# Biochemical Analysis Using High-Resolution Magic Angle Spinning NMR Spectroscopy Distinguishes Lipoma-Like Well-Differentiated Liposarcoma from Normal Fat

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Biochemical analysis of benign and malignant tissue specimens may provide an objective method of tissue classification and thus serve as an adjunct to conventional morphological assessment. Liposarcomas account for about 30% of all soft tissue sarcomas in adulthood,[1−3] and occur in three principle forms: well-differentiated/dedifferentiated, myxoid/round cell, and pleomorphic. The well-differentiated liposarcoma subtype often has a gross appearance similar to that of normal fat and immediate frozen section morphological analysis of fat tissue is not helpful in distinguishing normal surrounding fat from liposarcoma due to the presence of substantial freezing artifact seen in snap-frozen fatty tissue.[1−3] Thus, differentiating normal fat from well-differentiated liposarcoma, particularly in retroperitoneal locations, continues to be a significant problem even for clinicians with considerable expertise in liposarcoma. In this communication the utility of a quantitative NMR biochemical analysis as a method for distinguishing well-differentiated liposarcoma from normal surrounding fat is explored. High-resolution magic angle spinning proton NMR (HR-MAS-$^1$H NMR) has already been shown to provide a reliable, quantitative biochemical profile of human soft tissue specimens and may be used as an objective method to distinguish the principle forms of liposarcoma.[4−8] MAS of tissue specimens reduces the effects of dipolar coupling interaction on neighboring spins, and averages the susceptibility inhomogeneity to zero. This improves NMR sensitivity, resolution, and spectral quality compared to static conditions. From previous studies it was clear that the higher level of triglycerides (by 3 orders of magnitude, about 5 M) compared to levels of other cellular metabolites in both normal fat tissue and well-differentiated liposarcoma posed a significant dynamic range problem in resolving and quantifying the less abundant nontriglyceride metabolites.[5] A double pulsed field gradient selective echo (DPFGSE) technique is employed together with MAS to selectively excite a region of the proton spectra which contains important less abundant nontriglyceride metabolites such as phosphatidylcholine (PTC) and phosphocholine (PC). A quantitative analysis of PTC and PC is then used to distinguish well-differentiated liposarcoma from normal fat.

A combination of selective pulses and pulsed field gradients provides band selectivity with a flat baseline and simultaneous suppression of large surrounding peaks. Magnetization of all resonances is rotated to transverse by the hard 90° pulse and dephased by the first pulsed field gradient $G_1$. The second gradient is identical to the first one and only refocuses the magnetization that was inverted by the selective 180° RE-BURP pulse. The magnetization that was not touched by this selective pulse is further dephased by the second gradient and cannot be detected afterward. To obtain clean selectivity and to maintain an undistorted baseline, the same procedure is repeated once, with the gradient intensity changed from 8.5 G/cm to 11.5 G/cm. The advantages of this DPFGSE have been described by Huang et al.[10,11]

Normal fat and lipoma-like well-differentiated liposarcoma samples were obtained from surgical pathology specimens following complete surgical removal of the patients' tumor along with normal surrounding tissue at the Brigham and Women's Hospital, Boston, Massachusetts. Tissue samples were either immediately frozen and stored in liquid nitrogen or immediately prepared for NMR experiments. Frozen tissues were prepared by thawing a small amount of tissue in 3 mL of PBS/$D_2O$ (pD = 7.4) at room temperature for 2 min. Excess PBS/$D_2O$ was removed by blotting, and the sample was then placed into a 4 mm O.D. zirconium rotor. Ten microliters of 95 mM sodium trimethyl-silylpropionate-2,2,3,3-$d_4$ (TSP) was added to the rotor as an internal reference standard. Typically between 0.06 and 0.07 g of tissue was used. Fresh samples were prepared similarly. All NMR data were collected at 20 °C on a Bruker DRX 500 spectrometer equipped with a 4 mm high-resolution $^1$H/$^{13}$C MAS probe. A pulse field gradient can be applied along the sample spinning direction.[12] A MAS rate of 5 kHz was used for these data. Increasing the MAS rate to 10 kHz was found to decrease the line width of broad peaks but did not change the area. Chemical shifts were referenced to the internal TSP standard. Deconvolution, using the Lorentzian line shape, was done using the program 1D WIN NMR (Bruker). The integrals of PTC and PC were obtained from this procedure.

Figure 1 shows the efficiency of the DPFGSE for selective detection in the presence of strong dynamic range problem. Figure 1a is a typical nonselective spectrum of normal fat. Signals from triglyceride clearly dominate the spectrum. The insert shows the 128 times enlarged signals of some low-abundance metabolites. Figure 1b is the selective spectrum of the same sample acquired using DPFGSE. The RE-BURP was designed to work in a region of about 500 Hz, between the two dashed lines. The baseline in the selective region is flat, which allows for more reliable deconvolution and quantitative measurements.

PTC is the most abundant membrane phospholipid and also serves as a precursor to other membrane phospholipid species such as phosphatidylethanolamine and sphingomyelin. For cells to proliferate, they must double their phospholipid mass to form

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(1) Fletcher, C. *Diagnostic Histopathology of Tumors*, Edinburgh: Churchill Livingstone, 1995; pp 1049−1053.
(2) Fletcher, C.; Akerman, M.; Cin P.; Wever, I. D.; Mandahl, N.; Mertens, F.; Mitolman, F.; Rosai, J.; Rydholm, A.; Sciot, R.; Tallini, G.; Berghe, H.; Ven, W.; Vanni, R.; Willen, H. *Am. J. Pathol.* **1996**, *148*, 623−630.
(3) Mentzel, T.; Fletcher, C. *Virchows Arch.* **1995**, *427*, 353−363.
(4) Andrew, E R. *Nature* **1958**, *182*, 1695.
(5) Millis, K.; Weybright, P.; Campbell, N.; Fletcher, J. A.; Fletcher, C. D.; Cory, D. G.; Singer, S. *Magn. Reson. Med.* **1999**, *41*, 257−267.
(6) Cheng, L. L.; Ma, M. J.; Becerra, L.; Ptak, T.; Tracey, I.; Lackner, A.; Gonzalez, R. G. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 6408−6413.
(7) Griffin, J. L.; Troke, J.; Walker, L. A.; Shore, R. F.; Lindon, J. C.; Nicholson, J. K. *FEBS Lett.* **2000**, *486*, 225−229.
(8) Moka, D.; Vorreuther, R.; Schcha, H.; Spraul, M.; Humpfer, E.; Lipinski, M.; Foxall, P.; Nicholson, J.; Lindon, J. *J. Pharm. Biomed. Anal.* **1998**, *17*, 125−132
(9) Geen, H.; Freeman, R. *J. Magn. Reson.* **1991**, *93*, 93−141.
(10) Huang, T. L.; Shaka, A. J. *J. Magn. Reson.*, **1995**, *112*, 275−279.
(11) Jerschow, A. *J. Magn. Reson.* **1999**, *137*, 206−214.

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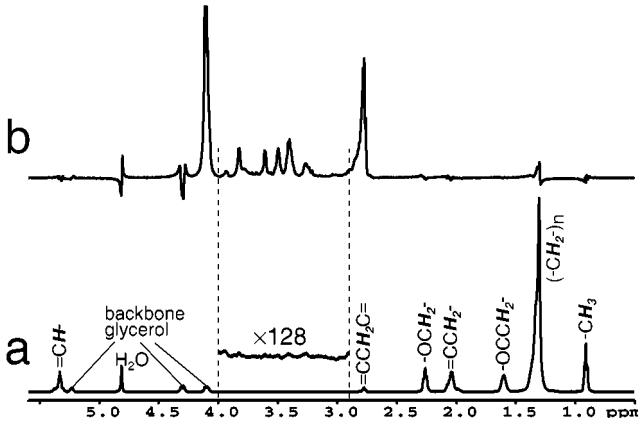


**Figure 1.** (a) Representative spectrum of normal fat. All the observable signals are from triglyceride except the residual water peak. (b) Spectrum acquired using double pulsed field gradient selective echo [90°−$G_1$−(RE-BURP)−$G_1$−$G_2$−(RE-BURP)−$G_2$−acq]. The gradients $G_1$ and $G_2$ were 1 ms with intensities of 8.5 and 11.5 G/cm, respectively. 11.6 ms RE-BURP was used.[9] The receiver gain used for (b) was increased 1024 times compared to (a).

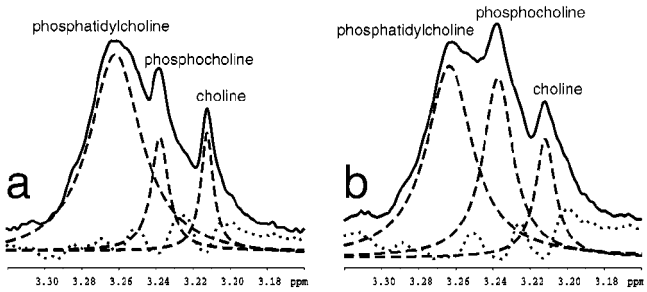


**Figure 2.** Selective spectra of normal fat (a) and lipoma-like well-differentiated liposarcoma (b) from one representative individual. Dashed lines show deconvolution. Dotted lines are the baseline which include some low abundant metabolites. The DPFGSE sequence with the RE-BURP for refocusing provides a uniform excitation over this observed frequency region.

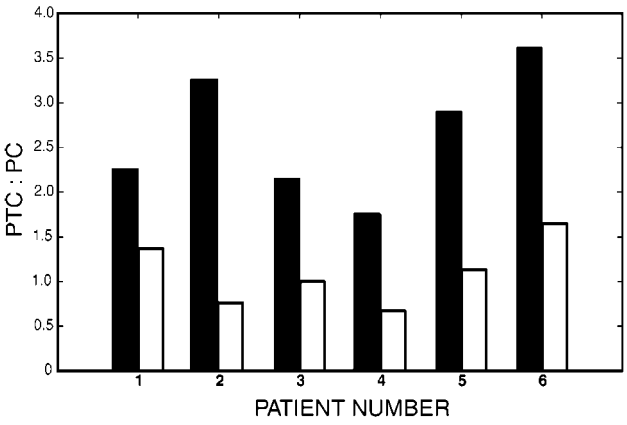


**Figure 3.** Ratios of phosphatidylcholine to phosphocholine in normal fat (filled bar) and in lipoma-like well-differentiated liposarcoma (open bar) for six pairs of samples from six patients. Each pair comes from a single individual. The ratio is an average of 2.4-fold larger in normal fat than in liposarcoma. ($n = 6$, $p < 0.001$)

new cells. Phospholipid metabolism must be carefully regulated throughout the cell cycle to produce daughter cells that are of normal cell size and intracellular lipid content. Cytidylyltransferase (CT) is a key regulatory enzyme in PTC biosynthesis and phospholipid formation.[13,14] PTC synthesis and CT activity is enhanced during the $G_1$ and S phases of the cell cycle in preparation for cell division. The $G_2$ and M phases are characterized by an inhibition of CT activity and a cessation of PTC synthesis. Thus, changes in PTC synthesis are carefully coordinated with the cell cycle and may be an important indicator of cell proliferation. The PTC-to-PC ratio is a measure of PTC synthesis and is largely a reflection of CT activity, the enzyme that converts phosphocholine to CDP-choline, since this is the rate limiting-step in PTC formation. A decrease in the PTC-to-PC ratio would indicate a reduction in PTC synthesis with an inhibition of the CT enzyme activity. From the selective spectrum, the ratio of PTC to PC can be determined and then used to distinguishing lipoma-like well-differentiated liposarcoma from normal fat (Figure 2). Deconvolution provides a measure of the proton signal from the three methyls that are bound to nitrogen in PTC, PC, and choline, respectively and are shown as dashed lines in Figure 2. Assignments were made on the basis of 2D experiments and from authentic materials.[16] Six pairs of normal fat and tumor tissues, each pair from the same individual, were analyzed. In each case of normal fat, the PTC-to-PC ratio was greater than that of the corresponding liposarcoma. The average ratio was $2.7 \pm 0.7$ in normal fat and $1.1 \pm 0.4$ in liposarcoma ($n = 6$, $p = 0.0008$). The data are summarized in Figure 3.

In summary, DPFGSE combined with MAS increase the efficiency of triglyceride suppression and at the same time minimize spectral artifacts over the selected region of interest. These methods have been used to distinguish well-differentiated liposarcoma from normal surrounding fat on the basis of ex vivo NMR determination of the PTC-to-PC ratio. We find that the ratio of PTC to PC is about 2.4-fold higher in normal fat than in corresponding lipoma-like well-differentiated liposarcoma. Cytidyltransferase is the rate-determining enzyme in the biosynthetic pathway for the synthesis of membrane phospholipids from choline. Activity of this enzyme is known to be cell-cycle dependent, with inhibition occurring in the $G_2$ and M phases of the cell cycle.[15] Our results are consistent with these observations in that normal fat, which is largely terminally differentiated, has a high PTC-to-PC ratio. In contrast, well-differentiated liposarcoma, which contains a greater fraction of cells that are actively dividing compared to normal fat, has a significantly lower PTC-to-PC ratio and a relative inhibition of cytidyltransferase activity compared to normal fat. The 1D selective MAS methods provide a rapid, objective technique for distinguishing well-differentiated liposarcoma from normal fat that is unable to be performed using conventional nonselective 1D methods. Ex vivo NMR biochemical classification of tissue type may serve a useful adjunct to conventional pathological techniques.

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**Supporting Information Available:** Detailed data on different spinning rate and confirmation of the assignment of peaks (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) Maas, W. E.; Laukien, F. H.; Cory, D. G. *J. Am. Chem. Soc*. **1996**, *118*, 13085−13086.
(13) Michal, G. *Biochemical Pathways: An Atlas of Biochemistry and Molecular Biology*; John Wiley and Sons: New York, 1998.
(14) Tronchère, H.; Record, M.; Tercé, F.; Chap, H. *Biochim. Biophys. Acta* **1994**, *1212*, 137−151.
(15) Jackowski, S. *J. Biol. Chem*. **1996**, *271*, 20219−20222.
(16) Millis, K.; Mass, W.; Cory, D.; Singer, S. *Magn. Reson. Med*. **1997**, *38*, 399−403.