Ex vivo HR-MAS Magnetic Resonance Spectroscopy of Normal and Malignant Human Renal Tissues

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Abstract. *The aim of the present study was to examine the metabolic profile of normal and tumoral renal tissues by ex vivo high resolution magic angle spinning magnetic resonance spectroscopy (HR-MAS MRS). Patients and Methods: Five patients, three affected by clear cell renal cell carcinoma (RCC) and two by papillary RCC, were examined. A radical nephrectomy was performed in each. In all patients, fresh tissue samples taken from normal cortex, normal medulla and tumor were collected and analyzed by mono-dimensional HR-MAS MRS. Results: The spectra of human normal cortex and medulla showed the presence of differently distributed organic osmolytes as markers of a physiological renal condition. The marked decrease or disappearance of these metabolites and the high lipid content (triglycerides and cholesteryl esters) is typical of clear cell RCC, while papillary RCC are characterized by the absence of lipids and very high amounts of taurine. Conclusion: This paper demonstrates that ex vivo HR-MAS MRS is a viable and powerful means of probing for molecular information in human normal and tumoral renal tissues. This research will constitute the basis for a biochemical classification of renal neoplastic pathologies, especially for RCCs, which can be thus evaluated by in vivo MRS for clinical purposes. Moreover, these data may contribute to a better knowledge of the molecular processes for the basis of the onset of renal carcinogenesis.*

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Renal cell carcinoma (RCC) is the sixth leading cause of cancer death. Approximately one-third of patients present with disease which is already metastatic and for which there is currently no adequate treatment, furthermore no biofluid screening tests exist for RCC. It is now well established that kidney cancer does not represent a single disease, but rather a collection of different types of cancer (1).

The identification of these different types of cancer requires the monitoring of their molecular signature and of their altered metabolic profile. Magnetic resonance spectroscopy (MRS), giving an accurate description of the molecular composition of a human tissue, represents an important tool for this analysis.

MRS is widely applied in biomedical research, with particular attention to molecular evaluations regarding diagnosis, treatment and prognosis of human neoplasms (2, 3). The clinical role of proton MRS in the evaluation of brain tumors, as well as breast and prostate cancer, has been under investigation for several years (4). However, the potential for application of MRS in the study of different organs is still in its infancy, although it shows great potential, especially for study of the human kidney (5). In this field, basic MRS studies are needed to elucidate the main metabolic profiles in the comparison between healthy and neoplastic kidney.

In the urological field, MRS has been applied to the study of the biochemical composition of biological fluids (6, 7), as well as renal tissue metabolism and function (8, 9-11). In our previous papers (12, 13), we described the biochemical composition of human renal tissues with regards to both aqueous and lipid extracts studied by monodimensional low field *in vitro* MRS. *In vitro* studies

Table I. *Patient characteristics.*

Patients	Age (years)	Tumor size (cm)	Histological type	Fuhrman grade	TNM group stage
1	76	4	Clear cell RCC	G3	pT3a Nx
2	58	5	Clear cell RCC	G3	pT1bN0
3	77	9	Clear cell RCC	G3	pT3bNx
4	86	4	Papillary RCC	G2	PT1aNx
5	70	10	Papillary RCC	G2	PT3aN0

yielded information on the marked decrease in renal osmolytes in nephrocarcinomas and on the cholesteryl ester content in malignant tumoral tissues.

Nowadays, the different metabolic profiles associated with functional, benign and/or malignant human tissues can be established through *ex vivo* high resolution magic angle spinning (HR-MAS) MRS (14-22). HR-MAS MRS is a powerful analytical tool for the investigation of human tissue molecular composition bridging the gap between *in vitro* (performed on tissue extracts) and the *in vivo* (performed directly on the patient) MRS. The first application of HR-MAS MRS was made possible by the commercial diffusion of MRS probe-heads capable of studying the samples in rapid rotation around an axis of 54.7° ("magic angle") tilted with respect to that of the static magnetic field. These probe-heads drastically reduce the contribution from dipolar couplings and chemical shift anisotropy providing high resolution spectra from semi-solid samples. The quality of these spectra is comparable to that obtained from aqueous extracts with the advantage of carrying out the measurements on intact tissue specimens without any pretreatment. *Ex vivo* HR-MAS MRS analysis is able to relate the biochemical tissue composition to the ultrastructural and histopathological findings on the same specimen in order to establish the metabolic profile typical of the pathological tissue (23). Correlations between some metabolites and proliferative markers to gain insight into the relationship between cellular proliferation and the metabolic changes associated with the presence and tumor aggressiveness are thus possible (17, 24). Moreover, information provided by *ex vivo* HR-MAS MRS may be fundamental for the development of *in vivo* MRS for the diagnosis of renal pathologies on clinical grounds (5, 8, 25-27).

Here, we report on *ex vivo* HR-MAS MRS study performed on human renal tumor tissue samples taken from five patients at the time of radical nephrectomy. A sampling of macroscopically normal renal cortex and medulla from the same kidney was also performed.

To our knowledge, this is the first HR-MAS MRS study differentiating the full metabolic pattern of human intact renal cortex, medulla and tumor tissues.

Patients and Methods

Clinical materials. Specimens were collected from five patients undergoing radical nephrectomy for solid renal tumors. The clamping and/or division of the main renal artery was performed carefully immediately before collecting the specimens to minimize the warm ischemia time. In all cases, sample of normal cortex, normal medulla and tumor were taken.

Pathology. On microscopy, renal tumors were classified according to the WHO classification (28) as clear cell (n=3) and papillary renal cell carcinoma (RCC) (n=2); tumor stage was based on TNM staging system (29) and grade was assessed according to the Furhman grading system (30) (Table I).

Tissue samples. The fresh tissue samples were quickly frozen in liquid nitrogen after surgery to avoid micro-crystallization of the water in the cells, which causes damage, and stored at –85°C until MRS analyses.

Magnetic resonance spectroscopy. Proton HR-MAS MR spectra were recorded with a Bruker Avance400 spectrometer (Rheinstetten, Germany) equipped with a $^1H/^{13}C$ HR-MAS probe operating at 400.13 and 100.61 MHz, respectively. Samples were spun at 4000 Hz and three different types of monodimensional (1D) proton spectra were acquired using: a) A composite pulse sequence (31) with 1.5 s water-presaturation during relaxation delay, 8 kHz spectral width, 32 k data point, 32 scans; b) A water-suppressed spin-echo Carr-Purcell-Meiboohm-Gill (CPMG) sequence (32) with 1.5 s water-presaturation during relaxation delay, 1 ms echo time and 360 ms total spin-spin relaxation delay, 8 kHz spectral width, 32 k data point, 256 scans; c) A sequence for diffusion measurements based on stimulated echo and bipolar-gradient pulses (33) with Δ 200 ms, eddy current delay T_e 5 ms, δ 2x2 ms, fine shaped gradient with 32 G/cm followed by a 200 μs delay for gradient recovery, 8 kHz spectral width, 8k data point, 256 scans.

Results

Ex vivo 1D proton HR-MAS MR spectra obtained from human normal cortex and medulla samples are shown in Figure 1 and 2, respectively.

The spectra shown in Figure 1a and 2a, acquired using a 1D ^1H sequence with a water-presaturation, shows the presence of narrow resonances derived from low molecular
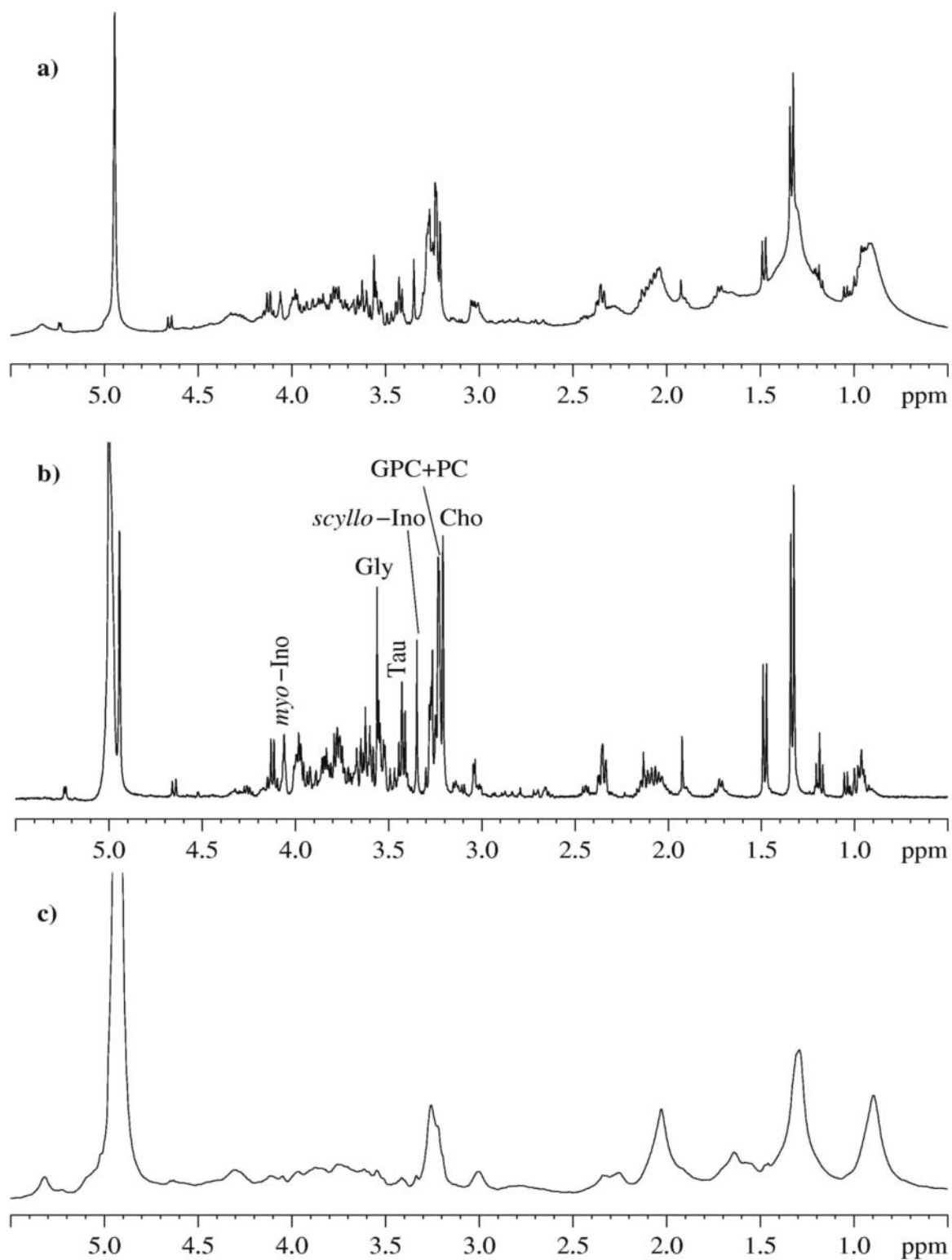
Figure 1. *1D ^{1}H MR spectra of normal human cortex: a) Conventional proton spectrum with water-presaturation (the broad resonances are assigned to lipids and oligopeptides); b) CPMG spectrum; c) Diffusion-edited spectrum (the resonance at ~3.22 ppm is assigned to the trimethylammonium residue of phosphatidylcholine and the broad resonances are assigned to FA (fatty acids) esterified in TG (triglycerides) and Ph (phospholipids) with a lesser contribution of oligopeptides). Cho (free choline), Gly (glycine), GPC (glycerophosphorylcholine), myo-Ino (myo-inositol), PC (phosphorylcholine), scyllo-Ino (scyllo-inositol), (Tau) taurine.*
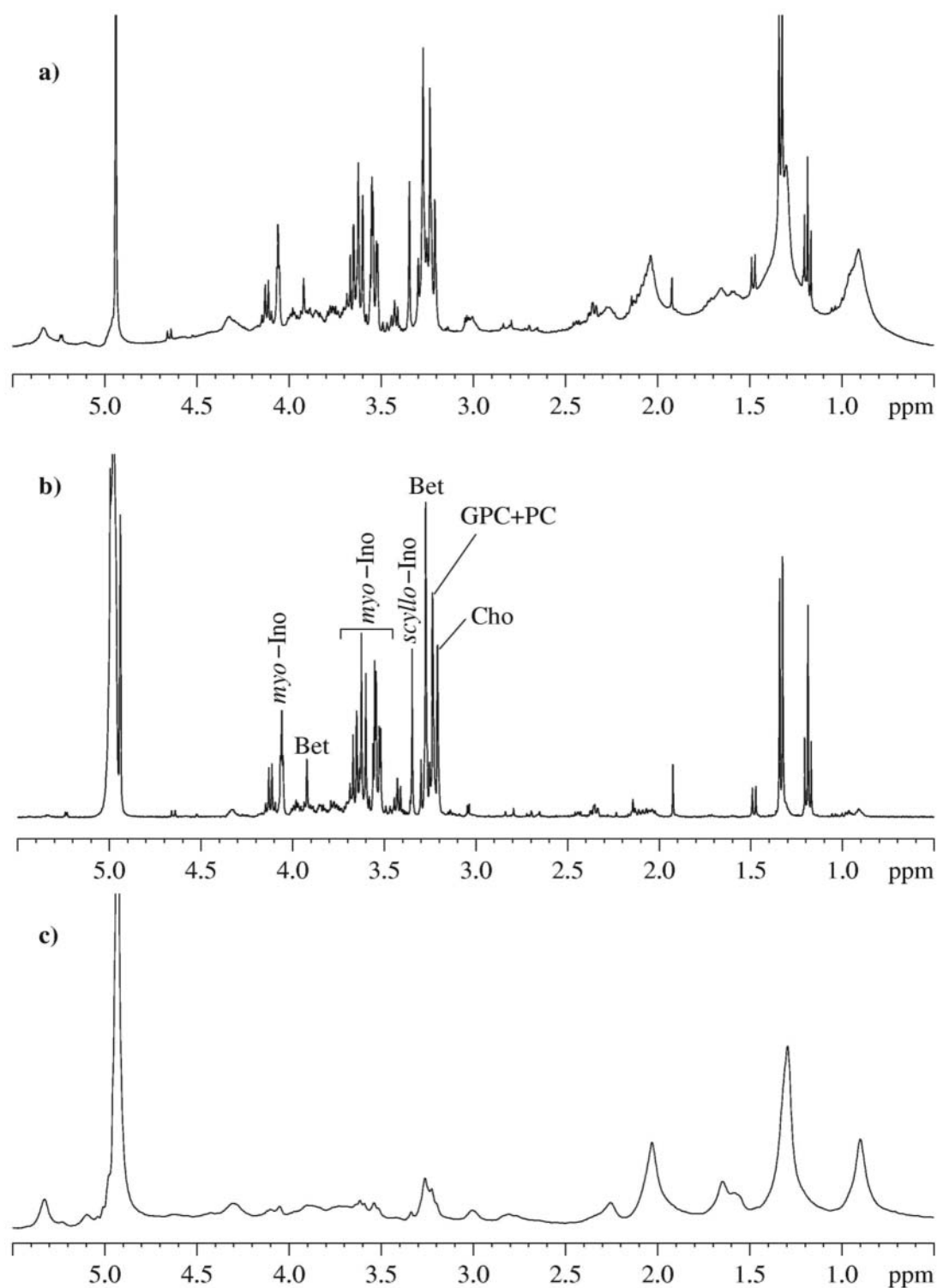
Figure 2. *1D ^{1}H MR spectra of normal human medulla: a) Conventional proton spectrum with water-presaturation (the broad resonances are assigned to lipids and oligopeptides); b) CPMG spectrum; c) Diffusion-edited spectrum (the resonance at ~3.22 ppm is assigned to the trimethylammonium residue of phosphatidylcholine and the broad resonances are assigned to FA (fatty acids) esterified in TG (triglycerides) and Ph (phospholipids) with a lesser contribution of oligopeptides). Bet (glycine-betaine), Cho (free choline), Gly (glycine), GPC (glycerophosphorylcholine), myo-Ino (myo-inositol), PC (phosphorylcholine), scyllo-Ino (scyllo-inositol), Tau (taurine).*

weight metabolites, and broad resonances mainly due to the presence of lipids and oligopeptides.

The MR spectra obtained by using a CPMG sequence are given in Figure 1b and 2b. These spectra allow the separation of the contribution to the spectrum of macromolecules and small metabolites. The cortex and medulla contain many small molecules with different distributions in the two kidney regions. Amino acids are present in higher amounts in the cortical tissue than in the medullary. In particular, the cortex is characterized by a high percentage of glycine (Gly) and taurine (Tau), while the medulla by glycine-betaine (Bet) and *myo*-inositol (*myo*-Ino). Other osmolytes, such as *scyllo*-inositol (*scyllo*-Ino) and the choline-containing compounds (ChoCC), glycerophosphorylcholine (GPC), phosphorylcholine (PC) and free choline (Cho), are present in approximately the same relative amounts in both kidney tissues.

Diffusion-edited spectra, acquired in order to observe components with low diffusion rates mainly deriving from lipids and small proteins, are shown in Figure 1c and 2c. The spectra of the cortex and medulla are quite similar and are dominated by the signals due to fatty acids (FA) of triglycerides (TG) and phospholipids (Ph), with a lesser contribution of oligopeptides.

The same analyses were also performed on two different subtypes of renal cancer, namely clear cell RCC and papillary RCC. The corresponding spectra are shown in Figure 3 and 4, respectively. A marked difference between the two spectra is evident: the spectrum of the clear cell RCC displays predominant resonances due to lipids, whereas the papillary RCC shows a profile characterized by the presence of a large amount of amino acids. The analysis of the CPMG spectra (Figure 3b and 4b) indicate that the resonances of *myo*-Ino, *scyllo*-Ino and Bet drastically decrease or disappear in both the renal neoplasms in comparison with the normal cortex and medulla (Figure 1b and 2b) and show that Tau is present in high amounts in papillary RCC (Figure 4b), whereas ChoCC is the predominant component in the clear cell RCC (Figure 3b). The diffusion-edited spectra, shown in Figure 3c and 4c, further confirm that the neoplastic renal tissues present a different metabolic profile. That of the clear cell RCC (Figure 3c), is characterized by the exclusive presence of FA esterified in TG and cholesterol (CholE), while that of the papillary RCC (Figure 4c) shows weak signals due to bonded amino acids (MM), FA esterified in Ph (phosphatidylcholine).

Discussion

In the biomedical field, *in vivo* volume-localized proton MRS has been used to assay the regional biochemistry in isolated perfused rat kidneys (34). The medullary region spectra were characterized by signals of osmolytes such as Bet, GPC and *myo*-Ino, while the spectra of the cortex were more complex and contained fewer osmolytes. In previous studies using ^{1}H HR-MAS MRS, a biochemical investigation of ten histologically unclassified human renal carcinomas has been reported (35) and a mere distinction between normal cortex and again histologically unclassified eleven renal carcinoma biopsy samples (including 2 renal metastases) has been also published (36). Moreover ^{1}H HR-MAS MRS studies on intact rat renal cortex and medulla have been performed (37).

In an our previous paper (12), we reported the low field *in vitro* [1]H MR spectra of the two different regions of healthy human kidney, *i.e.* the cortex and medulla. We showed that many osmolytes, markers of normal renal function, were present in the healthy kidney, with a different distribution between the cortex and medulla. In particular, Bet and *myo*-Ino predominated in the medulla, while tetramethylammonium (TMA) and succinate (Suc) were predominant in the cortex. We further reported that a marked decrease of these osmolytes was typical of clear cell RCCs with respect to the histological Fuhrman grade. On the contrary, the ChoCC signal intensity remained unchanged, assuming in the tumoral sample the significance of a cell proliferation marker.

Renal cells under physiological conditions are exposed to extracellular NaCl and urea and respond to hypertonic stress by accumulating small organic molecules, the so-called osmolytes. In the medulla of the concentrating kidney, the accumulation of osmolytes, which do not perturb cell function, permits the mantainance of normal intracellular concentrations of inorganic electrolytes and are thus termed "compatible osmolytes". It has been shown that in the mammalian kidney these include the trimethylamines Bet and GPC, the polyols sorbitol (Srb) and *myo*-Ino, and, quantitatively less important, free amino acids and their derivatives (38, 39).

Our *ex vivo* HR-MAS MRS data permitted the evaluation of the different distribution of the metabolites in intact specimens of the human kidney cortex and medulla. Tau and Gly are the more important markers of the cortical region, whereas Bet, *myo*-Ino and GPC characterize the medulla. In the spectra, there is no evidence of any substantial amounts of Srb detected, in contrast to that in intact rat renal medulla (37). Our data are in agreement with the findings of Schmolke *et al.* (40) who measured, by liquid chromatography, the osmolyte concentration in homogenates of five different human kidney zone from the cortex towards the papillary tips. The authors showed that the major osmolytes were *myo*-Ino, GPC and Bet, whereas Srb was detectable in negligible amounts. Our spectroscopic data show that both kidney regions present signals ascribable to *scyllo*-Ino and *myo*-Ino. To our knowledge, the presence of
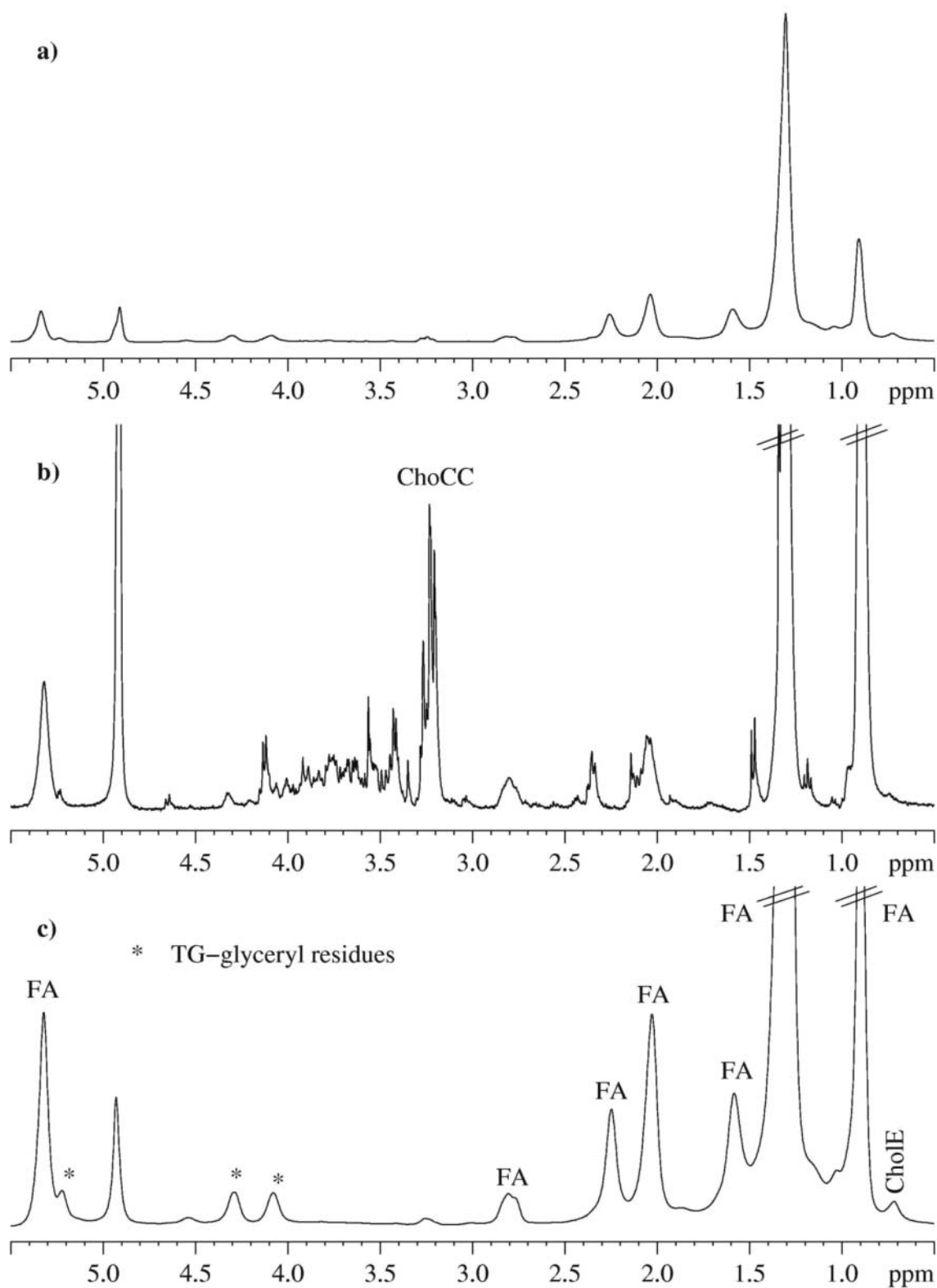
Figure 3. *1D ¹H MR spectra of human clear cell renal cell carcinoma: a) Conventional proton spectrum with water-presaturation (the broad resonances are assigned to lipids); b) CPMG spectrum; c) Diffusion-edited spectrum. ChoCC (choline-containing compounds), CholE (cholesteryl esters), FA (fatty acids), TG (triglycerides).*
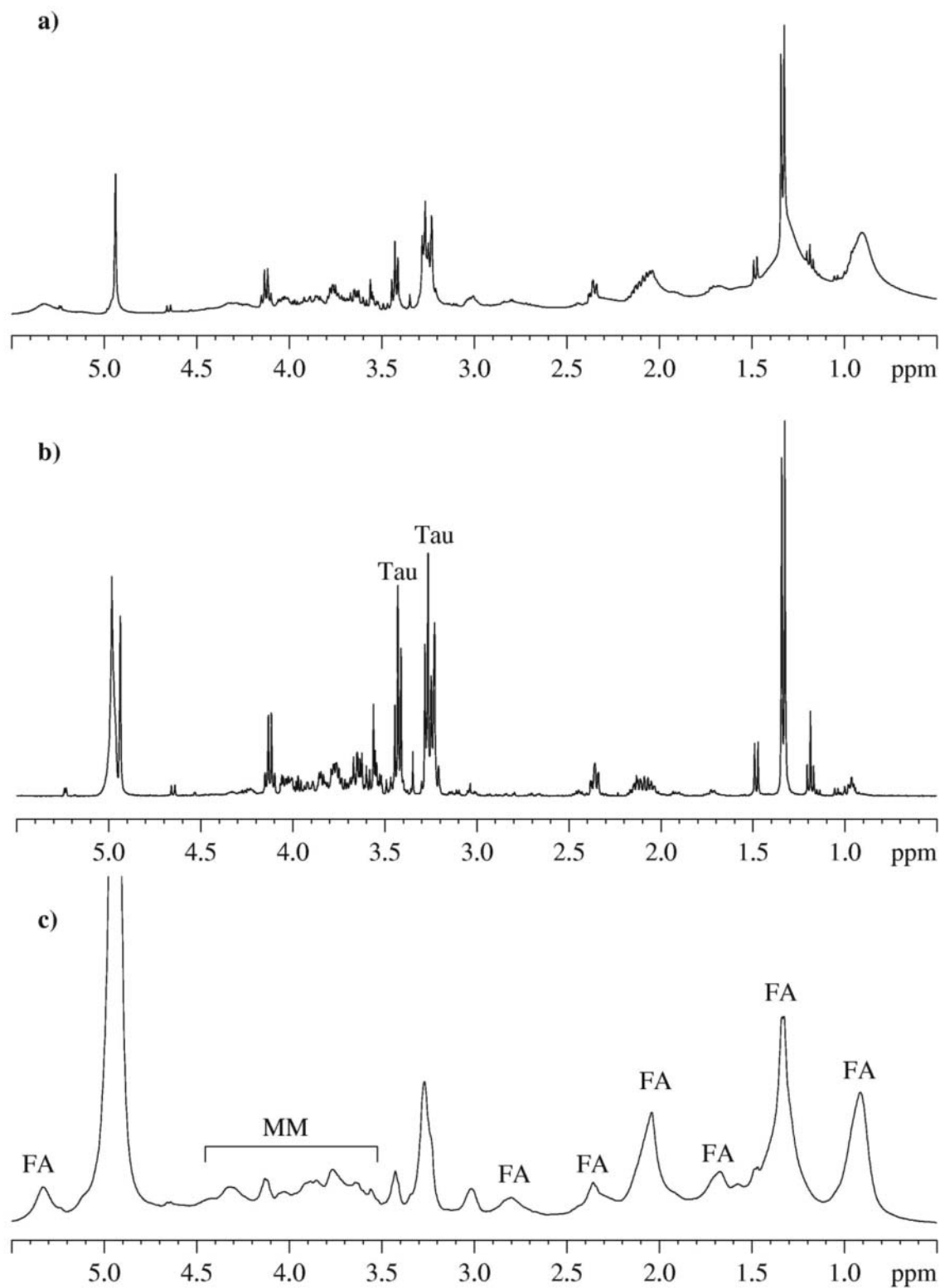
Figure 4. *1D ^1H MR spectra of human papillary renal cell carcinoma: a) Conventional proton spectrum with water-presaturation (the narrow resonances are assigned to low molecular weight metabolites, and the broad resonances are due to lipids and bonded amino acids); b) CPMG spectrum; c) Diffusion-edited spectrum (the broad resonance at ~3.22 ppm is assigned to the trimethylammonium residue of phosphatidylcholine). FA (fatty acids), MM (bonded amino acids), Tau (taurine).*

scyllo-Ino has not yet been reported in normal human kidney. This metabolite may be involved in osmoregulatory processes as more abundant stereoisomer *myo*-Ino.

As regards the presence of TMA and Suc, no traces were found in the intact cortical samples, indicating that they may have been generated by the extraction process.

Clear cell and papillary RCCs are characterized by a metabolic pattern markedly different from those of cortical and medullary tissues not macroscopically affected by tumor invasion (Figure 3 and 4). By inspection of Figure 3b and 4b, relative to the resonances pertaining to the low molecular weight metabolites, a marked decrease of Bet, *myo*-Ino and *scyllo*-Ino in the malignant lesions can be seen. The general decrease of the relative amounts of the osmolytes is a recurrent finding of all the tumors studied, thus confirming on intact specimens that these osmolytes are the markers of normal kidney function. Furthermore, ChoCC content is higher in clear cell RCC (Figure 3b) with respect to the papilla and the latter is characterized by a high level of Tau (Figure 4b), which participates in osmoregulation, stabilizes the membrane potential in skeletal muscle, affects calcium ion kinetics, has antioxidant and anti-inflammatory properties and acts as a neurotransmitter (38, 39).

The high amount of ChoCC may be attributed to the "non-physiological" condition of neoplastic renal cells, considering that changes in osmolyte levels are not uniform when renal function is impaired (38). ChoCC, GPC in particular, behave differently from the other osmolytes, substantially maintaining their relative intensity in the *ex vivo* MR spectra of intact normal and tumoral renal tissues. It is difficult to account for this phenomenon, which is probably caused by multiple concomitant factors, but we assume that in the renal tissues ChoCC are not only implicated in osmotic balance. Since these compounds are also involved in cell membrane synthesis and breakdown processes and are thus related to cell replication, high levels in clear cell RCCs could be ascribed to tumor cell proliferation, as observed in brain malignancies (2, 4, 17, 41).

Another important difference between the normal and the neoplastic tissues arises from the analysis of Figure 1c, 3c and 4c. The spectrum in Figure 3c relative to a clear cell RCC shows a strong increment of the lipid component due to TG and confirms the presence, in the intact biopsies, of CholE, which we have previously demonstrated to be present as cholesteryl oleate in the extracts (13, 42). These results are relevant in the light of theories that correlate the presence of CholE to cell proliferation in different experimental models and in several types of human neoplasms. Indeed, Batetta *et al.* (43) have hypothesized that "proliferative processes are characterized by a specific pattern of cholesterol metabolism, namely an increase in cholesterol synthesis and an accumulation of cholesterol esters in proliferating cells." The hypothesis that CholE play a leading role in the regulation of tumor growth, particularly within the tumor itself, thus find

a possible explanation. Indeed, abnormal CholE storage in clear cell RCCs has already been reported in literature (44) and is paralleled by an increase in AcylCoA-cholesterol acyltransferase (ACAT) activity.

Lipid components due to TG and CholE are present in a very low amounts or absent in the spectra of the papillary RCC (Figure 4 c). The spectroscopic comparison between clear cell and papillary RCC tissues shows important metabolic differences. Our previous data (12, 13, 45, 46) demonstrated that benign oncocytomas and malignant chromophobe RCCs are characterized by a typical and different metabolic pattern if compared with clear cell RCC, the latter having a worse prognosis. Indeed, metabolic differences can be related to the different histological RCC types and nuclear Fuhrman grade, which are two of the most important prognosticators for survival (1). Further studies are needed to confirm the hypothesis that the metabolic profile of renal neoplasms are correlated with their prognosis.

In conclusion, this paper demonstrates that *ex vivo* HR-MAS MRS is a viable and powerful means of probing for molecular information on human normal and neoplastic renal tissues. This technique allows the identification of metabolic indicators of general renal condition in both the physiological and pathological situation, such as depleted osmolyte concentrations and increased lipid components. These observations are consistent with evident changes in renal tissue metabolic profiles. These preliminary results encourage further investigations on a wider range of kidney tumors of different histhological type and grade. Such research will constitute the basis for a biochemical classification of renal neoplastic pathologies which can thus be evaluated by *in vivo* MRS for clinical purposes.

Moreover, these data contribute to a better knowledge of the molecular processes fundamental to the onset of renal carcinogenesis.

Acknowledgements

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