SUPPLEMENTARY INFORMATION

Differentiation between glioma and radiation necrosis using molecular magnetic resonance imaging of endogenous proteins and peptides

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Supplementary Figures

**Supplementary Figure 1.** Characteristics of radiation necrosis (a) and tumor recurrence (b) on conventional MR images for two patients, who both had glioblastoma multiforme treated with the same chemoradiation protocol. Both developed symptomatic, progressive contrast-enhancing disease, and progression of FLAIR hyperintensity, within 3–5 months after completing therapy. The standard MRI sequences for radiation necrosis and tumor recurrence had similar imaging features that were not predictive of the final pathology.
Supplementary Figure 2. \( T_2 \), \( T_1 \), ADC, blood flow, MTR at 2 kHz and APT images acquired pre-radiation and at 3 days and 6 days post-radiation (40 Gy) for a typical rat (the same as Fig. 5) implanted with a U87MG glioma. For experimental details and data processing methods, see Supplementary Methods below. The display windows are \( T_2 \) (0 to 100 ms), \( T_1 \) (0.5 to 2 sec), ADC (0 to \( 2 \times 10^{-9} \) m\(^2\)/sec), blood flow (0 to 200 ml/100g/min), MTR at 2 kHz (0 to 50% of the bulk water signal intensity) and APT (-10% to 10% of the bulk water signal intensity). The tumors continue to grow in size during the first days post-radiation. These irradiated tumors maintain their typical contrast on several conventional MRI sequences: hyperintense (\( T_2 \), \( T_1 \), ADC) or hypointense (MTR). In contrast, both blood flow and APT signals clearly show a decrease in intensity following therapy. On post-radiation day 6, the APT signal for the irradiated tumor is very heterogeneous with some almost isointense areas (pink arrow). Examples of tumor regions of interest for quantitative image analysis are shown with red dotted lines. Ventricles and peritumoral edema are always excluded.
Supplementary Figure 3. Histogram analysis of $T_2$, $T_1$, ADC, blood flow, MTR values at 2 kHz and APT intensities obtained pre-radiation and at 3–24 days post-radiation (40 Gy) for a U87MG glioma in a rat. For the experimental and data processing methods, see Supplementary Methods below. During the first days post-radiation, the tumor continues to grow in size, as reflected by increased areas under the histograms. Before irradiation, the tumor appears homogenous and all histograms (except blood flow) show a relatively narrow distribution. Starting from about 9 days post-radiation, the tumor becomes very heterogeneous, as characterized by widened histograms. The ADC histogram has a right shift, showing a similar pattern as that observed previously in the 9L brain tumor model following chemotherapy\(^1\). The APT histogram has a left shift. Both ADC and APT demonstrate large changes in response to therapy. The blood flow maps quantified by the arterial spin labeling (ASL) technique are somewhat noisy owing to low sensitivity (Supplementary Fig. 2); thus, the corresponding histograms have very wide distributions. Some apparently negative blood flow values are also due to low signal-to-noise ratios\(^2\) and not meaningful.
Supplementary Table 1. Quantitative comparison for radiation necrosis and tumor models for conventional and APT MRI sequences. $T_2$ hyperintensity and Gd-enhancement (%) were quantified as the ratios of the imaging intensities in tumor and the imaging intensities in contralateral normal-appearing white matter. APT imaging was quantified as % change of the bulk water signal intensity. Radiation necrosis (40 Gy, 163–188 days post-radiation, $n = 9$); SF188/V+ human glioma (9–35 days post-implantation, $n = 9$); 9L gliosarcoma (10–12 days post-implantation, $n = 9$). The statistical significance of the difference between the MRI parameters for radiation necrosis and those for the tumor models was marked (*** $P < 0.001$). $T_2$-weighted MRI can distinguish radiation necrosis from 9L tumors ($P < 0.001$), but not from SF188/V+ tumors ($P > 0.2$). Gd-enhanced $T_1$-weighted MRI is unable to distinguish radiation necrosis from the two tumor models studied (both $P > 0.2$). In contrast, APT MRI can distinguish radiation necrosis from these two tumor models reliably (both $P < 0.001$).

| MRI sequence | Radiation necrosis | SF188/V+ glioma | 9L gliosarcoma |
|--------------|--------------------|-----------------|---------------|
| $T_2$-weighted | 117 ± 9           | 118 ± 16        | 144 ± 13 *** |
| Gd-enhanced  | 164 ± 27          | 165 ± 38        | 169 ± 23      |
| APT          | -3.6 ± 0.4         | 12.2 ± 4.6 ***  | 2.7 ± 0.7 *** |
### Supplementary Table 2.

Changes in mean MRI variables for the U87MG tumors and contralateral normal-appearing brain tissue at 3 days and 6 days following radiation therapy ($n = 5$). For the experimental and data processing methods, see **Supplementary Methods** below. The statistical significance of the difference between the parameters at pre-radiation and those at 3 days or 6 days post-radiation was marked (* $P < 0.05$; ** $P < 0.001$). According to our experimental results, APT ($P < 0.05$ and $P < 0.001$, respectively) and blood flow (both $P < 0.001$) signals decrease significantly at 3 days and 6 days post-radiation; $T_2$, $T_1$, ADC and MTR do not show significant changes (most $P > 0.2$) at these two early time points following therapy.

| MRI parameter | Pre-radiation | Post-radiation day 3 | Post-radiation day 6 |
|---------------|---------------|----------------------|----------------------|
| $T_2$ (ms)    |               |                      |                      |
| Tumor         | 69.8±3.2      | 71.2±2.4             | 72.9±4.0             |
| Contralateral | 57.4±0.6      | 56.6±0.9             | 57.2±1.2             |
| $T_1$ (sec)   |               |                      |                      |
| Tumor         | 1.86±0.04     | 1.81±0.06            | 1.85±0.07            |
| Contralateral | 1.39±0.03     | 1.36±0.05            | 1.37±0.03            |
| ADC ($10^{-9}$ m$^2$/sec) |               |                      |                      |
| Tumor         | 1.06±0.03     | 1.04±0.03            | 1.11±0.11            |
| Contralateral | 0.80±0.02     | 0.77±0.02            | 0.77±0.04            |
| Blood flow (ml/100g/min) |               |                      |                      |
| Tumor         | 61.3±20.3     | 13.9±9.6             | 12.7±11.3***         |
| Contralateral | 95.7±26.5     | 45.2±13.4            | 66.6±34.0            |
| MTR at 2 kHz (%) |               |                      |                      |
| Tumor         | 22.9±0.9      | 22.6±0.9             | 21.8±1.3             |
| Contralateral | 31.9±0.8      | 31.7±1.4             | 31.0±1.4             |
| APT (%)       |               |                      |                      |
| Tumor         | 3.1±0.3       | 2.1±0.5              | 0.6±1.1***           |
| Contralateral | -1.6±0.5      | -1.5±0.5             | -1.8±0.1             |
Supplementary Discussion

It is important to note that many commonly used MRI parameters did not detect significant changes in irradiated tumors during the first days post-radiation (Supplementary Table 2). The one exception was the tumor blood flow, which showed a significant decrease \((P < 0.001)\) at these time points. Tumor blood flow has been suggested to be a marker for tumor in clinical settings\(^3\) and reduced blood flow in tumor following radiation may correlate with inactive tumor and the appearance of radiation necrosis. In this study, we used the ASL technique to quantify tumor blood flow. Both ASL and APT are non-invasive MRI techniques that seem equally applicable for evaluating viable tumors versus radiation necrosis. However, ASL is associated with a change of 1-2% bulk water intensity and APT is associated with a change of 3–5% bulk water intensity (as observed in the rat U87MG glioma model); thus, APT imaging has higher signal-to-noise ratios than ASL, as seen in Supplementary Fig. 2. At these two time points post-radiation, mean blood flow in contralateral brain decreased substantially. This could be due to increased intracranial pressure as the tumor grew and edema developed, but an exact mechanism needs to be further investigated.

It is widely realized that ADC is an effective early biomarker for tumor response to therapy, and successful treatment is associated with increases in ADC values (mainly due to tumor cell loss)\(^4\)\(^6\). We observed that the mean ADC values in tumor increased at the later time points (as expected\(^1\)), but tended to decrease at 3 days post-radiation (compared to pre-radiation) for most rats, corresponding to a slight left shift of the ADC histogram (Supplementary Fig. 3). This leads to insignificant changes in mean ADC at the two initial time points (3 days and 6 days; Supplementary Table 2), despite histologically verified necrosis, which is at variance with most available pre-clinical and clinical data. However, a few exceptions have been reported (decreased ADC in the early phase following therapy; see a recent review article\(^7\)). This transient decrease in ADC in response to therapy may depend on tumor type, treatment type, dose, and imaging time point. Currently, an exact biophysical understanding of this phenomenon is not available, but cell swelling and a reduction in blood flow have been suggested as possible reasons\(^7\)\(^8\).

The APT MRI signal can differentiate viable tumor from treatment-induced necrosis in a tumor-bearing animal. This can be very important in clinically relevant settings, which would help to predict glioma response to radiation therapy. In this regard, one should note that the presence of
spontaneous necrosis commonly observed in high-grade gliomas may be a confounder for radiation necrosis, as spontaneous necrosis would also demonstrate a low APT signal\textsuperscript{9}. This potential limitation may be eliminated by comparing APT images acquired pre-treatment and post-treatment, as done in our animal study.

The new standard of care for patients with glioblastoma multiforme is extensive surgery followed by concurrent radiation therapy and temozolomide chemotherapy\textsuperscript{10}. Although this regimen improves survival, it is associated with an increased incidence of brain injury, which most commonly appears within the first six months after completion of therapy and may stabilize or spontaneously resolve over time\textsuperscript{11-13}. Similar to radiation necrosis (that typically appears six months to several years after treatment), this early treatment-induced brain injury can mimic tumor recurrence both clinically and radiographically\textsuperscript{14}. Imaging features include signal abnormality on \(T_2\)-weighted or FLAIR images, Gd enhancement due to blood-brain barrier disruption, and mass effect. This has been dubbed “pseudoprogression,” and represents a major diagnostic challenge in neuro-oncology. After radiation therapy alone, the incidence of pseudoprogression was reported to be about 10\%\textsuperscript{15}. In recent studies of patients treated with radiation therapy and temozolomide, the incidence of pseudoprogression has ranged from 21–37\%\textsuperscript{16-18}. Our findings predict that pseudoprogression will be associated with low APT signal (similar to radiation necrosis). Thus, pseudoprogression may be distinguished from tumor recurrence in terms of APT MRI signal characteristics.

The inability of currently available MRI techniques to differentiate tumor progression from treatment-related injury complicates daily patient care and is a critical barrier to investigating the efficacy of new therapies for brain tumors. The extension of MR neuroimaging to the cellular and molecular level has introduced new possibilities for imaging malignant gliomas. Currently, however, most molecular and cellular MRI studies have been limited to the pre-clinical setting, because they rely on the administration of paramagnetic or super-paramagnetic metal-based substrates that could be potentially toxic. APT imaging is a totally non-invasive molecular MRI technique that can easily be translated to the clinic using existing hardware\textsuperscript{19}. If validated in patients who have more complex and histopathologically heterogeneous lesions, the results of this pre-clinical study could quickly be applied to improve non-invasive brain tumor diagnostics and patient care.
Zhou et al.

Supplementary Methods

SF188/V+ tumor implantation. Nine nude rats (female; 6–8 weeks; 150–200 g) were anesthetized by an intraperitoneal injection of 3–5 ml/kg of a stock solution, containing ketamine hydrochloride 25 mg/ml, xylazine 2.5 mg/ml, and 14.25% ethyl alcohol in 0.9% NaCl. A midline scalp incision was made, exposing the sagittal and coronal sutures. A small burr hole was made with an electric drill, centered 3 mm to the left of the sagittal suture, avoiding the sagittal sinus, and 5 mm posterior to the coronal suture. A needle was placed into the burr hole at a depth of 5 mm from the skull. Then, \(3 \times 10^6\) human SF188/V+ glioma cells in 4 \(\mu\)l media were injected stereotactically over 3–4 min. The scalp was re-approximated and the wound was closed with clamps. After surgery, buprenorphine hydrochloride (0.04 mg/kg body weight, intraperitoneal) was used for analgesia.

U87MG and 9L tumor implantation. Similar to the SF188/V+ cell implantation, five nude rats (male; 20–22 weeks; 300–350 g) and nine Fisher 344 rats (male; 8–10 weeks; 200–250 g) were anesthetized. A small burr hole was made with an electric drill, centered 3 mm to the right of the sagittal suture, and 1 mm anterior to the coronal suture. A needle was placed into the burr hole at a depth of 5 mm from the skull. There were \(10^6\) U87 human glioma cells in 4 \(\mu\)l media (for each nude rat) or 25,000 9L tumor cells in 2 \(\mu\)l media (for each Fisher rat) injected stereotactically over 3–4 min.

Animal care for MRI. Animals were re-anesthetized with 5% isoflurane in a mixture of 75% air and 25% oxygen in a box for about 5 min for induction, followed by breathing of 1.5–2.5% isoflurane through a noise cone fixed with an MRI coil setup during MRI procedures. While anesthetized, a PE-10 catheter was placed into the dorsal tail vein to administer Gd contrast agents prior to MRI. The rat head and body were fixed and taped to the coil and cradle to avoid motion artifacts. Rats in the magnet were monitored online through a small-animal respiratory-gating system connected with optic fibers, and the breathing rate of the animal was kept at 40 ± 5 breaths per minute by adjusting the isoflurane ratio (1.5–2.5%) in the breathing mixture.
Theory. Similar to other magnetization transfer-based MRI experiments\textsuperscript{20,21}, the magnetization transfer ratio (MTR) is defined as: $\text{MTR} = 1 - \frac{S_{\text{sat}}}{S_0}$, where $S_{\text{sat}}$ and $S_0$ are the signal intensities with and without radiofrequency irradiation, respectively. For APT imaging, the irradiation is selective on the backbone amide protons, located at $\sim$3.5 ppm downfield from the water resonance in the proton MR spectrum, which is arbitrarily assigned to 0 ppm for MT data. To reduce the interference by other saturation effects that are concurrent with APT measurements, such as the conventional magnetization transfer and direct water saturation effects, an MTR asymmetry (MTR\textsubscript{asym}) parameter is often used, which compares MTR values at 3.5 ppm downfield (+) and upfield (-) of the water signal\textsuperscript{22-24}:

$$\text{MTR}_{\text{asym}}(3.5\text{ppm}) = \frac{S_{\text{sat}}(3.5\text{ppm})}{S_0} - \frac{S_{\text{sat}}(-3.5\text{ppm})}{S_0}.$$ \hfill [1]

As demonstrated previously\textsuperscript{23,24}, MTR\textsubscript{asym}(3.5ppm) measured in tissue by APT imaging consists of two components. One is the amide proton transfer ratio (APTR) associated with mobile cellular proteins and peptides, and the other is the non-APT contributions, $\text{MTR}^\prime_{\text{asym}}(3.5\text{ppm})$, including the inherent asymmetry of the solid-phase magnetization transfer effect around the water signal\textsuperscript{25}, and possible exchange-relayed nuclear Overhauser effects of the aliphatic protons of mobile macromolecules and metabolites\textsuperscript{26}. Thus, Eq. [1] can be re-written as:

$$\text{MTR}_{\text{asym}}(3.5\text{ppm}) = \text{MTR}^\prime_{\text{asym}}(3.5\text{ppm}) + \text{APTR}.$$ \hfill [2]

According to Eq. [2], the measured MTR\textsubscript{asym}(3.5ppm) value in tissue is actually an apparent APT signal of endogenous cellular proteins and peptides. In the current experiment, with the saturation settings used, the presence of $\text{MTR}^\prime_{\text{asym}}(3.5\text{ppm})$ would cause a negative background signal for MTR\textsubscript{asym}(3.5ppm), as observed in the contralateral brain tissue. In humans with other saturation settings, normal tissue can be zeroed at this frequency or even positive\textsuperscript{27}. This is a typical issue in quantitative APT imaging that has to be always kept in mind. However, the comparison between tumor and tissue should provide the required contrast, with the magnitude depending on the power level for the RF. For convenience, the calculated MTR\textsubscript{asym}(3.5ppm) images are called the APT images.

Based on a two-pool exchange model, APTR can be written as\textsuperscript{28}:...
where \( k \) is the proton exchange rate for all amide protons participating in the effect, \([\text{amide proton}]\) is the amide proton concentration, \([\text{water proton}]\) (= 2 \( \times \) 55 M \( \times \) water content) is the water proton concentration, \( R_{1w} \) is the longitudinal relaxation rate of water, and \( t_{\text{sat}} \) is the saturation time used to irradiate the amide protons. Notably, the effects resulting from water content and \( R_{1w} \) changes are compensated for the most part\(^3\). Therefore, the APT imaging signal in tissue is primarily related to two factors: the mobile amide proton content and the amide proton exchange rate, a parameter that depends on tissue pH.

**Correction for \( B_0 \) field inhomogeneity.** The effect of \( B_0 \) field inhomogeneity on APT imaging is a major concern. When \( B_0 \) field homogeneity is poor, the water resonance signals for some voxels may not be centered properly at the offset of 0 ppm, often resulting in a change of a few percentage points in the asymmetry data when the asymmetry analysis is used. There are two possibilities: \( B_0 \) inhomogeneity inside the slices and the uniform \( B_0 \) shift of all voxels. On the 4.7T animal scanner, high-order, local shimming can give good \( B_0 \) homogeneity; however, a \( B_0 \) field shifting of tens of Hz is often observed in the brain because the original frequency setting may be affected by skull water. To reduce this, prior to the APT data acquisition, a magnetization transfer spectrum with a low saturation power (0.5 \( \mu \)T) and a narrow offset range (-0.6 to 0.6 ppm, interval 0.05 ppm) was acquired on the same slice. The variation of the magnetization transfer-spectrum center frequency was determined on the scanner and added to adjust the scanner transmitter frequency.

**Multi-parametric MRI acquisition and data analysis for Supplementary Figs. 2 and 3 and Supplementary Table 2.** The complete MRI protocol in time order included high-resolution \( T_2 \)-weighted (horizontal, 5 slices, matrix = 256 × 192, field of view = 42 × 32 mm\(^2\), slice thickness = 1.5 mm; coronal, 5 slices, matrix = 192 × 192, field of view = 32 × 32 mm\(^2\), slice thickness = 1.5 mm); quantitative MRI parameter mapping (\( T_2 \), \( T_1 \), isotropic ADC, blood flow, MTR values at 2 kHz, APT; coronal, single-slice, matrix = 64 × 64 (interpolated to 384 × 384), field of view = 32 × 32 mm\(^2\), slice thickness = 1.5 mm); high-resolution \( T_1 \)-weighted (horizontal, coronal, 5 slices) and Gd-enhanced \( T_1 \)-weighted (horizontal, coronal, 5 slices). For more details about other
Zhou et al.

Experimental parameters and data processing methods, see ONLINE METHODS or elsewhere\(^{29,30}\). Specifically, when serial MRI scans were performed, the follow-up coronal slices at a later time point were always positioned as much as possible at the same tumor location as before using intrinsic landmarks (corpus callosum, hippocampal commissure and ventricles) on the horizontal high-resolution T\(_2\)-weighted images that were first acquired for reference. For the quantitative image analysis, the signal abnormalities on the high-resolution T\(_2\)-weighted images and Gd-enhanced T\(_1\)-weighted images were used as a basis for defining regions of interest. For all cases, these tumor regions of interest covered most areas of the lesions with the signal abnormalities on MRI (Supplementary Fig. 2). The same tumor region was analyzed for all MRI variables at each time point. Ventricles and peritumoral edema were excluded. In addition to the average imaging intensities, the histogram analysis with each MRI parameter was performed to evaluate treatment response, as shown previously\(^1\).

**Histology.** In the terminal MRI experiments, histology was carried out immediately after MRI. When rats were deeply anesthetized (breathing stopped), the animals were dissected by fixing on a board. After euthanasia, brains were excised and preserved in 4\% paraformaldehyde at 4 °C for a week for proper tissue fixation. When the brains were sectioned, the acquired MR images and intrinsic landmarks, such as the corpus callosum and the ventricles, were used for reference. Ten-μm-thick cryostat sections were cut and processed for H&E staining for histopathological evaluation. Low-magnification pictures were obtained from the stained sections to compare with MR images.
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