# Identification of mobile lipids in human cancer tissues by *ex vivo* diffusion edited HR-MAS MRS

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Received June 10, 2009; Accepted July 23, 2009

DOI: 10.3892/or_00000592

**Abstract.** Magnetic Resonance Spectroscopy visible mobile lipids are considered important markers in the diagnosis of human cancer and are thought to be closely involved in various aspects of tumour transformation, such as cell proliferation, necrosis, apoptosis, hypoxia and drug resistance. A method allowing the straightforward identification of the lipid classes contributing to the mobile lipids in human malignant tissues is highly advisable. *Ex vivo* High Resolution Magic Angle Spinning Magnetic Resonance Spectroscopy was done directly on human cerebral, renal and colorectal malignant tissue specimens. A diffusion edited sequence, based on stimulated echo and bipolar gradient pulses, was used to characterize molecules with low diffusion rates, arising from mobile lipid components. Cholesterol, triglycerides and phosphatidylcholine are simultaneously detected and all contribute to the mobile lipid resonances present in malignant glioma and clear cell renal carcinoma tissue specimens spectra. On the contrary, papillary cell renal carcinoma spectrum is predominated by phosphatidylcholine resonances and that of colorectal adenocarcinoma is characterized by signals arising from triglycerides. *Ex vivo* diffusion edited High Resolution Magic Angle Spinning Magnetic Resonance Spectroscopy, done on intact tissue, is a powerful analytical tool to obtain a simple and immediate identification of mobile lipid components. This can offer a significant contribution to better understanding their involvement in cancer tissues. Furthermore, *ex vivo* high resolution spectroscopic measurements allow to improve the interpretation of *in vivo* Magnetic Resonance spectra, increasing its clinical potentiality.

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*Key words: ex vivo*, diffusion, High Resolution Magic Angle Spinning, Magnetic Resonance Spectroscopy, mobile lipids, cancer

## Introduction

Great interest has been dedicated to Magnetic Resonance Spectroscopy (MRS) visible mobile lipids (MLs), which significance in life and death of cells has been highlighted by Hakumaki and Kauppinen (1). Lipid deposits in human cells and tissues are revealed and identified by MRS. These compounds are thought to originate mainly from triglycerides (TG) fatty acid (FA) chains with a lesser contribution from cholesteryl esters (CholE) (1). In the early 80s, Mountford and Wright hypothesized that MLs arise from neutral lipids located in plasma membrane (2). Afterwards, other authors have speculated that MLs exist in cytosolic lipid droplets (3-5). Several MRS studies have clearly elucidated how these lipids are closely involved in various important aspects of tumour transformation, such as cell proliferation, necrosis, apoptosis, hypoxia and drug resistance (6-10). Moreover, MLs are supposed to have clinical potential in brain cancer treatment response detection (11-14). Despite the large number of MRS studies on experimental and human cancer (1-14), the exact MLs metabolic origin is still debated, therefore, a method allowing the straightforward characterization of these important tissue components, is highly advisable. In this study, the possibility to perform the simple and simultaneous identification of cholesterol (Chol) and/or CholE, TG and phospholipids (Ph) in human malignant neoplasms, is described. Cancer tissue specimens were studied by *ex vivo* High Resolution Magic Angle Spinning (HR-MAS) MRS, using a diffusion edited sequence, which allowed the separation of molecules characterized by low diffusion rates. To our knowledge, a similar experiment on different human cancer tissues, has not yet been reported in literature.

## Materials and methods

Magnetic Resonance Imaging (MRI) and localized single voxel *in vivo* $^{1}$H-MRS were performed with a 3 Tesla whole-body scanner (General Electric Medical Systems, Milwaukee, WI) on two patients affected by cerebral lesion, following the routine standard clinical protocol previously described (15). After MRI and *in vivo* MRS the patients underwent surgery and two adjacent samples were obtained. One section was used for routine histopathology and revealed a malignant glioma in both cases. The second sample was used for HR-MAS measurements. Specimens were also collected from three patients undergoing radical nephrectomy for solid masses. Renal lesions were sorted by microscopy: two were classified as clear cell and one turned out to be a papillary cell renal carcinoma. In this study, a tissue specimen obtained from a patient who underwent surgery for colorectal adenocarcinoma has been also included. All patients gave written informed consent to participate to the study which was approved by the local research Ethics Committees.

The six tissue samples were quickly frozen in liquid nitrogen and stored at -85˚C until MRS analyses. *Ex vivo* $^{1}$H HR-MAS MR spectra were recorded with a Bruker Avance400 spectrometer (Bruker BioSpin, Karlsruhe, Germany) equipped with a 4-mm dual $^{1}$H/$^{13}$C HR-MAS probe, operating at 400.13 MHz. Samples were spun at 4000 Hz and $^{1}$H MR spectra were acquired by using a sequence for diffusion measurements (16), based on stimulated echo and bipolar gradient pulses, with big delta 200 ms, eddy current delay $T_e$ 5 ms, little delta 2*2 ms, fine shaped gradient with 32 G/cm followed by a 200 $\mu$sec delay for gradient recovery, 8 kHz spectral width, 8k data point and 256 scans. The assignments were in agreement with the literature and were confirmed by comparison of the MR spectra of Sigma standard lipid compounds [Chol, CholE, TG, phosphatidylcholine (PhC), saturated fatty acids and poly-unsaturated fatty acids (PUFAs)].

## Results and discussion

*Ex vivo* HR-MAS MRS, introduced in 1997 by Cheng *et al* (17), has become essential to outline intact human tissues metabolic profiles (9,18-22). In a previous study performed in our laboratory (22), using HR-MAS technique, we characterized the human gastric adenocarcinomas biochemical profile. In particular, a diffusion edited sequence (16) allowed us to obtain spectra displaying broad resonances of molecules characterized by low diffusion rates, deriving from mobile lipids, which were identified as saturated and unsaturated FA chains, almost exclusively esterified in TG. Since the importance of MLs in cancer (1-14), we have undertaken a diffusion edited HR-MAS MRS study on different human neoplasms, in order to indentify accurately the lipid classes contributing to the MLs resonances in human malignant tissues.

Fig. 1 shows malignant glioma *ex vivo* diffusion edited $^{1}$H HR-MAS MR spectrum. In the window, the axial $T_2$-wheighted MR image and localized *in vivo* $^{1}$H MR spectrum of the same lesion, obtained before surgery, are also reported. In *in vivo* spectrum, two signals at 1.30 and 0.90 ppm are present, and are assigned to methylene -(C**H**$_2$)$_n$- and terminal methyl -C**H**$_3$ protons, respectively, in FA chains. These signals, in brain neoplasm *in vivo* MR spectra, are generally attributed to the presence of FA chains arising from MLs, which are associated with tumour aggression and are considered markers of high grade malignant lesions (23); however, these resonances do not give information on the lipid classes contributing to MLs composition.

The analysis of the *ex vivo* diffusion edited $^{1}$H HR-MAS MR spectrum is considered largely helpful. Indeed, in Fig. 1, besides several resonances pertaining to FA chains, signals arising from neutral (TG and Chol and/or CholE) and polar (Ph) lipid moieties, are clearly observed. Eight signals [proton assignments are reported in brackets] resonating at 0.90 [terminal methyls, y-CH$_2$-C**H**$_3$], 1.30 [methylenes, x-CH$_2$-(C**H**$_2$)$_n$-CH$_2$-y and x-C**H**$_2$-(CH$_2$)$_n$-C**H**$_2$-y], 1.58 [ß-methylenes, x-CH$_2$-C**H**$_2$-CH$_2$-CO-y], 2.05 [mono-allylic methylenes, x-C**H**$_2$-CH=CH-y and x-C**H**$_2$-CH=CH-C**H**$_2$-CH$_2$-y], 2.30 [α-methylenes, x-CH$_2$-CH$_2$-C**H**$_2$-CO-y], 2.70-2.80 [di-allylic methylenes, =CH-C**H**$_2$-CH= and x-CH=CH-(C**H**$_2$-CH=CH)$_n$-y] and 5.30 [vinyl protons, x-C**H**=C**H**-(CH$_2$-C**H**=C**H**)$_n$-y, x-CH$_2$-C**H**=C**H**-y and x-CH$_2$-C**H**=C**H**-CH$_2$-CH$_2$-y] ppm indicate the presence of saturated and unsaturated FA chains (19,24,25). Other resonances arising from Chol and/or CholE, Ph and TG moieties, are detected. These lipids are clearly identified, according to the literature (19,24,25) and to the comparison of single standard spectra, based on the following signals: 0.68 [Chol backbone, 18-C**H**$_3$], 0.90 [Chol backbone, 21-C**H**$_3$, 9-C**H**, 19-C**H**$_3$], 1.04 [Chol backbone, 1-C**H**$_2$, 14-C**H**, 17-C**H**, 22-C**H**$_2$, 23-C**H**$_2$ and 24-C**H**$_2$], 1.65 [Chol backbone, 7-C**H**$_2$, 15-C**H**$_2$ and 25-C**H**], 3.04 [phosphatidylethanolamine (PhE) plasmalogen, -CH$_2$-CH$_2$-N**H**$_2$], 3.25 [PhC, trimethyammonium, -N$^+$(C**H**$_3$)$_3$], 4.08 and 4.28 [TG, bonded glycerol, C**H**$_2$OCOR$_1$-CHOCOR$_2$-C**H**$_2$OCOR$_3$] and 5.22 [TG, bonded glycerol, CH$_2$OCOR$_1$-C**H**OCOR$_2$-CH$_2$OCOR$_3$].

Other malignant glioma *ex vivo* diffusion edited $^{1}$H HR-MAS MR spectra (data not shown) are quite similar to that reported in Fig. 1, the only difference being a more intense trimethylammonium -N$^+$(C**H**$_3$)$_3$ choline residue signal at 3.25 ppm, indicating a larger amount of PhC. In both cases, diffusion edited HR-MAS sequence, due to higher signal-to-noise ratio and to the fact that signals are not spread over the whole spectrum, as in *in vivo* MRS, allows to determine that MR visible MLs resonances receive contribution from: i) FA esterified in TG; ii) FA esterified in PhC; iii) Chol, which has been found to be present as CholE in experimental and human gliomas (11). This opportunity appears particularly important considering, for example, that lipid content, revealed by a conventional single pulse HR-MAS MRS experiment, only from the FA signals at 1.30 and 0.90 ppm, has been found to be an important factor to separate the metastasis spectra originating from different primary tumours and was also correlated with clinical outcome (19).

All the above-mentioned lipid resonances, arising from saturated and unsaturated FA chains, TG, PhC and Chol and/or CholE moieties, are also present in Fig. 2a, displaying the clear cell renal carcinoma *ex vivo* diffusion edited $^{1}$H HR-MAS MR spectrum. The profile of this lesion, both for signal intensities and frequencies, is very similar to the one of the other clear cell carcinoma. In the spectrum in Fig. 2a, signals arising from cholesterol backbone, which we have

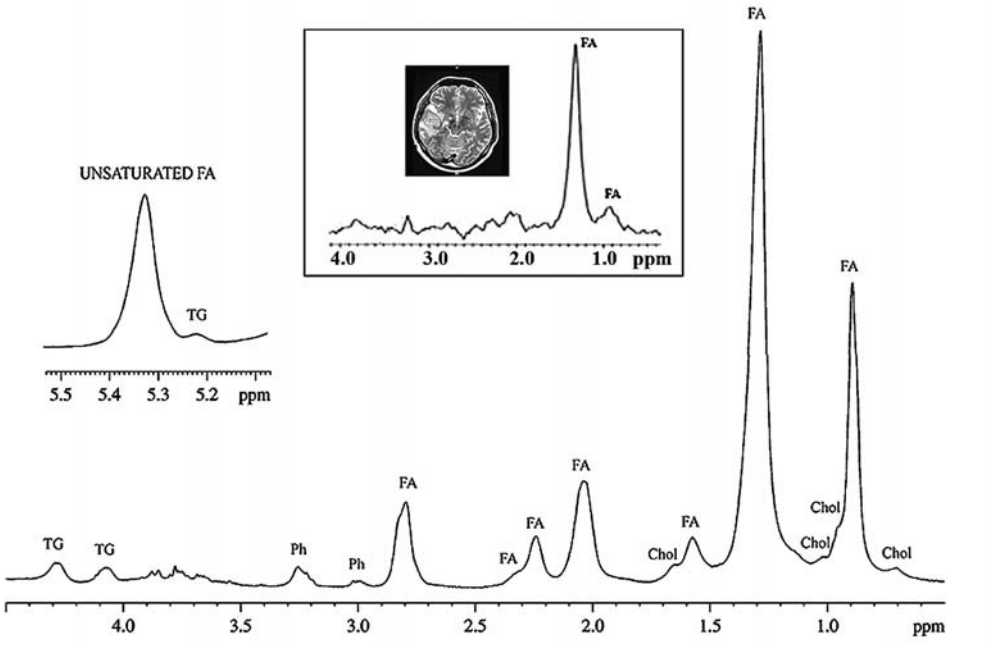


Figure 1. *Ex vivo* diffusion edited $^{1}$H HR-MAS MR spectrum of a malignant glioma; in the window, the axial FSE $T_2$-wheighted MR image and the localized *in vivo* $^{1}$H MR spectrum of the same lesion, are also reported.

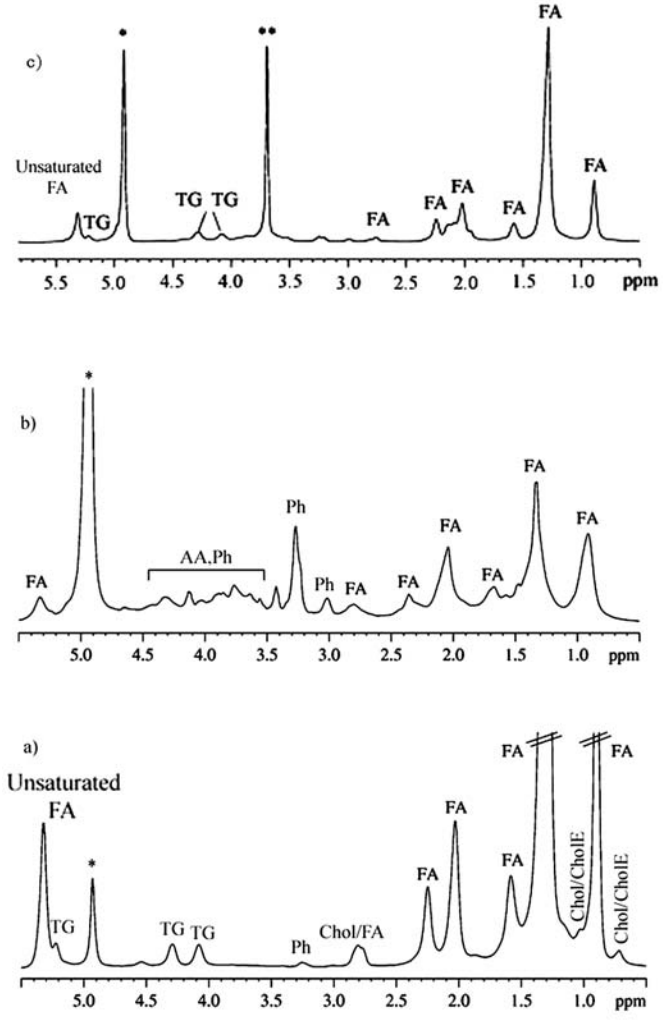


Figure 2. *Ex vivo* diffusion edited $^{1}$H HR-MAS MR spectra of: (a) clear cell renal carcinoma; (b) papillary cell renal carcinoma; (c) colorectal adenocarcinoma; *residual water peak; **PEG.

previously found to be present as cholesteryl oleate in the chloroform/methanol extracts (26), are particularly evident. On the contrary, resonances due to Chol and/or CholE are absent, and those arising from TG are negligible, in papillary cell renal carcinoma spectrum (Fig. 2b). In fact, the diffusion edited spectrum of this lesion shows signals due to saturated and unsaturated FA chains which are esterified in PhC, as inferred by the strong resonance at 3.25 ppm coming up from the trimethylammonium $-N^{+}(C\underline{\mathbf{H}}_3)_3$ choline residue. Weak resonances in the 3.50-4.50 ppm region are also present and assigned to bonded amino acids (AA) in small oligopeptides (22). It should be remarked that ratio between FA methyl [$-CH_3$] and FA methylene [$-(CH_2)_n-$] signal is bigger than the one present in other spectra, showing the contribution of methyl from AA in the 0.9 ppm resonance.

Colorectal adenocarcimoma $^{1}$H HR-MAS MR spectrum is reported in Fig. 2c. Signals due to saturated and unsaturated FA chains esterified in TG, as the main lipid component characterizing the malignant tissue, are observed. On the contrary, resonances typical of Ph and Chol moieties are negligible or absent, respectively. The strong signal at 3.70 ppm arises from polyethylene glycol (PEG), present in pharmaceutical preparation used for colon preparation.

As above described, a careful inspection of the human neoplastic lesions *ex vivo* diffusion edited HR-MAS MR spectra (Figs. 1 and 2), show clear signals arising from different groups of saturated and unsaturated FA chains. The resonance at 2.80 ppm, attributable to methylene protons between two double bonds (=CH-C$\underline{\mathbf{H}}_2$-CH=), is particularly intense in high grade glioma spectrum (Fig. 1) and indicates the presence of PUFAs (12,13,24,25,26). Monitoring these attracting markers could be of great utility. Indeed, PUFAs are strongly implicated in apoptotic processes occurring after anti-cancer gene therapy (12,13) and they are supposed to have clinical potential in brain cancer treatment response detection

(11-14). Even if TG and CholE are considered the predominant lipid groups contributing to the MRS visible MLs in human neoplastic tissue (1), from our *ex vivo* HR-MAS MRS findings it is evident that the contribution of mobile PhC cannot be overlooked.

Concluding, this preliminary study clearly indicates that diffusion edited HR-MAS MRS has the potential to bear novel implications for a better understanding of lipid biochemistry in cancer. Further studies on the direct correlation between all types of MLs components (FA, TG, Ph, Chol and/or CholE and PUFAs) characterizing human neoplastic tissues, with different aspects of tumour transformation, will be possible.

## Acknowledgements

This study was supported by a grant of MUR ex 60% to V.T. and S.B.

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