Identification of new markers for diagnosing 5-fluorouracil chemotherapy-induced brain damage using ultra-high field $^1$H-MR spectroscopy

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Aims and objectives

5-fluorouracil (5FU)-induced brain damage including leukoencephalopathy and most notably "chemo-brain" has recently attracted attention in clinical settings. 1-4 5FU is a principal drug for cancer chemotherapy and is frequently used in combinatorial chemotherapeutic regimens, e.g., FOLFOX for colorectal cancer as well as CAF for breast cancer. A subset of cancer patients treated with 5FU-based chemotherapy is well known to experience cognitive impairment during or shortly after the completion of treatment. Recently, clinicians became aware of more long-term cognitive decline in cancer survivors who had completed 5FU-based chemotherapy several years ago (chemo-brain). This type of cognitive disorder can produce subtle symptoms related to attention, concentration, memory, and executive function, but definitely deteriorates quality-of-life of cancer patients. In addition, a large number of patients are at risk as the number of cancer patients who received 5FU-based chemotherapy continues to increase. Therefore, appropriate clinical management is necessary. 5

While, cognitive decline in these patients is closely related to 5FU-induced brain damage, a diagnostic marker has not yet been established. We hypothesized that MRS-detectable metabolites could be such diagnostic markers. To test the hypothesis, we assessed the changes in brain metabolites in 5FU-administered rats with ultra-high field proton MR spectroscopy.

To the best of our knowledge, only single report has been published regarding MRS-detectable metabolite changes in 5FU-induced brain damage. 6 De Ruiter, et al. reported reduction of brain N-acetyl aspartate (NAA) in breast cancer survivors who received high-dose 5FU approximately 10 years earlier. In this study, we focus on other metabolites that can be altered in the earlier disease process.
Methods and materials

Animal experiment

We intravenously administered 25 mg/kg BW/day 5FU for four consecutive days (total dose 100 mg/kg BW) or saline to two groups of male Wistar rat (9 wks of age, n = 34). On day 9, we acquired in vivo MRI of the brain and \(^1\)H-MR spectra in the left hippocampus using a Bruker 9.4 T scanner (n = 22). We excised the brain and performed NMR analysis of whole brain perchloric acid (PCA) extracts using a Bruker 400 MHz spectrometer. Our institutional animal experimental committee approved the animal experimental protocol.

In vivo MRI and MRS

All MR images and MR spectra were acquired using a 9.4 Tesla horizontal scanner (BioSpec 94/20 USR; Bruker BioSpin). We used a 72 mm-quadrature volume coil (T9361V3; Bruker BioSpin) for RF transmission and 4-channel array coil (rat brain coil array, T10324V3; Bruker BioSpin) for signal reception. We anesthetized rats using a gas mixture of isoflurane (1-2%), oxygen, and nitrous oxide, acquired axial and coronal T\(_2\)-weighted MR images of the brain using turbo RARE sequence (TR/TE = 2500/33 ms, FOV = 4 x 4 cm\(^2\), matrix = 256 x 256, slice thickness = 1.0 mm, NEX = 1, scan time = 1 min 20 sec). Then we performed \(^1\)H MRS of the hippocampus using PRESS sequence with VAPOR water suppression (TR/TE = 2500/20 ms, voxel = 3 x 3 x 3 mm\(^3\), number of accumulations = 256, acquisition time = 10 min 50 sec). We acquired 4K (4096) data points with a spectral width of 5597 Hz (13.98 ppm). Data were zero-filled to 64K and then Fourier-transformed with 2 Hz line broadening to obtain MR spectra with a spectral resolution of 0.17 Hz. Spectra were automatically phased using the manufacturer's standard software (ParaVision version 5.1).

PCA extraction

We killed these rats by decapitation, immediately froze the heads with liquid nitrogen, excised and pulverized the whole brain samples, and subjected them to PCA extraction. We performed PCA extraction as reported previously.\(^7\) To correct metabolite loss during the PCA treatment and minimize inter-assay variance in metabolite quantification, we applied dual internal reference compounds, namely DL-valine-2,3-\(d_2\) (Val-\(d_2\)) and 3-(trimethylsilyl)-propionic-2,2,3,3-\(d_4\) acid (TSP-\(d_4\)) to each sample. We lyophilized the brain extract, dissolved it with D\(_2\)O, and neutralized to pH 7.0 ± 0.4.

NMR measurements
All NMR spectra were acquired using a standard-bore NMR spectrometer (AVANCE III 400; Bruker BioSpin) equipped with a tunable multinuclear probe (PA BBO 400S1 BBF-H-D-05 ZPLUS) operating at 400 MHz for $^1$H. The scan parameters consisted of a TR at 9 sec, a flip angle of 90°, and the number of excitation of 16. We acquired 64K (65536) data points with a spectral width of 8223.7 Hz (20.6 ppm). Data were Fourier-transformed without zero-filling and with 0.3 Hz line broadening to obtain NMR spectra with a spectral resolution of 0.25 Hz. Spectra were automatically phased and the baseline was flattened using the manufacturer's standard software (TopSpin version 2.1).

Metabolite quantification

**In vivo** $^1$H MRS: Eight metabolite concentrations including lactate (Lac), NAA, glutamate (Glu), glutamine (Gln), total creatine (creatine and phosphocreatine; tCr), choline compounds (Cho), taurine (Tau), and myoinositol (mIns) were measured by LCModel (version 6.2) using water signal as an internal reference. Concentration of water was assumed to be 35.6 M.

**In vitro** NMR: We measured integrals of eleven metabolites including TSP-$d_4$, Val-$d_2$, Lac, alanine, NAA, Glu, Gln, tCr, Cho, Tau, and mIns using the TopSpin software, determined metabolite quantities by comparing the integral of each metabolite resonance with that of TSP trimethylsilyl resonance at 0.0 ppm. We corrected metabolite quantities based on the recovery factor of Val-$d_2$ (90.6 ± 9.2%).

Histological assessment

We performed perfusion fixation with paraformaldehyde to 5FU- or saline-administered rats ($n = 12$). Excised brain samples were immunohistochemically stained with neuron-specific nuclear protein (NeuN) as well as glutamine synthetase (GS). The number of NeuN as well as GS-positive cells in the hippocampus was counted.

Statistical analysis

Difference in metabolite concentrations between 5FU- and saline-treated groups was compared using unpaired t-test and commercially available software (SPSS, version 19, IBM). Difference in the number of NeuN as well as GS-positive cells between 5FU- and saline-treated groups was analyzed in the same manner. $P <0.05$ was determined statistically significant.
Results

Figure 1 shows the representative in vivo $^1$H MR spectrum of a 5FU-administered rat. Our ultra-high field 9.4T scanner enables acquisition of high-quality MR spectrum with high signal-to-noise ratio as well as high spectral resolution. Figure 2 shows hippocampal metabolite concentrations in the 5FU- and saline-administered groups. We found for the first time that glutamine (Gln) and taurine (Tau) concentrations in the 5FU-administered group ($4.07 \pm 0.44$ and $6.36 \pm 0.49 \, \mu\text{mol/g}$) were lower than those in the saline-administered group ($5.09 \pm 0.69$ and $6.59 \pm 0.39 \, \mu\text{mol/g}$). We confirmed the reduction by in vitro quantitative $^1$H-NMR (Figure 3).

The reduction in Gln and Tau levels is possibly caused by glial injury by 5FU catabolites, specifically fluorooacetate and fluorocitrate. Both compounds are well known to damage glial metabolism related to Gln and Tau$^{8-10}$. We did not observe significant alteration in neuronal markers including N-acetyl aspartate (NAA) and glutamate (Glu) levels by MRS, thus we speculated that 5FU catabolites could mainly damage glia, but not neuron. Accordingly, our histological data showed 16% reduction in hippocampal GS-positive glial cells in the 5FU administered group ($P = 0.273$, $t$-test), but no reduction in hippocampal NeuN-positive neuronal cells (Figure 4). Taken together, these findings suggest that 5FU catabolites injure the glial cells, and that subsequent alternation of brain metabolism may be the underlying cause of 5FU-induced brain damage. The selectivity of 5FU catabolites into the glia could be explained by more rapid uptake of acetate and citrate by these cells than by neurons$^{11}$.

Noticeably, there were no abnormal findings in T$_2$-weighted MR images in 5FU-administered rats (Figure 5) even when MRS detected the reduction in brain metabolite levels. This implies higher sensitivity of MRS for the detection of 5FU-induced brain damage than MRI. Therefore, we contend that MRS is a reasonable approach for early diagnosis of 5FU-induced brain damage. In addition, MRS-detectable metabolites, specifically, Gln and Tau could be putative diagnostic markers.
Fig. 1: Representative in vivo 1H MR spectrum of the rat brain at 9.4 T ultra-high field. High-quality MR spectrum with high signal-to-noise ratio as well as high spectral resolution was acquired.

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Fig. 2: Metabolite concentration of the hippocampus in 5FU- (red bars) and saline- (blue bars) administered rats measured with in vivo MRS and LCModel. Glutamine and taurine concentrations in the 5FU-administered group (4.07 ± 0.44 and 6.36 ± 0.49 µmol/g) were lower than those in the saline-administered group (5.09 ± 0.69 and 6.59 ± 0.39 µmol/g). An asterisk indicates statistical significance (P <0.05, unpaired t-test).

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Fig. 3: Metabolite concentration of the whole brain PCA extracts in 5FU- (red bars) and saline- (blue bars) administered rats. Taurine and alanine levels were significantly reduced in 5FU-treated rats (P <0.05, unpaired t-test). Glutamine level was also reduced in 5FU-treated rats compared to saline-treated rats, however the difference did not reach statistical significance (2.77 ± 0.87 and 3.33 ± 0.41 µmol/g, P = 0.066).

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**Fig. 4:** Immunohistochemical staining of the hippocampus in 5FU- and saline-treated rats. The number of glutamine synthetase (GS) positive glial cells in 5FU-treated rats was slightly reduced compared to that in saline-treated rats, whereas the number of neuron specific nuclear protein (NeuN) positive neuronal cells was equivalent.

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Fig. 5: Representative T2-weighted axial and coronal images of the brain in 5FU- and saline-treated rats. No abnormal finding is noted on day 9 after 5FU treatment.

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Conclusion

MRS-detectable metabolites, namely glutamine and taurine levels could be diagnostic markers of 5FU-induced brain damage.
Personal information

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