HIGH RESOLUTION MAGIC ANGLE SPINNING ^{1}H NMR SPECTROSCOPIC ANALYSIS OF CHOLINE REGION IN RAT BRAIN GLIOMA UNDERGOING APOPTOSIS

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Introduction: High proliferation rate in cancer cell is associated with high membrane turnover and is expressed as high concentrations of both membrane precursor and breakdown choline products. Owing to the fact that the N-trimethyl group in choline molecule has nine equivalent protons, choline-containing compounds (Cho) give rise to a strong singlet resonance in the *in vivo* ^{1}H NMR spectrum. The Cho resonance centred at 3.23 ppm consists of at least three different choline subspecies, i.e. choline (Chl), glycerophophocholine (GPC) and phosphocholine (PC). The biochemistry of the *in vivo* ^{1}H NMR spectral region around 3.2 ppm is, however, much richer than indicated above, as protons from taurine (Tau) and myo-inositol (mI) resonate in the close proximity of Cho. Despite complex biochemistry of the peak at 3.23 ppm, ^{1}H NMR spectroscopy *in vivo* has been shown to bear great potentials in delineation of malignant solid tumour of several organs, including brain, breast and prostate. Similarly, previous studies indicate that ^{1}H NMR may provide information from positive treatment response in cancer tissue and distinguish between remission and necrosis assessed by MRI [1,2] These reports are very intriguing warranting closer biochemical assessment of ^{1}H NMR Cho peak *in situ*, since GPC, PC and Chl all have specialised roles in cell proliferation and growth arrest. As far as ^{1}H NMR spectroscopic quantification of Cho is concerned, tissue extraction procedures are routinely needed. In this regard it is important to realise that magic angle spinning (MAS) NMR analysis dramatically improves spectral resolution *ex vivo* owing to the fact that the method improves spectral resolution by removing large body of chemical shift anisotropy and some of dipolar interactions also in biological samples. It is believed that ^{1}H NMR HRMAS detectable metabolites represent biochemically active pools of given species, thus avoiding artefacts associated with tissue extraction procedures. In the present study we have used ^{1}H NMR HRMAS in a rat glioma model of herpes simplex viruses thymidine kinase – ganciclovir (HSVtk-GCV) gene therapy to assess biochemicals contributing to the peak at 3.23 ppm during tumour eradication. In the glioma HSVtk-GCV gene therapy -induced apoptosis leads to synchronous programmed cell death [3,4] is associated with accumulation of ^{1}H NMR detectable polyunsaturated fatty acids (PUFA) [5] and loss of creatine (Cr) along with reduced cell count [6].

Methods: BT4C gliomas transfected with viral HSV-tk gene were induced by implanting 10^4 cells into the corpus callosum of female BDIX rats (n = 23) [3]. Animals were treated with ganciclovir (GCV; 25 mg/kg, i.p., twice daily) for the duration the study. For MRI, rats were anesthetized with 0.8-1.0% halothane in 7/3 N_2O/O_2, and the core temperature of animals was maintained close to 37°C using a heated water blanket. MRI was performed in a horizontal 4.7T magnet interfaced to Varian ^{UNITY}*INOVA* console. A quadrature surface coil (Highfield Imaging, Minneapolis, MH) was used in a receive/transmit mode. Tumour volumes were determined from T_2-weighted multi-slice spin echo images (TR = 2 s, TE = 70 ms, field of view 35 mm, matrix size 256 x 128, 2 scans/line and contiguous slices of 1 mm thickness). Rats were funnel frozen *in situ* and tumours were dissected frozen on dry ice to obtain metabolically intact samples for *ex vivo* NMR spectroscopy [7]. For HRMAS ^{1}H NMR spectroscopy of tissue, samples were placed into the zirconium oxide MAS rotor and 10 µl of D_2O containing 10mM TSP was added. Spectra were acquired at 400 MHz, 600 MHz and 700 MHz with Bruker MAS instruments (Bruker Avance) at +4°C using a conventional solvent suppressed pulse/acquire sequence (5 kHz spinning rate). *Ex vivo* spectra were analyzed by the PERCH software (www.perchsolutions.com). The concentrations of the substances were calculated and normalized to the creatine concentration within the same sample [6]. Data are presented as mean ± SEM and Student's unpaired t-test was used for statistical analysis of the results.

Results: GCV treatment resulted in T_2 hyperintensity by day 4 and decline of tumour volume by day 8 as signs of gene therapy -induced apoptotic cell death. *Ex vivo* HRMAS ^{1}H NMR spectra acquired at 700 MHz from normal parietal cortex and untreated BT4C glioma are shown (Fig. 1). It is evident that all Cho subspecies were well resolved together with Tau and mI triplets. Downfield of Cho two broad resonances were revealed underneath Tau/mI peaks at 700 MHz, but in the HRMAS spectra acquired at 400 and 600 MHz only one broad resonance was resolvable in this region. Concentrations of biochemicals in question are shown for normal brain cortex and gliomas (Table 1). Interestingly, GPC, PC, mI and Tau increased by day 4 of treatment, a time point in apoptosis with no significant decrease in cell density [6]. By day 8 of treatment, with precipitous drop in cell density [6] Tau had declined substantially, yet Chl, GPC, PC and mI were largely unchanged relative to pre-treatment figures. PC/GPC ratio was lower in glioma than in normal brain cortex and this ratio was unaffected by the gene therapy.

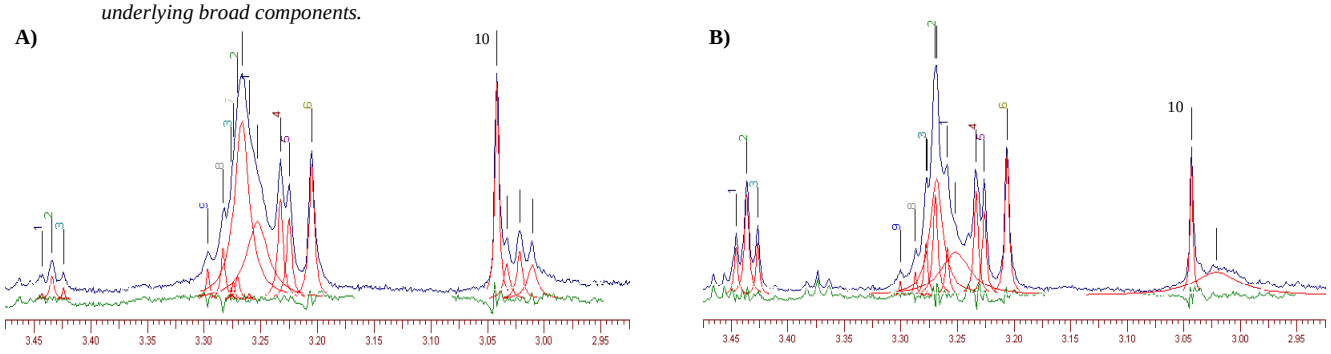
Conclusions: The present results demonstrate that the ^{1}H HRMAS reveals the major biochemicals of Cho region for quantification *in situ*. There appears to be broad resonance(s) underneath the Cho region showing field dependency in their appearance. Apoptosis, an active ATP-dependent cell death pathway, is associated with increase in concentrations of ^{1}H NMR HRMAS visible metabolites preceding actual cell eradication. These metabolite changes may result from several differing mechanisms, involving synthesis and/or degradation, altered cytoplasmic physico-chemical microenvironment as well as repartitioning of chemical species intracellularly. It is interesting to note that ^{1}H NMR Cho peak is a marker of advanced stage of apoptotic cell death *in situ*, differing from the situation in necrotic tissue.

Treatment	Chl	PC	GPC	TotalCho	PC/GPC	Tau	mI
Day 0	0.66 ± 0.10	0.48 ± 0.08	0.53 ± 0.10$^+$	1.68 ± 0.22$^+$	0.97 ± 0.19	5.73 ± 0.87$^+$	1.59 (n=1)
Day 4	0.79 ± 0.14	0.81 ± 0.08*$^+$	1.12 ± 0.19$^+$	2.72 ± 0.24*$^+$	0.94 ± 0.32	6.87 ± 0.75$^+$	4.60 ± 0.51*
Day 8	0.43 ± 0.08	0.43 ± 0.08	0.51 ± 0.06$^+$	1.38 ± 0.20	0.84 ± 0.07	2.90 ± 0.34*	2.44 (n=1)
Normal	0.42 ± 0.05	0.36 ± 0.06	0.19 ± 0.05	0.97 ± 0.06	2.35 ± 0.66	2.22 ± 0.45	4.32 ± 1.18

Table 1. *Concentrations (µmol/g) of the biochemicals in BT4C gliomas and in the normal brain tissue.*

**p<0.05, Student's t-test relative to Day 0, $^+$p<0.05, Student's t-test relative to Normal brain tissue.*

Fig. 1. *HRMAS NMR (at 700MHz) spectra from normal brain cortex (A), and from a glioma before GCV treatment (B). Peaks 1, 2 and 3 correspond to Tau; peak 4 to GPC; peak 5 to PC; peak 6 to Chl; peaks 7, 8 and 9 to mI (quantified from 3.63 ppm peak) and 10 to Cr. Peaks at 3.27 ppm and 3.25 ppm are from underlying broad components.*



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