Published in final edited form as:

J Vasc Interv Radiol. 2011 August ; 22(8): 1175–1180. doi:10.1016/j.jvir.2011.03.016.

Quantitative Proton MR Spectroscopy as a Biomarker of Tumor Necrosis in the Rabbit VX2 Liver Tumor

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Abstract

Purpose—To compare metabolic magnetic resonance (MR) imaging findings (ie, quantification of tumor choline concentration) with percentage of necrosis on pathologic examination in rabbits bearing VX2 liver tumors.

Materials and Methods—VX2 tumors were implanted in the livers of 16 rabbits. MR imaging was performed with a 1.5-T MR scanner and extremity coil, and a hydrogen-1 (^1H) proton MR spectroscopy (^1H MRS) imaging protocol was used. Rabbits were euthanized immediately after imaging, and the tumor was harvested and sliced at 4-mm intervals in the axial plane. Choline concentration was calculated and was compared with the percentage of tumor necrosis on pathologic examination.

Results—Mean tumor size at pathologic examination was 16 mm (range, 12–22 mm). Mean percentage of necrosis at pathologic examination was 22% (range, 4%–44%). Choline concentration showed a relatively high inverse correlation with percentage of necrosis on pathologic examination, with an r value of 0.78 ($P < .002$).

Conclusions—Choline concentration showed a relatively high inverse correlation with tumor necrosis on pathologic examination. Therefore, ^1H MRS may be useful to assess tumor necrosis.

Assessment of tumor response by imaging is conventionally based on tumor size and tumor enhancement on contrast-enhanced computed tomography or magnetic resonance (MR) imaging (1,2). In contrast to systemic treatment, locoregional therapy has been shown to decrease tumor size, but despite favorable clinical outcome, many responses do not qualify as complete according to the current guidelines (3). Moreover, in the early posttreatment period after locoregional therapy, tumors may be nonviable even though no change in tumor

size is observed. Therefore, treatment response after locoregional therapy cannot be fully assessed solely based on anatomic changes (4).

Tumor enhancement on contrast-enhanced MR imaging is a standard criterion based on which to assess tumor response after locoregional therapy (2,5). Enhancing portions of the tumor are presumed to be viable, whereas nonenhancing portions are presumed to be necrotic. One of the disadvantages of contrast-enhanced MR imaging is the incapability to distinguish viable cells from reactive granulation tissue. Contrast enhancement in granulation tissue is believed to be caused by increased capillary permeability and marked increase in the passive distribution of gadolinium (1). After locoregional therapies, an enhancing rim can appear on contrast-enhanced MR imaging, which can correlate to viable tumor as well as to reactive tissue (6,7).

In addition to these traditional modalities, diffusion-weighted MR imaging has increasingly been used to assess tumor response by measuring the apparent diffusion coefficient (ADC) value (8–10). However, changes in ADC values are transient and lag behind changes in tumor enhancement. Changes in ADC values are significant 1–2 weeks after treatment and become less apparent 3–4 weeks after therapy as a result of tissue dehydration (11). Therefore, to date, there is no reliable imaging technique for the monitoring of early tumor response to locoregional therapy.

Hydrogen-1 (^1H) proton MR spectroscopy (^1H MRS) is a noninvasive imaging technique that may be used to quantify biochemical metabolite concentrations. It has been successfully used as a diagnostic tool for tumors in the brain, breast, and prostate, and in the evaluation of treatment response to chemotherapy in tumors of the head and neck (12–17). In the liver, ^1H MRS has been used to evaluate diffuse hepatic disease such as hepatic steatosis, chronic hepatitis, and cirrhosis (18–20). However, the role of ^1H MRS in evaluating tumor response after locoregional therapy still has to be established.

In-vivo ^1H MRS can be used to differentiate between benign and malignant lesions based on the evaluation of choline levels detected in the lesion (21). Increased intensity of the choline peak is believed to represent an increased biosynthesis of membrane phospholipids and therefore cellular proliferation. Viable tumors consist of rapidly proliferating cells, causing a high choline peak, whereas necrotic tumors have decreased cellularity, causing the choline peak to diminish.

Quantification of choline concentration is essential to characterize changes after locoregional therapy. Several quantification techniques have been used for in-vivo ^1H MRS (22). Quantification is the procedure to estimate numeric values of metabolite concentrations by comparing in vivo signals from a volume of interest versus a standard signal from an internal or external reference. Several studies performed ^1H MRS using an external reference (23). Unfortunately, this procedure, which requires accurate calibration, is extensive and therefore impractical in the clinical setting. For this reason, water was used as an internal reference in the present study.

The aim of the present animal study was to compare metabolic MR imaging findings (ie, quantification of tumor choline concentration) with percentage of tumor necrosis on pathologic examination in rabbits bearing VX2 liver tumors.

Materials and Methods

Animals

This study was approved by the animal care and use committee at our facility, and was performed in accordance with our institutional guidelines. Adult New Zealand White rabbits weighing 3.5–4.2 kg (n = 16; Myrtle Rabbitry, Thompson Station, Tennessee) underwent implantation of rabbit VX2 tumor in the liver.

Study Design

A total of 16 New Zealand White rabbits were included in the study. Each animal received tumor implantation in the left lobe of the liver, and ^1H MRS imaging was performed 2 weeks after implantation in six animals and 3 weeks after implantation in 10 animals to reflect various degrees of necrosis within the tumors. All animals were euthanized immediately after MR imaging, and their livers were explanted and submitted to pathologic analysis.

Tumor Implantation

All rabbits were anesthetized with a mixture of acepromazine (2.5 mg/kg; Phoenix, St. Joseph, Missouri) and ketamine hydrochloride (44 mg/kg; Phoenix) administered intramuscularly. The VX2 tumor cell suspension was first injected into the hind legs of carrier rabbits and grown for 2 weeks. For the rabbits that were going to receive the VX2 tumor implanted in the liver, intravenous access was gained via a marginal ear vein and 0.1–0.2 mL (2.5–5 mg) of sodium pentobarbital (Abbott Laboratories, Abbott Park, Illinois) was given periodically to maintain anesthesia. The liver of the rabbit was exposed by a midline incision, and an aliquot of the tumor cell suspension (0.2 mL) was then injected directly into the left lobe of the liver with a 21-gauge angiocatheter, with the intent to develop a solitary lesion with adequate surrounding liver parenchyma. Tumor was allowed to grow in the rabbit livers for 11–21 days to induce variable degrees of necrosis. In our experience, the use of an interval shorter than 10 days results in tumors that are too small to be characterized by imaging, whereas an interval longer than 21 days may result in an unacceptable tumor burden.

MR Imaging

All 16 rabbits underwent MR imaging immediately before they were euthanized. All animals were anesthetized with a mixture of acepromazine (2.5 mg/kg) and ketamine hydrochloride (44 mg/kg) administered intramuscularly. Subsequently, the animals were intubated, intravenous access was gained via a marginal ear vein, and 0.1–0.2 mL (2.5–5 mg) of sodium pentobarbital was given periodically to maintain anesthesia during imaging. MR imaging was performed by using a 1.5-T whole-body clinical MR system (Avanto; Siemens, Erlangen, Germany) and dedicated extremity coils. Acquisitions included a T2-

weighted turbo spin-echo sequence with fat suppression (repetition time [TR]/echo time [TE], 4,000 ms/103 ms; field of view, 380 mm).

The spectroscopic voxel was positioned within the liver tumor based on a combination of three-dimensional gradient-recalled echo T1 images and T2 sagittal images, to cover the entire lesion with minimal inclusion of surrounding tissue (major blood vessels, intrahepatic bile ducts, and lateral margin of liver). After placement of the voxel, the spectra were acquired in the axial plane. Localized ^1H MRS was performed with a point-resolved spectroscopy spinecho sequence, which included a water suppression enhanced through T1 effects (WET)-based water suppression scheme (24). The field homogeneity was optimized over the selected voxel of interest by using an automated three-dimensional shim, followed by optimization with manual shimming as needed. The following acquisition parameters were used: repetition time, 1,500 ms; echo time, 30 ms; averages, 128; spectral width, 1,000 Hz; and vector size, 1,024 data points.

Image Analysis

Spectra were processed by a single experienced physicist (N.S.) with 20 years of experience in spectroscopic analysis, using a spectroscopy task card available on a workstation running the Syngo platform (Siemens). Processing included zero-filling to 2,048 data points, multiplication by a Gaussian filter with less than 1 Hz line broadening, Fourier transformation, and phase and baseline corrections. Automated curve fitting was performed, which yielded choline peak amplitude, line width, and peak integral (area) value. Choline peak area and line width were tabulated, and serial measurements were performed on separate days for each tumor. The choline concentration was calculated with the internal water reference technique by using the following equation:

$$[\text{Cho}] = \frac{S_{\text{Cho}}}{S_{\text{water}}} \times \frac{n_{\text{water}}}{n_{\text{Cho}} \times MW_{\text{water}}} \times \frac{fT1_{\text{water}}}{fT1_{\text{Cho}}} \times \frac{fT2_{\text{water}}}{fT2_{\text{Cho}}}$$

where [Cho] is the concentration of choline in the tumor; S_{Cho} is the integral value of choline at 3.22 ppm; S_{water} is the integral value of the unsuppressed water signal; n_{Cho} and n_{water} are the numbers of ^1H nuclei contributing to the choline and water resonances, respectively; MW_{water} is the molecular weight of water; $fT1$, or $(1 - \exp[-TR/T1])$, is the T1 correction factor for partial saturation; and $fT2$, or $(\exp[-TE/T2])$, is the correction factor for signal loss from T2 relaxation. Relaxation times T1 and T2 of choline and water in tumor voxels of two rabbits were measured. T1 was measured with a TE of 30 ms and eight values of TR between 500 ms and 6,000 ms. T2 was measured with a TR of 2,000 ms and eight TE values between 30 and 400 ms. The resulting relaxation times are T1Cho of $1,293 \text{ ms} \pm 75$, T1water of $912 \text{ ms} \pm 200$, T2Cho of $276 \text{ ms} \pm 30$, and T2water of $86 \text{ ms} \pm 11$.

Histologic Analysis

All animals were euthanized under deep anesthesia by slow injection of a lethal dose (100 mg/5 mL) of sodium pentobarbital intravenously after the completion of MR imaging. Immediately after euthanasia, livers of all rabbits were carefully removed and subsequently

placed in 10% formaldehyde for fixation. After fixation, the liver was examined and the liver tumor was dissected out of the nontumorous liver tissue. Tumors were sliced at 3- to 4-mm intervals in the axial plane to correspond to the plane of the MR images and placed in standard cassettes. To maintain proper orientation, the dorsal and medial sides of each slice were stained with different colors. All sections were submitted to pathology personnel for histologic preparation. The tissue slices were embedded in paraffin, and two sections of each paraffin block were stained with hematoxylin and eosin. A microscope (SMZ800; Nikon, Tokyo, Japan) was coupled with a digital sight camera (resolution, $1,376 \times 1,032$; DS-U1; Nikon). Images of the tumors were captured at a magnification of 10. Digital images were first converted to JPEG format and then imported into ImageJ software (version 1.37; National Institutes of Health, Bethesda, Maryland), which was used to estimate the percentage of necrosis within the tumor. For each liver tumor, the axial slide representing the part of the tumor with the largest diameter was selected. For each slide, a region of interest was delineated around the entire tumor and another region of interest was delineated around the necrotic part of the tumor. The area of each region of interest was calculated using ImageJ software, and the percentage of tumor necrosis was determined for each tumor by dividing the necrotic tumor area by the total tumor area. Tumor size was recorded as the maximum diameter in millimeters.

Statistical Analysis

Regression analysis was performed using Excel software (Microsoft, Redmond, Washington). A comparison was made between the choline concentration and the percentages of necrosis on pathologic examination. An r value greater than 0.7 was considered to represent a good correlation, an r value of 0.4–0.7 a moderate correlation, and an r value less than 0.4 a poor correlation.

Results

All implantations were successful, and solid tumor developed in all 16 rabbits. Liver spectra were acquired in all rabbits (Fig 1a), all of whom tolerated the imaging procedure and related deep anesthesia without complications. Results of experiments are presented in the Table. Mean duration between implantation and MR imaging was 17 days (range, 11–21 d).

On ^1H MRS, the spectroscopic voxel size ranged from 1.7 to 6 cm^3 , depending on the size of the tumor. Although measurements in the liver are known to be susceptible to motion artifacts, a good spectral quality was consistently observed (Fig 1b). A choline resonance was detected at 3.22 ppm in 13 of the 16 spectra (81%). However, in three animals, no spectra were detected. According to the equation provided earlier, the calculated choline levels ranged from 0.21 to 8.96 mmol/kg.

At pathologic analysis, a solid tumor was identified in all livers (Fig 1c). Tumors were undifferentiated and consisted of necrotic and viable cells. For each liver tumor, the axial slide representing the part of the tumor with the largest diameter was successfully selected and used for further evaluation. Mean tumor size at pathologic examination, as measured on this axial slide, was 16 mm (range, 11–22 mm). Mean percentage of necrosis at pathologic examination, measured on the same slide, was 22% (range, 4%–44%).

Choline concentration had a relatively high inverse correlation with percentage of necrosis at pathologic examination, with an r value of 0.78 ($P < .002$; Fig 2).

Discussion

Assessment of tumor response after locoregional therapy has become increasingly important, as these therapies have become the mainstay of treatment for the growing population of patients with unresectable hepatocellular carcinoma (25). The ability to assess early tumor response after treatment is of great particular importance, because treatments may be repeated before disease progression occurs if treatment failure is identified early. Additionally, overtreatment may be prevented by early identification of tumor response. Therefore, development of novel imaging methods that focus on the early molecular changes after locoregional therapy remains significant.

Proton MR spectroscopy is a powerful, noninvasive tool for the biochemical characterization of normal and abnormal tissues *in vivo*. In the present study, we used the VX2 liver cancer model to perform a precise comparison between choline concentration as calculated by ^1H MRS and percentage of tumor necrosis measured by pathologic examination, and showed that tumor choline concentrations correlate with the amount of tumor necrosis on pathologic study. Our results are in line with those from a study by Chen et al (8), who measured the choline/lipid ratios of the viable part of liver tumors in 18 patients with HCC before and after chemoembolization. They showed that the choline/lipid ratio decreased significantly early after chemoembolization. However, for obvious ethical reasons, they had no pathologic confirmation of their findings.

The increase of choline levels in a lesion detected with ^1H MRS can be used to distinguish malignant from benign lesions. Choline is one of the components of phosphatidylcholine, an essential element of phospholipids in the cell membrane. Malignant tumors usually exhibit a high proliferation of cells and are therefore associated with increased metabolism of cell membrane components. This biochemical background will lead to an increased presence of choline in viable cancer cells. On the contrary, necrotic tumors have a lower cell density, which leads to a decrease of the choline concentration (21). In the present animal study, a choline resonance was detected in 13 of 16 liver tumors. We were not able to detect a choline peak in three animals, which may have been a result of breathing and cardiac movement. In patients, however, breath-hold acquisitions can be obtained, thereby improving the identification of small amounts of choline within the tumors.

In the clinical setting, quantification of the choline concentration is of great importance. Various approaches have been used to calibrate ^1H MR signals based on internal or external standards (26). Internal-standard methods have most often been used to calibrate liver spectra. Their use is based on the assumption that the concentrations of some metabolites such as tissue water are relatively constant. The use of water as an internal reference has found widespread use in single-voxel ^1H MRS (27). Acquisition of the large water signal as an internal reference requires few averages and a short scan time. Measurements with and without water suppression are acquired from the same selected volume without the need for additional shimming between measurements. In the present study, we observed a

consistently high-intensity water peak, which was positioned at 4.7 ppm, used a reference with in-vivo ^1H MRS.

The present study showed that the choline concentration measured with ^1H MRS had a relatively high inverse correlation with tumor necrosis at pathologic examination ($n = 13$, $r = 0.78$, $P < .002$). A high percentage of necrosis at pathologic examination corresponded with a low choline concentration measured on ^1H MRS, whereas a low percentage of necrosis corresponded with a high choline concentration. Therefore, ^1H MRS may be useful in determining tumor response after locoregional therapy.

The present study has several limitations. First, the VX2 tumor used in our study is of nonhepatic origin. However, it has proven to be convenient to study liver cancer in animal models because of the similarities in blood supply, genotype, and metabolism to advanced human hepatocellular carcinoma (28). Second, because it is difficult to use breath-holding or respiratory gating techniques in rabbits, respiratory movement during abdominal MR imaging might degrade image quality to some extent and cause variations in measurements. Third, the use of an internal reference standard may be influenced by the variation in concentration under pathologic conditions. Additionally, the choline level detected by ^1H MRS in the present study is the average concentration within the whole tumor, and in some cases, this might represent a combination of solid tumor and central liquefactive necrosis. Last, we used hematoxylin and eosin staining, which can accurately and reliably distinguish tumor necrosis, but staining with triphenyltetrazolium chloride may be more accurate in determining tissue viability. Future studies would, therefore, benefit from the addition of a second stain.

In conclusion, the present study demonstrated that the quantification of choline concentrations in liver tumors by using ^1H MRS with water as an internal reference was feasible. The calculated choline levels showed a relatively high inverse correlation with the percentage of tumor necrosis on pathologic examination. Therefore, that quantitative ^1H MRS measurement might be of additional value in the MR imaging of liver tumors.

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Abbreviations

ADC	apparent diffusion coefficient
¹H MRS	hydrogen-1 proton MR spectroscopy
TE	echo time
TR	repetition time
WET	water suppression enhanced through T1 effects

Figure 1.

(a) Typical example of a rabbit VX2 tumor (arrow) in the left lateral lobe of the liver 2 weeks after implantation. The lesion appears heterogeneously bright on T2-weighted images. The lesion size in the superior–inferior direction was approximately 15 mm. The spectroscopic voxel (size, $12 \times 11 \times 13 \text{ mm}^3$) is superimposed on the hyperintense lesion on the T2 image. **(b)** Water-suppressed spectrum from a rabbit liver obtained 2 weeks after implantation of the tumor. The spectrum consists of a choline (cho) resonance at 3.2 ppm and a lipid resonance at 1.3 ppm. It is acquired with a TR of 1,500 ms, TE of 135 ms, 128 averages, 3 min 13 s measurement time, and a voxel size of $12 \times 11 \times 13 \text{ mm}^3$. **(c)** Hematoxylin and eosin staining of the tumor. The entire tumor is outlined in black, and the necrotic parts of the tumor are outlined in white. (Available in color online at www.jvir.org.)

Figure 2. Correlation between choline concentration and percentage of necrosis ($n = 13$; $r = 0.78$; $P < .002$).

Table 1
Imaging and Pathologic Variables for All 16 Rabbits

Animal No.	Interval from Implantation to MR Imaging (d)	Pathologic Finding		Choline (mmol/kg)
		Size (mm)	Necrosis (%)	
1	21	20	27	4.25 ± 1.06
2	21	13.5	33	3.01 ± 0.75
3	21	21.5	27	1.89 ± 0.47
4	21	21.5	30	ND
5	19	12	44	0.21 ± 0.06
6	19	13	28	2.9 ± 0.72
7	19	17.5	15	3.26 ± 0.82
8	20	13	31	1.26 ± 0.32
9	20	11	11	7.26 ± 1.81
10	20	13	20	8.95 ± 2.24
11	11	14	18	6.62 ± 1.66
12	11	21	7	ND
13	12	12	4	ND
14	12	19	19	4.14 ± 1.04
15	12	20	16	8.96 ± 2.24
16	12	16	13	8.71 ± 2.18
Mean	17	16	22	4.72 ± 1.18

Note.—ND = not detected.