1%, however the increase was not significant ($p$-value = 0.084). No significant changes were observed from $\nu_c$ (extravascular extracellular space) measured from Gd $^1$H DCE or $\nu_c$ (extravascular space) measured from hyperpolarized $^{13}$C urea.
Figure 6.12 shows an approximately linear correlation (Spearman’s $\rho$ of 0.86, $p$-value of 0.0003) between tumor perfusion measured by hyperpolarized $^{13}$C urea and tumor $K_{trans}$ measured by Gd $^{1}$H DCE.

Figure 6.9. Representative multi-parametric MRI data acquired at pre-radiation, 1 day, and 4 days post-radiation. Top row: Hyperpolarized lactate-to-pyruvate total area under the time curve ratio. Middle row: Urea perfusion calculated from hyperpolarized $^{13}$C urea time curve. Bottom
row: $K_{\text{trans}}$ calculated from $^1$H DCE images. The [1-$^{13}$C] pyruvate, lactate, and $^{13}$C urea images have been zero-filled and resized for display.

Figure 6.10. Lactate-to-pyruvate ratio calculated from TRAMP tumors comparing pre-radiation to 1 day, and 4 days post-radiation. Lactate-to-pyruvate ratio decreased 1 day post-radiation with a $p$-value of 0.050. Lactate-to-pyruvate ratio significantly decreased from pre-radiation 4 days post-radiation. The statistical significance compared with pre-radiation: *$p < .05$. 

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Figure 6.11. Quantitative analysis of tumor perfusion using hyperpolarized $^{13}$C urea and Gd $^1$H DCE at different time points (pre-radiation, and at 1, and 4 days post-radiation). Top left: Tumor perfusion measured from hyperpolarized $^{13}$C urea. Perfusion significantly increased from pre-radiation 1 day post-radiation. Top right: Tumor $K_{trans}$ measured from Gd $^1$H DCE. $K_{trans}$ increased 4 days post-radiation with a $p$-value of 0.080. Bottom left: Extravascular space calculated from hyperpolarized $^{13}$C urea. Bottom right: Extravascular extracellular space calculated from Gd $^1$H DCE. The statistical significance compared with pre-radiation: *$p < .05$ and not marked = not significant.
Figure 6.12. Correlation between tumor $K_{\text{trans}}$ (calculated from Gd $^1$H DCE) and tumor perfusion (calculated from hyperpolarized $^{13}$C urea) across treatment time points, which had a nonparametric Spearman’s $\rho$ of 0.86.

**Discussion**

In this chapter a flyback spectral-spatial excitation pulse sequence was developed and implemented on a 14T micro-imaging scanner to dynamically image [1-$^{13}$C]pyruvate-lactate exchange and obtain a dynamic measurement of perfusion using hyperpolarized $^{13}$C urea in cancer. A variable flip angle scheme was designed for each metabolite to provide optimum SNR for an appropriate duration of time (45 -60 seconds) to accurately image pyruvate to lactate metabolism and model changes in perfusion before and after radiation therapy. Flip angles at early time points were kept small for pyruvate and urea to preserve magnetization and increase observable time, while flips angles for lactate were larger to increase SNR since lactate is constantly being generated from pyruvate. The design of the flip angle scheme also took into
consideration in vivo T₁s and previously measured pyruvate to lactate flux measurements. In addition, the excitation pulse width was kept small, ≈ 6 ms, to minimize the impact the effect of the hyperpolarized molecule T₂* s, which are in the range of a couple of hundred milliseconds.

The sequence was used in conjunction with the same TRAMP radiation model used in Chapter 5 to monitor dynamic changes in tumor perfusion and metabolism with radiation dose. Perfusion measured by hyperpolarized ¹³C urea was compared with K_{trans} calculated from Gd ¹H DCE. Hyperpolarized ¹³C urea is metabolically inactive and has shown great potential for perfusion imaging(13). This comparison is of interest since urea is an endogenous compound that does not have the safety concerns associated with the paramagnetic Gadolinium chelates(15). Also, the in vivo urea signal is directly proportional to tracer concentration and ¹³C urea and can be copolarized with [¹-¹³C]pyruvate to allow the assessment of tissue metabolic and perfusion status in a single hyperpolarized MR study.

As shown in Equation 6.11 and 6.12, perfusion (F) measured by hyperpolarized ¹³C urea and K_{trans} measured by Gd ¹H DCE are obtained using the same equation. Hyperpolarized urea was acquired over a time interval of 65 s after injection. Presumably urea at this early time was still getting taken up into the tumor tissue. Perfusion measured by hyperpolarized ¹³C urea significantly increased 4 days post-radiation by 51.7% (p-value = 0.021). While, K_{trans} measured by Gd ¹H DCE also shown an increase of 27.4% 4 days post-radiation, however, the increase was not significant. Note the Gd ¹H DCE was acquired only from 4 out of 5 mice, therefore, more samples would need to be acquired to determine the significance of the increase in K_{trans}. Previous studies have shown increased tissue perfusion/permeability early after radiation therapy
in patients and preclinical animal models(3,16). Chapter 5 also showed an increase in perfusion after radiation therapy as measured by single time point hyperpolarized $^{13}$C urea measurement. Perfusion measured by hyperpolarized $^{13}$C urea was much higher than $K_{\text{trans}}$ measured by Gd $^1$H DCE, possibly due to the much lower molecular weight of urea (60 g/mole) as compared to Gd-DTPA is (938 g/mol). Due to the smaller molecular size, urea may be able to leak into the interstitial space faster than Gd. Possibly due to the same reason, the percentage increase of perfusion measured by hyperpolarized $^{13}$C urea almost doubles the percentage increase of $K_{\text{trans}}$ measured by Gd $^1$H DCE within TRAMP tumors after radiation therapy.

In addition, we observed a strong correlation between tumor perfusion measured by hyperpolarized $^{13}$C urea and tumor $K_{\text{trans}}$ measured by Gd $^1$H DCE. This observation suggests that the perfusion measured by hyperpolarized $^{13}$C urea could provide similar information as perfusion measured by Gd $^1$H DCE. $v_e$ measured by Gd $^1$H DCE represents the extravascular extracellular space since Gd does not enter the cell. However, the cellular membrane is semi-permeable to urea, therefore, further analysis is needed the define $v_e$ measured by hyperpolarized $^{13}$C urea. Also errors can arise in $v_e$ measured by hyperpolarized $^{13}$C urea, as $v_e$ can be affected by the hyperpolarized $^{13}$C urea $T_1$ decay.

The ratio of total areas under the time curve of pyruvate to total area under the time curve of lactate decreased by 1 day post-radiation and continued to decreased 4 days post-radiation, which is consistent with the single time point $^{13}$C MRSI results from Chapter 5. A model-free approach based on the ratio of total areas under the time curve was used to avoid errors associated with input function for the injected metabolite. Kinetic modeling of hyperpolarized $[1^{-13}]$C pyruvate-
to-lactate requires prior knowledge of the reaction mechanism and accurate estimate of the AIF. It has been shown that errors in the AIF critically influence estimates of the apparent rate constants from kinetic modeling(17). Prior study(11) shows that the ratio of the total areas under the dynamic curves, which is independent of AIF, is proportional to the forward apparent rate constant. Measurement of apparent reaction rate constants governing pyruvate-to-lactate exchange reflects lactate dehydrogenase (LDH) activity. LDH is a key metabolic enzyme that is commonly uprelated in cancer, and is central to the altered energy metabolism evident in cancer. Changes in LDH activity has been shown to be a marker of radiation response in Chapter 5.

**Conclusion**

We have presented a method to dynamically acquire hyperpolarized $^{13}$C data *in vivo* on the 14T scanner using spectral-spatial imaging pulse sequence. Variable flip angle schemes were tailor designed for individual metabolite to improve SNR and increase the duration of time courses. This method allows for observation of spatial variation of the substrate perfusion, uptake, and metabolite kinetics.

Spatial assessment of metabolism and perfusion has been demonstrated using co-polarized [1-$^{13}$C]pyruvate and $^{13}$C urea. High correlation was observed between perfusion measured by hyperpolarized $^{13}$C urea and $K_{\text{trans}}$ measured by Gd $^{1}$H DCE, suggesting urea might be able to provide similar information as Gd $^{1}$H DCE. Both Gd $^{1}$H DCE and hyperpolarized $^{3}$C urea increased following radiation therapy.
Reference:


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