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Assessing Amide Proton Transfer (APT) MRI Contrast Origins in 9 L Gliosarcoma in the Rat Brain Using Proteomic Analysis

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Abstract

Purpose—To investigate the biochemical origin of the amide proton transfer (APT)-weighted hyperintensity in brain tumors.

Procedures—Seven 9 L gliosarcoma-bearing rats were imaged at 4.7 T. Tumor and normal brain tissue samples of equal volumes were prepared with a coronal rat brain matrix and a tissue biopsy punch. The total tissue protein and the cytosolic subproteome were extracted from both samples. Protein samples were analyzed using two-dimensional gel electrophoresis, and the proteins with significant abundance changes were identified by mass spectrometry.

Results—There was a significant increase in the cytosolic protein concentration in the tumor, compared to normal brain regions, but the total protein concentrations were comparable. The protein profiles of the tumor and normal brain tissue differed significantly. Six cytosolic proteins, four endoplasmic reticulum proteins, and five secreted proteins were considerably upregulated in the tumor.

Conclusions—Our experiments confirmed an increase in the cytosolic protein concentration in tumors and identified several key proteins that may cause APT-weighted hyperintensity.

Keywords

APT imaging; CEST imaging; Glioma; Mobile protein; Proteomics

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Introduction

Despite advances in surgery, radiotherapy, and chemotherapy, malignant brain tumors remain rapidly fatal, with a median survival of 12–15 months for glioblastoma and 2–5 years for anaplastic astrocytoma [1]. The accurate detection and localization of brain tumors are crucial for improved treatment and management. Magnetic resonance imaging (MRI) is an extremely versatile technology that employs water content and water relaxation properties to image the basic anatomy and functions of many organs in the body. MRI became available in the clinic in the 1980s and is now a standard modality for imaging brain tumors. However, the existing standard MRI techniques, such as T₂-weighted and gadolinium-enhanced T₁-weighted sequences, are not sufficiently tissue-specific and suffer from some limitations [2, 3], particularly with regard to specificity in the differential diagnosis of tumor core vs. peritumoral edema, and tumor recurrence vs. treatment effects, which greatly complicate the clinical management of patients with gliomas and impede efficient testing of new therapeutics.

Proteins are not only the building blocks of cells but also execute almost all cellular functions. Proteins in tissues can roughly be classified by their mobility into two types: semisolid proteins (such as nuclear proteins and membrane proteins) that possess solid-like properties and have a very short proton transverse relaxation time T₂ (10 μs) and mobile proteins (such as cytosolic proteins, many endoplasmic reticulum (ER) proteins, and secreted proteins) that rotate rapidly and have a relatively long proton T₂ (tens of ms). Amide proton transfer (APT) imaging [4, 5] is an important molecular MRI technique that is sensitive to endogenous mobile proteins and peptides in tissue [6]. Prior data suggest that there are consistent APT-weighted hyperintensities in animal glioma models [7, 8] and in high-grade gliomas in patients [9–11]. These studies show that APT imaging for malignant brain tumors has much potential, for example, to differentiate between tumor and peritumoral edema, to separate high-grade from low-grade gliomas, and to detect high-grade gliomas that do not show gadolinium enhancement, all without exposure to contrast agents. APT imaging can also distinguish viable malignancy from radiation necrosis and predict tumor response to therapy [12–14].

Although APT imaging is quite unique in the detection and diagnosis of gliomas, the contrast mechanism underlying APT-MRI remains a matter of some controversy. The cell density is generally higher in tumor than in normal tissue (a histopathological characteristic that is evident in malignant gliomas [15]). It was previously assumed that APT-weighted hyperintensity in gliomas and other cancers [16–20] is associated with high tumor cellularity and depends on increased mobile protein and peptide content in the tumors, as well as several other factors, such as the upfield nuclear Overhauser enhancement (NOE) effect and tissue pH [21, 22]. In principle, only mobile proteins and peptides (such as those in the cytoplasm) can result in a characteristic amide proton resonance at 8.3±0.5 ppm (or 3.5 ppm downfield from water) [23–25], enabling selective radiofrequency (RF) irradiation for APT imaging. However, which protein(s) or peptide(s) dominate APT hyperintensity in tumors is still totally unknown.

Proteomics is a powerful biochemical technique that can be used for the large-scale identification and quantification of proteins in cells and tissues [26-28]. This approach allows a comparison of normal vs. diseased samples or diseased vs. treated samples at the global protein expression level [29-32]. For example, Li et al. [31] identified 17 proteins that were uniquely expressed and verified that protein expression patterns can reliably distinguish between normal white matter and astrocytoma and between tumor grades. In addition, Hobbs et al. [30] found that there was a correlation between protein expression profiles and gadolinium enhancement in human glioblastoma. The higher protein number and concentration were seen in gadolinium-enhancing regions than in non-enhancing regions.

In this article, we determined the protein concentrations in total tissue homogenate and the cytoplasmic subproteome and investigated changes in protein profiles of gliomas and the origin of APT-weighted hyperintensity using the rat 9 L gliosarcoma model, by means of an efficient and accurate proteomics method, two-dimensional difference gel electrophoresis (2D DIGE). This technique allows one to compare abundant complex protein mixtures by separating proteins, according to their isoelectric point and molecular weight. We aimed to identify some key mobile proteins that likely correlate with the APT-weighted imaging hyperintensity of this brain tumor model.

Materials and Methods

9 L Tumor Implantation

The Johns Hopkins Animal Care and Use Committee approved all experiments. Seven Fischer 344 rats (male; 8–10 weeks; 150–200 g) were anesthetized by an intraperitoneal injection (3–5 ml/kg, ketamine hydrochloride 25 mg/ml, xylazine 2.5 mg/ml). A midline scalp incision was made, exposing the sagittal and coronal sutures. A small burr hole was made approximately 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 surface. 9 L tumor cells (25,000 in 2 µl media for each rat) were injected over 3–4 min. After infusion, the needle was left in place for 5 min before withdrawal. The skull wound was sutured with wound clips.

MRI Experiments

MRI data were acquired using a horizontal bore 4.7 T BioSpec animal imager (Bruker BioSpin, Billerica, MA), with an actively decoupled cross-coil setup (a 70-mm body coil for RF transmission and a 25-mm surface coil for signal reception). Thirteen days after tumor implantation, rats were re-anesthetized with 5 % isoflurane in a mixture of 75 % air and 25 % O₂ for 5 min, followed by breathing of 1.5–2.5 % isoflurane through a nose cone during MRI procedures. The rat head and body were fixed and taped to the coil and cradle to avoid motion artifacts. Rats that were prone in the magnet were monitored online through a small-animal respiratory gating system connected fiber optically, and the breathing rate of the animal was kept at 40±5 breaths per min by adjusting the isoflurane ratio (1.5–2.5 %) in the breathing mixture.

Several different MRI sequences were acquired for each animal. First, high-resolution, T₂-weighted imaging in both the horizontal plane (matrix=256×192; field of view=42×32 mm²) and the coronal plane (matrix=192×192; field of view=32×32 mm²) was acquired (fast spin echo; echo train length=8; repetition time=3 s; effective echo time=64 ms; five slices; slice thickness 2 mm; number of averages=2). Then, APT-weighted imaging was acquired (offsets=±3.5 ppm; repetition time=10 s; echo time=30 ms; saturation power=1.3 μT; saturation time=4 sec; 16 averages). To correct for B₀ inhomogeneity effects on APT imaging, slab shimming (thickness 7.5 mm) around the image slice and adjustment of the scanner transmitter frequency were performed prior to APT data acquisition. Conventional magnetization transfer ratio (MTR) images at an RF saturation frequency offset of 10 ppm (2 kHz at 4.7 T) were acquired, which had the same experimental parameters as the APT scans. Single-shot, spin-echo, echo-planar imaging was used for data acquisition (matrix=64×64; field of view=32×32 mm²; single slice; slice thickness=2 mm). The image geometry was imported from one of the coronal T₂-weighted images that showed the largest tumor.

Brain Image Data Analysis

All data processing procedures were performed using Interactive Data Language (IDL; Version 7; Exelis Visual Information Solutions, Inc., Boulder, CO). The acquired raw images were interpolated to 384×384. The APT-weighted images were quantified by the MTR asymmetry at ±3.5 ppm with respect to water: $MTR_{asym}(3.5 \text{ ppm}) = \frac{S_{sat}(-3.5 \text{ ppm})/S_0 - S_{sat}(+3.5 \text{ ppm})/S_0}{S_0}$ (where S_{sat} and S_0 are the signal intensities with and without RF irradiation), thresholded to remove pixels outside the brain based on the S_0 image, and displayed using a window of -8 to 8 %. The MTR map was obtained at an offset of 10 ppm, using the equation: $MTR(10 \text{ ppm}) = 1 - S_{sat}(10 \text{ ppm})/S_0$. The quantitative analysis of APT imaging was performed in areas where tissues were sampled for proteomics (25 to 30 pixels on the APT-weighted images). The signal abnormalities on the high-resolution, T₂-weighted images were used as a reference to define regions of interest. Contralateral normal-appearing, relatively homogeneous brain tissue was used for comparison. The average APT imaging intensities for the tumors and the corresponding contralateral brain regions were calculated, and the results were represented as mean±standard deviation. In addition, tumor volumes were manually measured as the sum of all tumor voxels in all slices on the high-resolution T₂-weighted images. A paired *t* test was used to determine whether the observations were significant. The level of significance was set at $P < 0.05$.

Tissue Sample Preparation

This study required an accurate method for dissecting the same volumes of tissue samples from both tumor and normal regions [33]. Figure 1 shows some special tools used for tissue sample preparation: a rat brain slicer matrix at an interval of 2 mm and a 3-mm-diameter biopsy punch (Zivic Instruments, Pittsburg, PA). After APT scanning, the rat was anesthetized and decapitated. The brain was rapidly removed, rinsed with ice-cold phosphate-buffered saline (PBS), and placed into the rat brain matrix. Precise 2-mm-thick coronal brain slices were cut corresponding to preoperative MRI scans. Small tissue pieces from normal and tumor regions were excised with the 3-mm-diameter biopsy punch and

frozen with liquid nitrogen immediately. All the tissue samples were stored at -80°C before the protein extraction.

Protein Extraction and Concentration Determination

Total protein extraction was performed using the PlusOne Sample Grinding Kit (GE Healthcare, Piscataway, NJ) according to the manufacturer's recommended protocol. Each tissue sample (~15 mg) was lysed with 1 ml lysis buffer (8 M urea, 2 M thiourea, 4 % CHAPS, 1 % dithiothreitol (DTT)). After centrifuging for 10 min at maximum speed (12,500 rpm), cellular debris was removed, and the clear supernatant was carefully collected. Total protein extracts were aliquoted and stored at -80°C for the further use. Cytosolic proteins were extracted with ReadyPrep™ Protein Extraction Kit (Bio-Rad Laboratories, Inc., Hercules, CA). Total protein and cytosolic protein concentrations were determined using the CB-X Protein Assay (Geno Technology Inc., Maryland Heights, MO). The average concentration value for each sample was obtained from triplicate measurements.

2D DIGE

The 2D DIGE method labels protein samples with fluorescent dyes before 2D electrophoresis, enabling the accurate analysis of differences in protein abundance between samples, which we exploited to compare the tumor and contralateral normal brain region of each individual animal. In this study, protein labeling was performed using the CyDyes DIGE Fluors, developed for fluorescence 2D DIGE technology (GE Healthcare). Protein samples (50 μl) were labeled with the respective fluorophores: Cy2 blue for an equal mixture of normal and tumor extracts (unused in this study), Cy3 green for normal extract, and Cy5 red for tumor extract. Thus, yellow spot 2D DIGE images would represent the equal protein amounts in the normal brain and tumor tissues (adding red to green yields yellow). The labeled protein samples (50 μl of each, total 150 μl) were combined, and rehydration buffer was added (8 M urea, 2 % w/v CHAPS, 0.1 % bromphenol blue, 20 mM DTT, and 1 % IPG buffer) (GE Healthcare) to a final volume 340 μl . Immobiline Dry Strips (18 cm, pH 4–7) (GE Healthcare) were rehydrated at 50 V for 12 h and then gradually increased to 10,000 V and kept constant until 100,000 V h. Ten percent Bis-Tris polyacrylamide gels were used for the second dimension sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, CyDye-labeled proteins were visualized using a Typhoon™ 9410 imager (GE Healthcare). The scanned gels were analyzed using the DeCyder™ 2D 6.5 software (GE Healthcare). The spot volumes were detected using the differential in-gel analysis (DIA) mode according to the manufacturer's user manual. To prepare the gel spots for protein identification purposes, 200 μl of proteins were subjected to 2D gel electrophoresis as indicated above, without CyDye labeling. At the end of the run, the gels were removed and fixed in a solution containing acetic acid/methanol/water (5:45:50, v/v/v) for 1 h, followed by another hour of methanol/water (1:1, v/v) and three washes for 5 min in water. Staining was performed with silver nitrate. An Epson 10000XL Fastsilver Imager (Agilent, Wilmington, DE) was used to visualize silver-stained gels.

In-Gel Tryptic Digestion and Peptide Desalting

After gel image acquisition, the interesting, large protein spots were excised and subjected to in-gel tryptic digestion. After digestion, peptide samples were dissolved with 10 μ l 0.1 % trifluoroacetic acid (TFA) water for desalting with ZipTip C18 pipette tips (Millipore, Billerica, MA). The eluted samples were used directly for mass spectrometry analysis with a matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF)-TOF mass spectrometer or dried by Speed Vac and redissolved in 1 % TFA for analysis using the electrospray on an LTQ-Orbitrap mass spectrometer.

Mass Spectrometry Analysis and Protein Identification

Mass spectra were acquired with a 4800 Plus MALDI-TOF/TOFTM mass spectrometer (Applied Biosystem, Foster City, CA) and LTQ-Orbitrap mass spectrometer (ThermoElectron, San Jose, CA). The samples were first analyzed using the MALDI-TOF-TOF mass spectrometer. Then, 0.5 μ l of sample solution were placed on the plate and covered with 0.5 μ l of 50 mM alpha-hydroxycinnamic acid in 0.1 % TFA, 70 % acetonitrile, and then dried before being put into the instrument. Spectra were recorded by accumulating 100–200 laser shots, depending on the quality of the sample. Peptide mass fingerprints were internally calibrated to monoisotopic trypsin autolysis peaks (m/z =515.33, 842.51, 1045.56, 2211.11). Mascot search programs from www.matrixscience.com were used to obtain protein(s). The instrument used to acquire tandem mass spectrometry was an LTQ ion trap/orbitrap mass spectrometer equipped with an online nano2D HPLC (Eksigent, CA). Peptides were trapped on a peptide trap (3 cm, 75 μ m, packed with irregular size particles of 5–15 μ m, 120 Å, YMC ODS-AQ) and further separated on a reverse-phase analytical column packed with 10 cm of C18 beads (360 $\times$ 75 μ m, 5 μ m, 120 Å, YMC ODSAQ, Waters, Milford, MA), with a 10 μ m emitter tip (New Objective, Woburn, MA) attached. The HPLC gradient was 5–40 % B for 30 min (A, 0.1 % formic acid; B, 90 % acetonitrile in 0.1 % formic acid) and then quickly increased to 90 % B before returning to initial conditions. The flow rate was 300 nl/min. The LTQ-Orbitrap-Velos data were searched against the SwissProt database using the Mascot Deamon search engine (www.matrixscience.com).

Results

Figure 2 shows the T₂-weighted, APT-weighted, and MTR images of 9 L gliosarcoma in a rat (post-implantation day 13). Tumor volumes for all rats were roughly estimated to be 172.8 $\pm$ 26.4 mm³. Compared to the contralateral normal brain region, the tumor showed hyperintensity on both the T₂-weighted and APT-weighted images and hypointensity on MTR images. Table 1 quantitatively compares the APT-weighted and MTR signal intensities measured from the tumor and contralateral normal brain regions (n =7). The average APT-weighted signal intensities were significantly higher in the tumor tissue than in the contralateral normal brain tissue (3.06 $\pm$ 1.14 % vs. -1.27 $\pm$ 0.75 %; P <0.001), as reported previously [7, 8]. The negative APT-weighted value indicated the contribution to the measured signal from the NOE. However, the average MTR intensities were significantly lower in the tumor tissue than in the contralateral normal brain tissue (26.1 $\pm$ 12.3 % vs. 36.1 $\pm$ 9.8 %; P <0.001).

Immediately after MRI scanning, fresh rat brains were rapidly removed, and tumor and contralateral normal tissue (primarily gray matter in this study) samples with the same volume (2 mm thickness and 3 mm diameter) were obtained. The total proteins ($n=4$) and cytosolic proteins ($n=3$) were extracted, and their concentrations were determined. As shown in Table 1, there was no significant difference between the measured total protein concentrations of tumor and normal tissues (0.99 ± 0.02 vs. 1.02 ± 0.10 $\mu\text{g}/\mu\text{l}$; $P=0.7$). However, the measured cytosolic protein content in tumor tissue samples was significantly higher than that in normal tissue samples (0.88 ± 0.12 vs. 0.63 ± 0.12 $\mu\text{g}/\mu\text{l}$; $P<0.05$).

2D DIGE was carried out on total protein samples to identify key proteins that may cause the APT signal difference between tumor and normal tissues. Figure 3 shows the protein expression profiles from tumor and normal regions within the same brain for four rats. Protein profiles for both tumor and normal regions showed very similar patterns between all four rats; however, there were remarkable proteomic pattern differences in protein species and concentration between the tumor and normal brain tissues of the same volume. Over 1000 protein spots were detected on all 2D gels. The 72 most abundant protein spots were chosen for mass spectrometry studies, and 45 proteins were identified successfully (Supplementary Fig. S1 and Table S1). Some proteins appeared at multiple spots, suggesting the presence of posttranslational modifications.

Table 2 shows the relative volumes of all identified protein spots, in which tropomyosin alpha-3 chain or TPM3 in the normal tissue (that had a moderate volume) was assigned to be one. Roughly, mobile proteins in biological tissues include cytosolic proteins, ER proteins, and secreted proteins, all of which remain in relatively liquid cell compartments. The measured cytosolic protein content in Table 1, which was higher in tumor tissue than in normal tissue, may include some secreted proteins. However, semisolid proteins include nuclear proteins, membrane proteins, and mitochondrial proteins, which might not contribute to measured APT-weighted signals.

Supplementary Table S2 shows 32 abundant, significantly changed proteins (± 1.5 -fold; 16 upregulated and 16 downregulated) in the tumor, compared to the contralateral brain tissue, based on their subcellular localization. Figure 4 shows these 16 significantly upregulated proteins on the 2D DIGE image. In particular, six cytosolic proteins, including ANXA5, TPT1, CRABP1, TXN, S100A4, and S100A6; four ER proteins, including HSP90B1, HSPA5, PDIA6, and PDIA3; as well as five secreted proteins, including ALB, SERPINA3L, SERPINA3K, SERPINA1, and LGALS1, were significantly upregulated in the tumor. Some of these proteins were identified and reported as tumor markers in previous studies, including ANXA5, S100A4, S100A6 [34], HSPA5 [35], PDIA6, ALB [36], and LGALS1 [37].

Discussion

In standard proteomics studies, equal amounts of protein are loaded onto 2D gels to compare the expression of specific proteins. In this study, to match the MRI observations, we compared proteins that were extracted from equal volumes of normal and tumor tissue samples [33]. Although the total protein concentrations in the tumor and contralateral

normal brain tissue were essentially the same in the 9 L brain tumor model, this may not be the case for other models and patients. Similar to some early methods [38, 39], we used a rat brain slicer matrix and a tissue biopsy punch, both made from high-grade stainless steel. To avoid the protein degradation, it is crucial to perform the procedures quickly and keep the equipment on ice at all times.

It is important to keep in mind that APT imaging is designed to detect “mobile” proteins and peptides in tissue, while conventional MT detects “semi-solid” macromolecules (including proteins and lipids). Only mobile proteins (such as cytosolic proteins, many ER proteins, and secreted proteins) could potentially be detected by APT-MRI (where a characteristic amide resonance is required). Thus, the chemicals that contribute to APT and conventional MT signals are totally different. In a recent study (also using the 9 L tumor model) [40], the total protein content in tissues, measured by standard biochemical methods, was incorrectly used to elucidate the APT-MRI results.

Notably, the measured total protein concentrations of tumor and normal (primarily gray matter in this study) tissues were similar (0.99 ± 0.02 vs. 1.02 ± 0.10 $\mu\text{g}/\mu\text{l}$, respectively), and the cytosolic protein concentrations were significantly higher in tumor tissue samples than in normal tissue samples (0.88 ± 0.12 vs. 0.63 ± 0.12 $\mu\text{g}/\mu\text{l}$, respectively), suggesting that the semi-solid protein concentration was likely lower in the tumor. This is consistent with a few previous quantitative MT studies [41, 42], showing that the semi-solid proton pool sizes are significantly smaller in the tumor than in the normal brain tissue, although the conventional MT imaging is also sensitive to various semisolid lipids in the tissue. These biochemical experimental results were in good agreement with the increased APT-weighted signal and decreased MTR signal in the tumor, as reported in this and many previous studies [7, 8, 20]. In addition, Howe et al. [43] quantified the metabolic profiles of human brain tumors using in vivo proton magnetic resonance spectroscopy, and the results showed that the mobile macromolecular proton concentrations for several upfield resonances were higher in human brain tumors than in normal white matter and increased with tumor grade.

All mobile proteins (including cytosolic proteins, ER proteins, and secreted proteins) may contribute more or less to the observed APT effects (primarily depending on the amide proton concentration and the amide proton exchange rate). One can assume that each protein in the cytosolic, ER, and secreted groups contributes to APT equally per the spot volume. Based on the spot volumes in Table 2, it was roughly estimated that about 93.6, 2.9, and 3.6 % of the APT effect in the normal tissue and about 52.0, 9.2, and 38.8 % of the APT effect in the tumor were contributed by the cytosolic proteins, ER proteins, and secreted proteins, respectively. Notably, the contributions from the mobile proteins in the normal tissue (green spots on the 2D DIGE images) to the observed APT-weighted signal are cancelled out by the increased mobile proteins in the tumor tissue (red spots), leading to the observed APT-weighted hyperintensity in the tumor. It seems that tumor tissues contain more complicated protein profiles (including more protein spots or species) than normal brain tissues. Thus, it is very likely that many tumor proteins were not identified due to the relatively low abundance and missed in Table 2. In addition, small proteins below the 10 kDa cutoff of the 2D DIGE method were not included in Table 2. However, according to our results (Figs. 3 and 4), these small proteins may not have a significant concentration. The cytosolic protein

content reported in Table 1 may have included some secreted proteins. Based on our experimental results, the secreted proteins, including ALB, LGALS1, and several serine protease inhibitors, were remarkably upregulated in the tumor tissue. These proteins are associated with the aggressiveness of tumors and might contribute to APT contrast enhancement.

Based on the MTR asymmetry analysis, the quantified APT-weighted signal in glioma is influenced by both the downfield APT and upfield NOE effects. The upfield NOE includes the narrow and wide components, and the former usually shows a visible dip in the z-spectrum [44]. The narrow component resulted from mobile biomolecules in tissue (including mobile proteins and peptides), and the wide component, previously thought to be the inherent semi-solid MTR asymmetry [45], resulted from relatively less mobile biomolecules. Our recent study at 4.7 T [44] has shown that the APT-weighted signal is comparable to or predominantly contributed by the APT effect at relatively higher saturation power levels ($>1.3 \mu\text{T}$). Notably, the NOE is actually a positive confounding factor in the APT-weighted image contrast in the tumor. Namely, the APT-weighted image contrast between the tumor and contralateral brain tissue would increase at all saturation powers due to the presence of the NOE [42].

According to the theory [4], the APT effect in tissue is primarily related to the mobile amide proton content, the amide proton exchange rate (a parameter that depends on tissue pH), the water content, and the water T_1 . Although the water content is usually higher, and water T_1 is enhanced in tumor, it is extremely important to understand that the effects of these two changes on the APT measurements are mostly compensated for in many diseases [4]. Therefore, assessing the influence of water T_1 in vivo on APT (and also NOE) imaging should be performed cautiously. Very recently, simply multiplying by water R_1 in the APT measurements was improperly used to correct for the influence of water T_1 , leading to the unexpected APT-MRI results ($\text{AREX}_{\text{tumor}} \approx \text{AREX}_{\text{normal}}$, insignificantly different between tumors and normal tissues) [40]. Previous ^{31}P MRS studies demonstrated that the intracellular pH of solid tumors remains neutral to a little alkaline [46]. As the amide proton exchange rate is base-catalyzed in the physiological pH range, the exchange rate increases with pH, thus increasing the APT effect (if the fast-exchange regime is not reached). However, the increased mobile protein concentration in the 9 L glioma, as observed in this study, would make the most contribution to the APT-weighted hyperintensity, because only a small intracellular pH increase (<0.1 unit) is often detected in the tumor, with respect to normal brain tissue.

Finally, it should be pointed out that this study is only an initial step in our quest to understand the mechanism behind the APT contrast for differentiating normal from malignant tissue. This study was conducted using only one rat model (9 L gliosarcoma model). Although it is not clear whether these results are specific to the tumor model that we investigated, we believe that the conclusions in this study could be extended to many other brain tumor types that have similar APT intensities, such as the U87 glioma model [13]. Further studies using other tumor models and, particularly, human tumors are needed to further elucidate the biochemical origin of the APT-MRI signal in tissue.

Conclusions

Compared to the contralateral brain tissue, 9 L gliosarcomas showed significant hyperintensity on both the T₂-weighted and APT-weighted images and significant hypointensity on MTR images. The measured total protein concentrations of tumor and normal tissues were similar, and the measured cytosolic protein concentrations were significantly higher in tumor tissue samples than in normal tissue samples. The protein expression profiles of tumor and normal tissues showed remarkable qualitative and quantitative differences. Several overexpressed proteins in tumors include some tumor markers, such as ANXA5, S100A4, S100A6, PDIA6, HSPA5, ALB, and LGALS1. It is highly possible that the APTMRI signal is related to these proteins and can be used as an imaging biomarker for predicting tumor progression and therapy responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Stupp R, Mason WP, van den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med*. 2005; 352:987–96. [PubMed: 15758009]
2. Chang SM, Nelson S, Vandenberg S, et al. Integration of preoperative anatomic and metabolic physiologic imaging of newly diagnosed glioma. *J Neuro-Oncol*. 2009; 92:401–15.
3. Wen PY, Macdonald DR, Reardon DA, et al. Updated response assessment criteria for high-grade gliomas: response assessment in neuro-oncology working group. *J Clin Oncol*. 2010; 28:1963–72. [PubMed: 20231676]
4. Zhou J, Payen J, Wilson DA, et al. Using the amide proton signals of intracellular proteins and peptides to detect pH effects in MRI. *Nature Med*. 2003; 9:1085–90. [PubMed: 12872167]
5. Zhou J, Lal B, Wilson DA, et al. Amide proton transfer (APT) contrast for imaging of brain tumors. *Magn Reson Med*. 2003; 50:1120–6. [PubMed: 14648559]
6. Kauppinen RA, Kokko H, Williams SR. Detection of mobile proteins by proton nuclear magnetic resonance spectroscopy in the guinea pig brain *ex vivo* and their partial purification. *J Neurochem*. 1992; 58:967–74. [PubMed: 1738003]
7. Salhotra A, Lal B, Laterra J, et al. Amide proton transfer imaging of 9 L gliosarcoma and human glioblastoma xenografts. *NMR Biomed*. 2008; 21:489–97. [PubMed: 17924591]
8. Grossman R, Tyler B, Brem H, et al. Growth properties of SF188/V+ human glioma in rats in vivo observed by magnetic resonance imaging. *J Neuro-Oncol*. 2012; 110(3):315–23.
9. Zhou J, Blakeley JO, Hua J, et al. Practical data acquisition method for human brain tumor amide proton transfer (APT) imaging. *Magn Reson Med*. 2008; 60:842–9. [PubMed: 18816868]
10. Wen Z, Hu S, Huang F, et al. MR imaging of high-grade brain tumors using endogenous protein and peptide-based contrast. *Neuro-Image*. 2010; 51:616–22. [PubMed: 20188197]
11. Zhao X, Wen Z, Zhang G, et al. Three-dimensional turbo-spinecho amide proton transfer MR imaging at 3-Tesla and its application to high-grade human brain tumors. *Mol Imaging Biol*. 2013; 15:114–22. [PubMed: 22644987]

12. Zhou J, Tryggstad E, Wen Z, et al. Differentiation between glioma and radiation necrosis using molecular magnetic resonance imaging of endogenous proteins and peptides. *Nature Med.* 2011; 17:130–4. [PubMed: 21170048]
13. Hong X, Liu L, Wang M, et al. Quantitative multiparametric MRI assessment of glioma response to radiotherapy in a rat model. *Neuro Oncol.* 2014; 16:856–67. [PubMed: 24366911]
14. Sagiya K, Mashimo T, Togao O, et al. In vivo chemical exchange saturation transfer imaging allows early detection of a therapeutic response in glioblastoma. *Proc Natl Acad Sci U S A.* 2014; 111:4542–7. [PubMed: 24616497]
15. Burger PC, Dubois PJ, Schold SC, et al. Computerized tomographic and pathologic studies of the untreated, quiescent, and recurrent glioblastoma-multiforme. *J Neurosurg.* 1983; 58(2):159–69. [PubMed: 6294260]
16. Jia G, Abaza R, Williams JD, et al. Amide proton transfer MR imaging of prostate cancer: a preliminary study. *J Magn Reson Imaging.* 2011; 33(3):647–54. [PubMed: 21563248]
17. Zhou J, Zhu H, Lim M, et al. Three-dimensional amide proton transfer MR imaging of gliomas: initial experience and comparison with gadolinium enhancement. *J Magn Reson Imaging.* 2013; 38:1119–28. [PubMed: 23440878]
18. Dula AN, Arlinghaus LR, Dortch RD, et al. Amide proton transfer imaging of the breast at 3 T: establishing reproducibility and possible feasibility assessing chemotherapy response. *Magn Reson Med.* 2013; 70:216–24. [PubMed: 22907893]
19. Togao O, Kessinger CW, Huang G, et al. Characterization of lung cancer by amide proton transfer (APT) imaging: an in-vivo study in an orthotopic mouse model. *Plos One.* 2013; 8:e77019. [PubMed: 24143199]
20. Desmond KL, Moosvi F, Stanisz GJ. Mapping of amide, amine, and aliphatic peaks in the CEST spectra of murine xenografts at 7 T. *Magn Reson Med.* 2014; 71:1841–53. [PubMed: 23801344]
21. Zhao X, Wen Z, Huang F, et al. Saturation power dependence of amide proton transfer image contrasts in human brain tumors and strokes at 3 T. *Magn Reson Med.* 2011; 66:1033–41. [PubMed: 21394783]
22. Togao O, Yoshiura T, Keupp J, et al. Amide proton transfer imaging of adult diffuse gliomas: correlation with histopathological grades. *Neuro Oncol.* 2014; 16:441–8. [PubMed: 24305718]
23. Chen W, Hu J. Mapping brain metabolites using a double echo-filter metabolite imaging (DEFMI) technique. *J Magn Reson.* 1999; 140:363–70. [PubMed: 10497044]
24. van Zijl PCM, Zhou J, Mori N, et al. Mechanism of magnetization transfer during on-resonance water saturation. A new approach to detect mobile proteins, peptides, and lipids. *Magn Reson Med.* 2003; 49:440–9. [PubMed: 12594746]
25. Zhou JY, Yan K, Zhu H. A simple model for understanding the origin of the amide proton transfer MRI signal in tissue. *Appl Magn Reson.* 2012; 42(3):393–402. [PubMed: 23243339]
26. Westermeier, R.; Naven, T. *Proteomics in practice.* Wiley-VCH; Weinheim: 2002.
27. He QY, Chiu JF. Proteomics in biomarker discovery and drug development. *J Cell Biochem.* 2003; 89(5):868–86. [PubMed: 12874822]
28. Zhu H, Bilgin M, Snyder M. Proteomics. *Annu Rev Biochem.* 2003; 72:783–812. [PubMed: 14527327]
29. Stoeckli M, Chaurand P, Hallahan DE, Caprioli RM. Imaging mass spectroscopy: a new technology for the analysis of protein expression in mammalian tissues. *Nature Med.* 2001; 7:493–6. [PubMed: 11283679]
30. Hobbs SK, Shi G, Homer R, et al. Magnetic resonance imaging-guided proteomics of human glioblastoma multiforme. *J Magn Reson Imag.* 2003; 18:530–6.
31. Li J, Zhuang Z, Okamoto H, et al. Proteomic profiling distinguishes astrocytomas and identifies differential tumor markers. *Neurology.* 2006; 66:733–6. [PubMed: 16534112]
32. Shen J, Behrens B, Wistuba II, et al. Identification and validation of differences in protein levels in normal, premalignant, and malignant lung cells and tissues using high-throughput Western array and immunohistochemistry. *Cancer Res.* 2006; 66:11194–206. [PubMed: 17145864]
33. Yan, K.; Fu, Z.; Van Eyk, J.; Wang, S.; Zhou, J., editors. Identification of endogenous proteins correlated with amide proton transfer (APT) imaging contrast using proteomic analysis. *Proc 19th Annual Meeting ISMRM; Montreal.* 2011.

34. Tabuchi K, Moriya Y, Furuta T, et al. S-100 protein in human glial tumours. Qualitative and quantitative studies. *Acta Neurochir (Wien)*. 1982; 65(3–4):239–51. [PubMed: 6295075]
35. Iwadata Y, Sakaida T, Hiwasa T, et al. Molecular classification and survival prediction in human gliomas based on proteome analysis. *Cancer Res*. 2004; 64(7):2496–501. [PubMed: 15059904]
36. Odreman F, Vindigni M, Gonzales ML, et al. Proteomic studies on low- and high-grade human brain astrocytomas. *J Proteome Res*. 2005; 4(3):698–708. [PubMed: 15952716]
37. Zhang RL, Tremblay TL, Mcdermid A, et al. Identification of differentially expressed proteins in human glioblastoma cell lines and tumors. *Glia*. 2003; 42(2):194–208. [PubMed: 12655603]
38. Palkovits M. Isolated removal of hypothalamic or other brain nuclei of the rat. *Brain Res*. 1973; 59:449–50. [PubMed: 4747772]
39. Jacobowitz DM. Removal of discrete fresh regions of the rat brain. *Brain Res*. 1974; 80(1):111–5. [PubMed: 4425300]
40. Xu J, Zaiss M, Zu Z, et al. On the origins of chemical exchange saturation transfer (CEST) contrast in tumors at 9.4 T. *NMR Biomed*. 2014; 27:406–16. [PubMed: 24474497]
41. Quesson B, Bouzier A-K, Thiaudiere E, et al. Magnetization transfer fast imaging of implanted glioma in the rat brain at 4.7 T: interpretation using a binary spin-bath model. *J Magn Reson Imag*. 1997; 7:1076–83.
42. Heo H-Y, Zhang Y, Lee D-H, et al. Quantitative assessment of amide proton transfer (APT) and nuclear Overhauser enhancement (NOE) imaging with extrapolated semi-solid magnetization transfer reference (EMR) signals: Application to a rat glioma model at 4.7 T. *Magn Reson Med*. 2014 In press.
43. Howe FA, Barton SJ, Cudlip SA, et al. Metabolic profiles of human brain tumors using quantitative in vivo ^1H magnetic resonance spectroscopy. *Magn Reson Med*. 2003; 49:223–32. [PubMed: 12541241]
44. Zhou J, Hong X, Zhao X, et al. APT-weighted and NOE-weighted image contrasts in glioma with different RF saturation powers based on magnetization transfer ratio asymmetry analyses. *Magn Reson Med*. 2013; 70:320–7. [PubMed: 23661598]
45. Hua J, Jones CK, Blakeley J, et al. Quantitative description of the asymmetry in magnetization transfer effects around the water resonance in the human brain. *Magn Reson Med*. 2007; 58:786–93. [PubMed: 17899597]
46. Griffiths JR. Are cancer cells acidic? *Br J Cancer*. 1991; 64:425–7. [PubMed: 1911181]

Fig. 1.

Illustration of the fresh rat brain tissue sample preparation. The brain was placed into a rat brain matrix with intervals of 2 mm (Zivic Instruments, Pittsburgh, PA) and then cut by blades (*red lines*). Two tissue samples from tumor (*red arrow*) and contralateral normal (*green arrow*) regions were punched with a 3-mm-diameter biopsy punch (Zivic Instruments, Pittsburgh, PA). Tissue samples from the tumor (*red circle*) and contralateral normal (*green circle*, primarily gray matter) regions of equal volumes (2 mm thickness and 3 mm diameter) were obtained.

Fig. 2. MR images of 9 L gliosarcoma in a representative rat (13 days after implantation). The tumor (*red arrows*) was visible on T₂-weighted, APT-weighted, and MTR images.

Fig. 3. 2D DIGE images of normal brain and tumor regions for four rats (labels A–D). *Green spots* indicate proteins from the normal brain tissue; *red spots* indicate proteins from the tumor tissue, and *yellow spots* represent the equal protein amounts in the normal brain and tumor tissues. Protein profiles showed similarities in both tumor and normal regions from different rats but substantial differences between tumor and normal regions within the same rat.

Fig. 4. 2D DIGE image of 16 upregulated (± 1.5 -fold) proteins in the tumor tissue compared to the contralateral normal brain tissue. *Green spots* indicate proteins from the normal brain tissue; *red spots* indicate proteins from the tumor tissue, and *yellow spots* represent the equal protein amounts in the normal brain and tumor tissues.

Table 1

APT-weighted and MTR signal intensities, total protein concentrations, and cytosolic protein concentrations of 9 L tumor tissue samples and the contralateral normal brain samples (mean $\pm$ SD; $n=7$)

	Contralateral	Tumor
APT-weighted signal (%)	-1.27 ± 0.75	3.06 ± 1.14
MTR (10 ppm) (%)	36.1 ± 9.8	26.1 ± 12.3
Total protein concentration ($\mu\text{g}/\mu\text{l}$)	1.02 ± 0.10	0.99 ± 0.02
Cytosolic protein concentration ($\mu\text{g}/\mu\text{l}$)	0.63 ± 0.12	0.88 ± 0.12

Table 2
Protein alterations in the 9 L brain tumor compared to the contralateral normal brain tissue (mean±SD; n=4)

No. ^a	Gene name	Ratio ^b	Normal spot volume	Tumor spot volume	No. ^a	Gene name	Ratio ^b	Normal spot volume	Tumor spot volume
Cytosol									
4	HSPA8	-0.97±1.38	0.81±0.19	0.62±0.28	Endoplasmic reticulum lumen				
5	HSPA8	-1.56±0.14	5.43±1.90	3.58±1.39	1	HSP90B1	2.54±1.02	0.45±0.34	1.12±0.79
6	ANXA6	1.23±0.22	1.00±0.36	1.18±0.34	2	HSPA5	2.82±0.46	0.94±0.19	2.61±0.24
11	NEFL	-10.44±4.60	0.75±0.51	0.07±0.05	31	PDIA6	9.47±3.48	0.26±0.12	2.35±1.28
18	INA	-4.03±1.24	0.34±0.16	0.10±0.07	35	PDIA3	5.29±2.69	0.44±0.25	1.99±0.77
19	INA	-9.84±3.05	0.73±0.45	0.07±0.03	Secreted				
20	INA	-3.56±1.33	0.31±0.12	0.10±0.06					
29	TUBB2C	-6.78±0.33	5.00±6.83	0.74±1.01					
30	TUBA8	-7.00±0.53	7.97±7.05	1.12±0.95	8	ALB	22.18±4.32	0.13±0.06	2.86±1.21
33	GFAP	-2.14±0.70	0.24±0.12	0.12±0.07	9	ALB	20.60±5.27	0.49±0.53	8.21±7.54
34	GDA	-0.01±1.45	0.50±0.09	0.52±0.23	10	ALB	28.37±1.04	0.30±0.24	8.46±6.82
36	DPYSL2	-2.29±0.67	0.24±0.06	0.11±0.04	12	SERPINA3L	12.68±6.92	0.05±0.04	0.45±0.22
37	DPYSL2	-8.78±3.27	0.21±0.08	0.03±0.02	13	SERPINA3L	8.88±1.02	0.08±0.03	0.73±0.23
38	DPYSL2	-10.33±1.59	1.13±0.46	0.11±0.05	14	SERPINA3L	8.15±1.65	0.13±0.04	0.99±0.22
39	CCT2	0.18±1.51	0.24±0.06	0.29±0.13	15	SERPINA3L	10.69±1.86	0.10±0.04	1.03±0.23
40	ENO1	0.10±1.69	0.66±0.31	0.75±0.44	16	SERPINA3L	9.65±3.67	0.12±0.09	0.91±0.20
41	ENO1	-1.40±0.24	3.22±1.10	2.30±0.71	17	SERPINA3L	6.97±1.05	0.09±0.06	0.60±0.34
44	ENO2	-8.86±0.95	0.30±0.17	0.03±0.02	22	SERPINA3K	9.76±3.22	0.13±0.07	1.13±0.28
45	ENO2	-20.94±6.64	3.32±1.79	0.17±0.09	23	SERPINA3K	10.27±2.59	0.14±0.04	1.41±0.24
46	ENO2	-2.97±3.06	0.59±0.44	0.55±0.93	24	SERPINA3K	8.43±1.65	0.16±0.05	1.33±0.28
47	ACTB	0.32±1.60	3.09±3.96	3.37±3.77	25	SERPINA3K	6.47±1.96	0.14±0.04	0.92±0.43
48	ACTB	0.70±1.21	9.88±6.26	12.29±8.16	26	SERPINA3K	6.08±1.71	0.25±0.21	1.64±1.51
49	ACTB	-2.08±0.37	4.94±1.01	2.42±0.68	27	SERPINA1	13.30±4.94	0.05±0.02	0.62±0.22
50	CKB	-5.42±1.44	0.70±0.50	0.14±0.11	28	SERPINA1	12.89±5.55	0.06±0.02	0.75±0.23
51	CKB	-13.82±5.15	2.47±1.43	0.20±0.12	68	LGALS1	11.14±4.57	0.19±0.13	1.86±0.88
52	TPM4	0.62±1.11	1.72±0.72	1.85±0.63	Nucleus				
53	TPM3	-0.04±1.26	1.00±0.00	0.97±0.11					

No. ^a	Gene name	Ratio ^b	Normal spot volume	Tumor spot volume	No. ^a	Gene name	Ratio ^b	Normal spot volume	Tumor spot volume
54	ANXA5	3.05±1.01	0.29±0.12	0.80±0.15	42	VIM	30.33±11.94	0.21±0.05	6.28±2.77
57	LDHB	-10.72±2.65	0.95±0.53	0.10±0.06	43	VIM	14.15±6.28	0.34±0.29	3.63±1.11
58	MDH1	-7.08±1.98	1.07±0.68	0.15±0.07					
59	YWHAB	-2.14±0.06	1.34±0.65	0.62±0.28					
60	YWHAB	-1.92±0.26	2.00±0.71	1.07±0.46					
61	YWHAB	-2.39±0.42	1.33±0.38	0.58±0.22					
62	YWHAB	-2.22±0.45	0.82±0.36	0.38±0.18					
63	ARHGDIA	0.76±1.25	0.92±0.18	1.11±0.14					
65	PEBP1	-2.14±0.65	0.95±0.15	0.47±0.11	3	NDUFS1	-3.76±0.55	0.23±0.05	0.06±0.01
66	TPT1	1.79±0.38	0.46±0.08	0.80±0.09	7	HSPA9	-1.27±0.11	0.87±0.18	0.81±0.37
67	PRDX2	-1.17±0.14	1.15±0.11	1.00±0.21	21	HSPD1	-1.73±0.47	1.36±0.39	0.90±0.56
69	CRABP1	6.51±2.69	0.23±0.10	1.35±0.42	32	UQCRC1	-3.26±0.97	0.43±0.12	0.13±0.01
70	TXN	2.53±0.11	0.34±0.08	0.87±0.22	56	PDHB	-2.07±0.67	0.44±0.29	0.28±0.30
71	S100A4	21.92±11.17	0.07±0.04	1.30±0.61	55	NAPB	-6.79±2.66	0.27±0.26	0.04±0.04
72	S100A6	37.13±10.32	0.03±0.02	1.05±0.58	64	NDUFV2	-8.80±2.40	1.15±0.58	0.14±0.06

^a See Supplementary Fig. S1 and Table S1

^b For upregulated proteins, ratio=tumor spot volume/normal spot volume; for downregulated proteins, ratio=normal spot volume/tumor spot volume