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T₂ relaxation times of ¹³C metabolites in a rat hepatocellular carcinoma model measured *in vivo* using ¹³C-MRS of hyperpolarized [1-¹³C]pyruvate

Yi-Fen Yen^{a,*}, Patrick Le Roux^b, Dirk Mayer^{c,d}, Randy King^c, Daniel Spielman^c, James Tropp^a, Kim Butts Pauly^c, Adolf Pfefferbaum^{d,e}, Shreyas Vasanawala^c, and Ralph Hurd^a

^a Global Applied Science Laboratory, GE Healthcare, CA, USA

^b Global Applied Science Laboratory, GE Healthcare, Europe

^c Department of Radiology, Stanford University, Stanford, CA, USA

^d SRI International, Neuroscience Program, Menlo Park, CA, USA

^e Psychiatry and Behavioral Sciences, Stanford University, Stanford, CA, USA

Abstract

A single-voxel Carr-Purcell-Meibloom-Gill sequence was developed to measure localized T₂ relaxation times of ¹³C-labeled metabolites *in vivo* for the first time. Following hyperpolarized [1-¹³C]pyruvate injections, pyruvate and its metabolic products, alanine and lactate, were observed in the liver of five rats with hepatocellular carcinoma and five healthy control rats. The T₂ relaxation times of alanine and lactate were both significantly longer in HCC tumors than in normal livers (*p* < 0.002). The HCC tumors also showed significantly higher alanine signal relative to the total ¹³C signal than normal livers (*p* < 0.006). The intra- and inter-subject variations of the alanine T₂ relaxation time were 11% and 13%, respectively. The intra- and inter-subject variations of the lactate T₂ relaxation time were 6% and 7%, respectively. The intra-subject variability of alanine to total carbon ratio was 16% and the inter-subject variability 28%. The intra-subject variability of lactate to total carbon ratio was 14% and the inter-subject variability 20%. The study results show that the signal level and relaxivity of [1-¹³C]alanine may be promising biomarkers for HCC tumors. Its diagnostic values in HCC staging and treatment monitoring are yet to be explored.

Keywords

hyperpolarized ¹³C; T₂ relaxation time; hepatocellular carcinoma; liver metabolism; *in vivo* metabolic MRS; Morris HCC model; alanine transaminase

Introduction

T₂ relaxation time has an impact on signal to noise ratios (SNRs) and image contrast, and is a critical consideration in the design of optimized pulse sequences. Recent progress in hyperpolarized ¹³C metabolic imaging (1–12) has demonstrated its potential as a technique to interrogate metabolic pathways, monitor metabolic changes in disease progression, and follow treatment. However, *in vivo* T₂ relaxation times of metabolites detectable using

* Correspondence to: Y.-F. Yen, GE Healthcare, 333 Ravenswood Avenue, Building 307, Menlo Park, CA 94025, USA. yi-fen.yen@ge.com.

hyperpolarized ^{13}C -MRS methods remain largely uninvestigated. An attempt to measure *in vivo* T_2 relaxation times of hyperpolarized $[1-^{13}\text{C}]\text{pyruvate}$ (noted herein as ^{13}C -pyruvate) and its metabolic products was first reported by Yen *et al.* (13) with multiple T_2 components found for each metabolite. However these T_2 data were acquired from a whole slice, hence including signals from vasculature as well as the targeted organs, which may explain the multiple T_2 components. Also noted from that study (13), the transgenic adenocarcinoma of mouse prostate (TRAMP) tumor seemed to have longer pyruvate, lactate, and alanine T_2 relaxation times than rat kidney or dog prostate. However, due to limited number of animals (one for each animal/organ), results were inconclusive. In order to more accurately measure T_2 values from both normal and diseased tissue, we developed a sequence to measure localized T_2 from a single voxel and compared the localized T_2 of hepatocellular carcinoma (HCC) tumor to normal liver.

The sequence consists of voxel-localization RF pulses followed by a Carr-Purcell-Meibloom-Gill (CPMG) echo train. LASER (14) sequence uses 3 pairs of adiabatic RF pulses (15–19) for voxel selection. However, we found that adiabatic pulse pairs were too long for this application. In order to shorten the time to localize a voxel, we used a Shinnar–Le Roux (SLR) (20) 90° pulse for slice-selective excitation and two pairs of quadratic-phase SLR 180° pulses to refocus the slices (21,22) on orthogonal planes to localize a voxel. Details of the sequence are described in the Experimental Section. With this sequence, we measured both *in vivo* signal intensities and T_2 relaxation times of ^{13}C -labeled metabolites in rat livers after injection with hyperpolarized ^{13}C -pyruvate solution, and investigated these measures as potential metabolic markers of HCC.

Hepatocellular carcinoma is a primary liver cancer, one of the most common tumors worldwide (23,24). Most HCC cases arise in the cirrhotic liver, which has a heterogeneous structure that makes detection challenging with conventional CT, MRI, or ultrasound. The cirrhotic liver has regenerative nodules, each of which may have a mass-like appearance, limiting the value of anatomic features to detect malignancy. As a result, currently, the most helpful feature for detecting malignancy is the Gd enhancement characteristics of lesions, though even this approach is hindered by poor specificity (25). We were interested in exploring potential metabolic biomarkers of HCC using hyperpolarized ^{13}C MRS technique in an animal model. Rat HCC model developed by H. P. Morris (26) is a popular animal model. It is prepared by transplantation of different cell lines of hepatoma and has different gradations from types widely divergent from normal liver to types approaching normal liver. These models provide opportunities to study biological and biochemical parameters associated with different growth rates and energy metabolism regulations (27–31). Here, we used a fast growing HCC model, Morris hepatoma 7777 (or McA-RH7777), to induce focal HCC lesions in the rat liver (32).

Elevated lactate in malignant tumors has been observed by proton spectroscopic imaging, as well as by hyperpolarized ^{13}C techniques (2,6,8–10). Recent studies showed increasing $[1-^{13}\text{C}]\text{lactate}$ (noted herein as ^{13}C -lactate) production, following the bolus injection of hyperpolarized ^{13}C -pyruvate, correlated with disease progression in TRAMP model (2,6). Another study (8) demonstrated that TRAMP mice responding to hormone treatment exhibited reduction of ^{13}C -lactate signal, whereas those not responding to treatment continued having high ^{13}C -lactate signal. The increased ^{13}C -lactate signal in these tumor models may be due to increased perfusion of pyruvate into these highly vascular tumors in combination with elevated lactate dehydrogenase (LDH) (11). High LDH reflects elevated anaerobic metabolism and increases pyruvate-to-lactate conversion rate, consistent with the ‘Warburg effect’ (33). Changes in lactate production can be quantified by estimating metabolic conversion rate constants using kinetic modeling techniques (11,34).

In contrast, studies (29,30) on metabolism regulations in Morris models showed that the LDH level in the fast growing HCC models, such as the Morris hepatoma 7777 model used here, is

similar to the LDH level in normal liver. This is different from the LDH findings of other tumor models mentioned above. Therefore, we hypothesized that the ^{13}C -lactate signal relative to the injected ^{13}C -pyruvate, which was quantified as the total ^{13}C signal (or total carbon) in this work, may not differentiate HCC tumor and normal liver. However, in some liver diseases, including hepatoma (35,36), the enzyme catalyzing pyruvate conversion to alanine, Alanine Transaminase (ALT), is elevated and we postulated a potential difference in ^{13}C -alanine. In this study, hyperpolarized ^{13}C -pyruvate was injected into both normal and HCC rats to measure both the signal levels and T_2 's of ^{13}C -lactate and ^{13}C -alanine.

Experimental

Animal preparation

The experiment was performed using four male Wistar rats (283–459 g) with liver tumors prepared by using Morris hepatoma 7777 model (hereafter referred to as HCC rats), and five healthy male Wistar rats (361–578 g) as controls. The Morris 7777 model is a transplantable hepatoma rat model. The tumor cells were prepared *in vitro* at a growth rate of 80% confluency in 3 to 5 days. Approximately 1 million tumor cells were surgically implanted into the rat liver. The tumor growth was monitored weekly for MRI detectable tumors. Approximately 3 weeks after the implantation when at least one tumor had grown to 1 cm size, the HCC rat underwent the hyperpolarized ^{13}C study. Immediately after the ^{13}C study, the HCC rat was euthanized and necropsy was performed. Multiple tumor nodules and ascites were found in each of the HCC rats and the tumor sizes typically ranged from 2–3 mm to 1 cm. HCC rats were fasted 12–22 h prior to ^{13}C scans.

Because the liver of Morris 7777 tumor model was highly heterogeneous and consisted multiple tumor lesions and nodules, we were not able to collect normal liver data from the HCC rats. Therefore, we acquired normal liver data from five healthy control rats. Two of the five controls underwent repeated hyperpolarized ^{13}C scans for reproducibility test were fasted for 20 h and the remaining three were free feeding. Respiration, rectal temperature, heart rate, and oxygen saturation were monitored throughout each of the experiment, and animal was kept warm by using a water blanket regulated at 37°C. Animal preparation and physiological monitoring all followed a protocol approved by the Stanford University *Administrative Panel on Laboratory Animal Care* (APLAC).

Hyperpolarized ^{13}C -pyruvate solution

A typical dose consisted of 40 mg [$1\text{-}^{13}\text{C}$]pyruvic acid and 15 mM trityl radical mixture with 3 μl of a 1:25 aqueous dilution of ProHance[®] (Gadoteridol; Bracco Diagnostics, Inc., Princeton, NJ, USA). The mixture was polarized using Dynamic Nuclear Polarization technique and dissolved (37) in 4.6 g of TRIS/EDTA (trishydroxymethylaminomethane/ethylene diamine tetraacetic acid)-NaOH solution using a HyperSense[™] polarizer (Oxford Instruments Molecular Biotools, Oxford, UK). The dissolved, hyperpolarized ^{13}C -pyruvate solution was then transferred into a syringe, delivered to the animal inside the scanner, and administered manually into a tail-vein catheter. Each rat was injected with a 3 mL of 100 mM ^{13}C -pyruvate bolus lasting 12–13 s. For each animal, injections were repeated two to three times at 2-h intervals. Since a desktop spectrometer was not available to measure liquid-state polarization for each injection, we relied on calibrations of liquid-state polarization by using the 3 T MRI scanner and the reproducibility of solid-state polarization on HyperSense. The ^{13}C -pyruvate liquid-state polarization was approximately 15% to 20%, and the pH of the injected pyruvate solution was 7.4 ± 0.6 .

MR Hardware and pulse sequence

A custom-built dual-tuned $^1\text{H}/^{13}\text{C}$ quadrature rat coil (38) was used for both RF excitation and signal reception. The ^{13}C resonator had eight rungs with 8 cm inner diameter and approximately 9 cm length of homogeneous B_1 . All experiments were performed on a 3 T SignaTM MR Scanner (GE Healthcare, Waukesha, WI, USA) equipped with self-shielded gradients (40 mT/m, 150 mT/m/ms).

The pulse sequence consisted of voxel-localization pulses followed by a CPMG echo-train. The pulse sequence diagram is shown in Figure 1. Voxel selection was achieved by using an SLR (20) 90° RF pulse (1.75 kHz bandwidth) to selectively excite a slice, followed by two pairs of quadratic-phase SLR 180° pulses (5 kHz bandwidth) to selectively refocus slices in the other two orthogonal planes. All RF pulses have a pulse width of 5 ms. For each of the quadratic-phase pulses, a crusher gradient was applied before and one after the RF pulse on the axis where the slice-selective gradient was applied in order to dephase the spins in the RF transition band. For this study, the 90° selective-excitation was consistently applied on the axial plane (i.e. in the inferior to superior direction). The minimum voxel size was 1.1 cm (inferior to superior) $\times$ 1.2 cm (left to right) $\times$ 1.2 cm (anterior to posterior), equivalent to 1.6 cc. Depending on the metabolite chemical shift, the voxel location shifts slightly for different metabolites. The chemical shifts of ^{13}C -lactate, ^{13}C -alanine, and ^{13}C -pyruvate are 185, 178, and 173 ppm, respectively. At 3T, the frequency difference is 225 Hz between ^{13}C -lactate and ^{13}C -alanine and 161 Hz between ^{13}C -alanine and ^{13}C -pyruvate. For data acquisition, we set the center frequency midway between ^{13}C -lactate and ^{13}C -pyruvate. Therefore, the ^{13}C -alanine voxel was very close to the prescribed voxel location. The ^{13}C -lactate voxel and the ^{13}C -pyruvate voxel were shifted in the opposite directions from the prescribed location by 11% of the slice thickness in the inferior to superior direction and 4% in the other two directions.

A CPMG echo-train of 192 non-selective SLR 180° refocusing pulses (42 ms echo spacing) was employed to measure signal intensity and T_2 decay from the localized voxel. Each refocusing pulse had a bandwidth of 1.74 kHz and pulse width of 5 ms. The first spin-echo signal was acquired 78 ms after the 90° -excitation RF pulse. This sequence was first validated on a ^{13}C -enriched acetate spherical phantom (non-hyperpolarized) of 2 cm diameter, in comparison with T_2 measurements by using a slice-selective CPMG sequence. Consistent T_2 relaxation time of 4.7 ± 0.2 s was obtained from both methods.

MR Imaging protocol

Prior to each animal experiment, the ^{13}C transmit gain was calibrated by using an 8 M ^{13}C -urea syringe phantom placed near the center of the coil. Also placed inside the coil with the ^{13}C -urea phantom was a 60 mL syringe filled with saline to properly load the coil. After the calibration, the animal was positioned inside the coil with its liver close to the middle of the coil to ensure homogeneous RF transmission. Proton T_2 -weighted ($T_2\text{W}$) and spoiled gradient-echo images were collected for graphic voxel prescription. Then proton shimming was performed manually on a volume of approximately $2.5\text{ cm} \times 2.5\text{ cm} \times 2.5\text{ cm}$ covering the voxel of interest. For the hyperpolarized ^{13}C acquisition on healthy rats, a voxel of $1.1\text{ cm} \times 1.2\text{ cm} \times 1.2\text{ cm}$ was prescribed in the liver avoiding major vessels such as the aorta and vena cava. For HCC rats, a voxel of the same size was placed on the 1 cm focal tumor. The ^{13}C acquisition started 20 s after the start of the hyperpolarized ^{13}C -pyruvate injection. A total of 192 echoes were acquired in 8 s with 604 points per echo and spectral bandwidth of 19.2 kHz. The acquisitions with hyperpolarized ^{13}C -pyruvate injections were repeated three times on two of the control rats to assess intra-subject reproducibility.

Data analysis

The data were apodized in spectral domain with a 60 Hz Gaussian filter and zero-filled twice. Magnitude peak height was used to derive T_2 -decay curve of each metabolite. Because noise in the magnitude spectrum is not white, the true signal amplitude is biased when the signal level is low. A correction was performed following the algorithm in Ref. (39) in order to obtain accurate estimates of signal amplitudes in magnitude spectra. Ref. (39) suggested that for magnitude images of regions with no signal, the ratio of the average value to the standard deviation of the signal over this region should be given by 1.91. In our case, we tested the ratio of the average value to the standard deviation of the magnitude signal over a large spectral region away from metabolic peaks. From all spectra acquired in this study, we obtained 1.94 ± 0.01 which is close to 1.91, supporting the validity of applying this correction algorithm to our data. The correction factor as a function of magnitude SNR from Ref. (39) was used to determine the appropriate amount of bias to subtract from the magnitude signal in order to obtain the final T_2 -decay curve. When the magnitude SNR was greater than 30, the subtracted amount was less than 0.1% of the magnitude signal. Without this noise correction, overestimation of the T_2 relaxation time is likely. Finally, mono-exponential curve fitting to the noise-corrected T_2 -decay data was performed using Nelder-Mead nonlinear least-square method in Matlab (The MathWorks, Inc., Natick, MA, USA) to estimate T_2 relaxation time.

To compare signal intensities of metabolites between the HCC and control groups, the spectrum of the first spin-echo was fitted by four Gaussian peaks, one for each of the pyruvate, alanine, pyruvate-hydrate and lactate resonances. The alanine signal was estimated as the area of the fitted alanine peak, corrected for the respective T_2 decay between the 90° excitation and the first spin-echo (78 ms), scaled for the amount of pyruvate solution injected per body weight (ml/kg), and corrected for the hyperpolarized ^{13}C -pyruvate T_1 relaxation between dissolution and injection. Lactate signal was estimated similarly. T_1 of 58 s was used for the T_1 relaxation correction. This value was obtained from a separate *in vitro* experiment, in which T_1 was measured from a syringe of 3 mL hyperpolarized ^{13}C -pyruvate solution using a pulse-and-acquire acquisition with constant 5° flip angles and TR of 3 s over 4 min. Total carbon signal was estimated as the sum of the four Gaussian peak areas, corrected for T_2 decay, scaled by the injection dose (mL/kg) and corrected for ^{13}C -pyruvate T_1 relaxation before injection. For simplicity, an average value of the alanine T_2 and lactate T_2 was used for the T_2 decay correction of the total carbon signal; that is 1.05 s for the HCC group and 0.45 s for the control group.

Results

The prescription of a representative voxel in an HCC rat is illustrated in Figure 2a. This rat had two liver tumors, both exhibiting hyper-intense signal on the T2W proton image. Two ^{13}C T_2 data sets were acquired on this rat, one acquisition from each tumor. The corresponding spectra are shown in Figures 2b and 2c. Voxels acquired from control rats were prescribed consistently on the right lobe of the liver above the right kidney (Fig. 3a). A syringe containing an 8M ^{13}C -urea solution was placed above the rat (Fig. 3b) and served as a reference for carbon transmit-gain calibration. A representative spectrum of normal liver (Fig. 3c) shows less ^{13}C -alanine signal compared with the ^{13}C -alanine signals in the tumor spectra (Figs 2b, 2c).

We checked the effect of fasting on the control data. A liver metabolism study published previously by using hyperpolarized ^{13}C -pyruvate MRSI showed significant increase of lactate to alanine ratio in fasted rats (40). The average alanine signal of (two) fasted rats in this study was $(6 \pm 2) \times 10^{12}$ and the average alanine of (three) free-fed rats $(8 \pm 1) \times 10^{12}$. The average lactate signal was $(1.01 \pm 0.04) \times 10^{13}$ for fasted rats and $(8 \pm 3) \times 10^{12}$ for free-fed rats. The sample sizes in this study were too small to draw any significant conclusion comparing fasted to free-fed rats. However, our finding shows perhaps a trend of smaller alanine and larger

lactate signal on fasted rats than free-fed rats, consistent to the published results (40). Since there is no significance separating the fasted and free-fed controls, we will report the results combining all five controls in this paper.

Intra-subject variation was estimated from repeated measurements performed on two of the control rats (both fasted). Inter-subject variability was assessed from independent measurements of the five different rats in the control group and only the first measurement of the repeated data sets was used. The intra- and inter-subject variations of the alanine T_2 relaxation time were 11% and 13%, respectively. The intra- and inter-subject variations of the lactate T_2 relaxation time were 6% and 7%, respectively. The intra-subject variability of alanine to total carbon ratio was 16% and the inter-subject variability 28%. The intra-subject variability of lactate to total carbon ratio was 14% and the inter-subject variability 20%.

The ^{13}C metabolites in HCC tumors had longer T_2 relaxation times than those in the normal livers, as demonstrated by the mean and $\pm$ standard-deviation (SD) of the T_2 curves of each group in Figure 4 for (a) ^{13}C -alanine, (b) ^{13}C -lactate, and (c) ^{13}C -pyruvate. For the two control rats on which repeated scans were performed, only the first data set was included in the analysis of the control group. The ^{13}C -alanine T_2 of HCC tumor (1.2 ± 0.1 s) was longer than that of normal liver (0.38 ± 0.05 s). Two-sample one-tail t -test comparing ^{13}C -alanine T_2 values between the HCC and control groups yielded $p < 3 \times 10^{-5}$. The ^{13}C -lactate T_2 was 0.9 ± 0.2 s in the HCC tumor and 0.52 ± 0.03 s in the normal liver. The t -test of the ^{13}C -lactate T_2 values between the two groups yielded $p < 2 \times 10^{-3}$. The T_2 differences between the two groups were significant.

The ^{13}C -pyruvate signal decay curves were very noisy (Fig. 4c) and the noise frequency matched with the frequency of cardiac cycles. Following high dose injection of hyperpolarized ^{13}C -pyruvate, substantial amount of ^{13}C -pyruvate circulated in blood and experienced B1 inhomogeneity during the (non-selective) refocusing pulse train. The T_2 signals were poorly refocused and the T_2 curves cannot be described well by mono-exponential curve fitting. Inter-subject deviations were large. There seems to be no difference between control and HCC groups.

Figure 5 shows a scatter plot of total carbon signal vs individual metabolite signal. Corrections for T_2 decay, injection dose and T_1 relaxation before injection were applied. Four out of five tumors had higher total carbon signal than normal livers. On average, the total carbon signal found in a HCC rat tumor was 1.7 ± 0.5 times the total carbon signal observed in normal liver. There was no evidence for an effect of tumor size on the levels of lactate and alanine. All tumors had higher alanine signals than control livers ($p < 3 \times 10^{-3}$, tumor/control = 2.6 ± 0.9). All but one tumor had higher lactate signal than normal livers ($p < 7 \times 10^{-3}$, tumor/control = 1.9 ± 0.7). However, because the total carbon in the HCC tumors was also higher than that in normal livers, it is possible that the individual metabolite signals may be high in tumors even though the production relative to the injected ^{13}C -pyruvate (as quantified by the total carbon signal) may not be significantly different from normal livers. This is indeed the case observed for labeled lactate (Fig. 6a). The lactate signal relative to the total carbon in HCC tumors was 1.1 ± 0.3 times of that in normal livers, not a significant difference ($p < 3 \times 10^{-1}$). On the other hand, the alanine to total carbon ratio in HCC tumors was significantly higher (Fig. 6b), about 1.5 ± 0.5 times of that in normal livers ($p < 6 \times 10^{-3}$). The pyruvate to total carbon ratio is relatively low in HCC tumors comparing to normal livers most likely because relatively more labeled pyruvate was converted to alanine in tumors.

Discussion

We report a technique to measure localized T_2 relaxation times of ^{13}C -labeled metabolites *in vivo*, and the comparison between an HCC rat model and normal rat liver. To the best of our knowledge, this is the first report on *in vivo* localized T_2 measurements of ^{13}C metabolites. The large signal enhancement afforded by the hyperpolarized ^{13}C MRS technique made such measurements possible *in vivo*. Atomic motion influences the rate of transverse relaxation, and such motion varies in different microscopic environment depending on viscosity, temperature, local magnetic fields, and surrounding molecules. Therefore, the T_2 measured *in vivo* may be particularly relevant to the design of imaging sequences and contrast generation strategies for *in vivo* hyperpolarized ^{13}C applications. The ^{13}C -alanine and ^{13}C -lactate T_2 relaxation times were found to be about half a second in the normal liver. For comparisons, the relaxation time for ^{13}C -lactate due to spin-spin coupling (J_{CH}) was about 25 ms (41) and a similar J_{CH} coupling for ^{13}C -alanine. Imaging sequences acquiring T_2 signal may have advantages in SNR and/or large matrix size. The large difference in T_2 relaxation time between HCC tumor and normal liver could provide an opportunity to develop novel strategies for enhancing image contrast and improving cancer detection.

One of the limitations with the *in vivo* measurement presented here was that metabolic conversions continued during data acquisition. Typical conversion rates from pyruvate to lactate or pyruvate to alanine range from 0.004 to 0.07 s^{-1} (11,34), corresponding to time constants longer than 14 s. Hence, during the 8-s T_2 acquisition time, a very small number of ^{13}C labels may have exchanged from one metabolite to another.

Another limitation of this technique is the possible variations in voxel placement due to respiratory motion. Although no appreciable liver displacement relative to the voxel was observed in repeated T2W proton images, contribution of extrahepatic tissues such as fat during respiration was almost unavoidable when measuring T_2 *in vivo*. Extend of this variability contributed to the intra and inter subject variations reported in this work. The partial volume effect due to respiratory motion may also affect tumor T_2 measurements, resulting an underestimate of T_2 if non-cancerous liver tissue contributed to the measured signal, or overestimate of T_2 if vasculature contributed to the measured signal. Given the highly heterogeneous nature of this tumor model as examined by necropsies and the limited spatial resolution of this approach, further uncertainty was added to this circumstance. Gd enhanced images (or SPIO images) of the same hepatic tumors, after ^{13}C MRS, would have provided a more clear picture of tumor heterogeneity and partial volume effects, helping a better interpretation of the results. For future studies, respiratory gating can be used to improve the consistency of voxel placements between proton scout scans and ^{13}C acquisitions.

We found that T_2 of ^{13}C -alanine and ^{13}C -lactate in rat HCC tumors were longer than those in the normal liver tissue. This may be related to tumor cell morphology and leaky vessels in the fast growing tumors. The improved localization of T_2 in this voxel-based study eliminated some of the complexity in the previous whole-slice study (13). Most notably, a very long T_2 component (2–3 s) previously observed in the whole-slice study was not observed in this study even when we tried bi- or tri-exponential fittings (results not shown here). This may not be surprising since significant signal from the intravascular space was included in the whole-slice data, and the proper noise correction (39) was not applied in the data analysis of the whole-slice study. The sensitivity of this technique to very short T_2 components (100 to 250 ms as previously reported (13)) was somewhat limited by the delayed acquisition (78 ms) due to the voxel selection pulses.

Besides variations in T_2 relaxation, injection dose and T_1 relaxation from dissolution to injection, which were corrected in the data analysis, the total carbon signal could also be

affected by the DNP solid-state build-up of the ^{13}C -pyruvate sample, variations in the liquid state polarization due to the dissolution process, and/or the voxel position and heterogeneity of the liver. Despite these variations, there is a trend towards higher total carbon signal in HCC tumors than that in normal livers. Since all detectable ^{13}C signal came from the injected ^{13}C -pyruvate, the high total carbon signal found in tumors may be due to the high metabolism expected in tumor cells or the high perfusion in these highly vascular tumors, or both. Techniques capable of differentiating signals from within cells versus signals in blood (42) will be very important for more detailed characterization of cancerous tumors using hyperpolarized ^{13}C -MRS.

The ratio of individual metabolite signals to the total carbon signal is, however, independent of many of the variations in experimental conditions mentioned above; and therefore, the ratio is a good 'first-order' indicator of enzymatic activity. We found the alanine to total carbon ratio is significantly higher in HCC tumors than in normal livers. This may be related to the reported high ALT level in the Morris HCC rat model (35,36). Even though the same amount of hyperpolarized ^{13}C -pyruvate solution was injected in each animal, the up-take of ^{13}C -pyruvate by each HCC tumor varies, as indicated by the wide range of total carbon signal found in HCC tumors (Fig. 5). The up-take of ^{13}C -pyruvate may depend on the tumor vasculature, monocarboxylate transporters, and metabolic activity. But the almost linear relationship between the total carbon and the labeled alanine signals observed in HCC tumors (red stars in Fig. 5) is very interesting. This may imply that the enzymatic activity in the HCC tumor cell is so high that it stays 'unsaturated' even with the high doses of ^{13}C -pyruvate injected in this study. An alternative interpretation as proposed by Day *et al.* (11) is that the observed differences in alanine to total carbon ratios between HCC tumor and normal liver result from passive exchange with a pre-existing alanine pool. Based on this interpretation, our data showing higher alanine than lactate production in the Morris 7777 tumors may imply there is more alanine than lactate in this tumor model. Unfortunately, we are not able to find alanine and lactate concentrations measured in Morris 7777 tumors in literature. This may be of future interest but is outside of the scope of this work.

This study showed similar lactate to total carbon ratios between HCC tumors and normal livers (Fig. 6b); very different from the elevated lactate to total carbon ratios previously reported by other tumor studies (2,6,8–10). This may also be due to the particular animal model used here. Studies done by Weber *et al.* (29,35) and Shonk *et al.* (30) on metabolism regulations in Morris HCC models found that in spite of correlation between the increasing growth rate and increased lactate production, the LDH level in the fast growing HCC model [approximately 135 $\mu\text{mole}/\text{min/g}$ w-w in average (30)] is not higher than the LDH level in the normal liver [162 $\mu\text{mole}/\text{min/g}$ w-w (30)]. In another study comparing a fast-growing HCC model to a slow-growing one, Fields *et al.* (31) found that the fast-growing model (Morris hepatoma 7777, in particular) is not able to oxidize fat. Therefore, it must derive all of its calories from protein or carbohydrate sources. We postulate that the Morris hepatoma 7777 model may have elevated lactate production due to high consumption of pyruvate as a fuel but the pyruvate to lactate conversion rate may be similar to normal liver, as indicated to the first-order by the similar lactate to total carbon ratios found here (Fig. 6b) in the presence of a pyruvate concentration higher than the normal physiological level. A better experiment to evaluate the pyruvate to lactate conversion *in vivo* using hyperpolarized ^{13}C technique would be to perform a localized dynamic acquisition from the start of hyperpolarized ^{13}C -pyruvate injection and to extract the apparent V_{max} (the maximum reaction rate) from the Michaelis-Menten kinetics (43). Such an experiment is beyond the scope of this study but will be of future interest.

It is important to note that in addition to the LDH activity, the labeled lactate signal to the total carbon ratio also depends on in flow and transport. The monocarboxylate transporters will equilibrate pyruvate and lactate, as determined by the intra- and extracellular pH gradient,

exchanging these metabolites much faster between the intracellular and extracellular spaces than LDH activity. This exchange between different vascular, extracellular and intracellular environments in control and HCC may be an additional source of the wide range of total carbon and lactate signals found in HCC tumors.

The similar ratio of labeled lactate to total carbon discovered here was after injections of unphysiologically high concentration of pyruvate. Under physiological conditions, LDH is not a rate-limiting enzyme of glycolysis. The glycolytic flux and lactate production are determined upstream in the glycolytic pathway at the hexokinase, phosphofructokinase and pyruvate kinase steps. Thus the finding of similar ratio of ^{13}C labeled lactate to the total ^{13}C in normal liver and HCC tumor does not imply that the HCC tumor will not produce more lactate from glucose than the normal liver under physiological conditions since all the rate limiting steps prior to LDH in tumor may be different or altered.

Conclusion

A single-voxel CPMG sequence was developed to measure both signal intensities and T_2 relaxation times of ^{13}C -labeled metabolites *in vivo* following hyperpolarized ^{13}C -pyruvate injections in rats with hepatocellular carcinomas and healthy control rats. The ^{13}C -alanine T_2 was 1.2 ± 0.1 s in HCC tumor and 0.38 ± 0.05 s in normal liver. The ^{13}C -lactate T_2 was 0.9 ± 0.2 s in HCC tumor and 0.52 ± 0.03 s in normal liver. The T_2 of alanine and lactate were both significantly longer in HCC tumors than in normal livers. The alanine to total carbon ratio in HCC tumors was also significantly higher (1.5 ± 0.5 times) than that in normal livers, but the lactate to total carbon ratio was comparable between the tumors and normal livers. The intra- and inter-subject variabilities were 11% and 13%, respectively, for alanine T_2 estimates and 6% and 7%, respectively, for lactate T_2 estimates. The intra-subject variability of alanine to total carbon ratio was 16% and the inter-subject variability 28%. The intra-subject variability of lactate to total carbon ratio was 14% and the inter-subject variability 20%. We conclude that ^{13}C -alanine may be a promising biomarker for hepatocellular carcinoma tumors, but its full diagnostic value in hepatocellular carcinoma staging and treatment monitoring is yet to be explored. The quantitative T_2 relaxation times reported here are important parameters for pulse sequence designs and a potential source of image contrast in hyperpolarized ^{13}C metabolic imaging applications.

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Abbreviations used

ALT	Alanine transaminase
APLAC	Administrative Panel on Laboratory Animal Care

CPMG	Carr–Purcell–Meiboom–Gill
CT	Computed tomography
EDTA	Ethylene diamine tetraacetic acid
GE	General Electric
HCC	Hepatocellular carcinoma
LASER	Localization by Adiabatic Selective Refocusing
LDH	Lactate dehydrogenase
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
RF	Radio frequency
SLR	Shinnar–Le Roux
SNR	Signal to noise ratio
std	Standard deviation
T2W	T2 weighted
TRAMP	Transgenic adenocarcinoma of mouse prostate
TRIS	trishydroxymethylaminomethane

Figure 1.

Pulse diagram of the voxel-selective CPMG sequence used to measure T_2 relaxation time of ^{13}C metabolites. Voxel selection is achieved by using an SLR 90° slice-selective RF pulse of 1.75 kHz bandwidth, followed by two pairs of quadratic-phase SLR 180° slice-selective pulses of 5kHz bandwidth to excite the second and third spatial dimensions. The CPMG echo train consists of 192 non-selective 180° refocusing RF pulses (1.74 kHz bandwidth), of which only two are shown here.

Figure 2.

(a) Voxel prescription based on a T2W proton coronal image of a rat with two HCC tumors (white arrows); (b) and (c) representative ^{13}C spectra of the tumors. Here P, A, P-H, and L indicate pyruvate, alanine, pyruvate-hydrate, and lactate peaks, respectively. The spectral line width is a combination of T_2^* (~ 100 ms for pyruvate) or J_{CH} (~ 25 Hz for lactate and alanine), and 60 Hz Gaussian filter (spectral apodization) applied in post-processing of the data. Since the spectral readout time was only 42 ms centered at each spin-echo, the spectral line width shown here is dominated by the 60 Hz spectral apodization.

Figure 3.

(a) Voxel prescription on T2W proton coronal image of a healthy control rat; (b) proton sagittal image shows the voxel location and the ^{13}C -urea reference syringe; (c) representative ^{13}C spectrum of the normal liver. Here P, A, P-H, and L indicate pyruvate, alanine, pyruvate-hydrate, and lactate peaks, respectively.

Figure 4.

Mean (red solid lines) and $\pm$ one standard-deviation (red dashed lines) T₂ decay curves acquired from 5 HCC tumors compared to mean (blue circles) and $\pm$ one standard-deviation (blue dotted lines) T₂ decay curves from livers of 5 healthy control rats. a) ¹³C-alanine, b) ¹³C-lactate, and c) ¹³C-pyruvate.

Figure 5. Scatter plot of total carbon signal vs. individual metabolite signal of ^{13}C -alanine (red stars: HCC tumor, blue circles: normal liver), ^{13}C -lactate (pink crosses: HCC tumor, green triangles: normal liver) and ^{13}C -pyruvate (black pluses: HCC tumor, cyan diamonds: normal liver). Corrections for T_2 decay, injection dose and T_1 relaxation before injection had been applied.

Figure 6.

(a) alanine to total carbon ratio of HCC tumors (red stars) and normal livers (blue circles); (b) lactate to total carbon ratio of HCC tumors (pink crosses) and normal livers (green triangles); (c) pyruvate to total carbon ratio of HCC tumors (black pluses) and normal livers (cyan diamonds).