Precise ex vivo histological validation of heightened cellularity and diffusion-restricted necrosis in regions of dark apparent diffusion coefficient in 7 cases of high-grade glioma

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See the editorial by Gerstner, on pages 1563–1564.

Background. Recent conflicting reports have found both brain tumor hypercellularity and necrosis in regions of restricted diffusion on MRI-derived apparent diffusion coefficient (ADC) images. This study precisely compares ADC and cell density voxel by voxel using post-mortem human whole brain samples.

Methods. Patients with meningioma were evaluated to determine a normative ADC distribution within benign fluid attenuated inversion recovery (FLAIR) T2/hyperintensity surrounding tumor. This distribution was used to calculate a minimum ADC threshold to define regions of ADC-FLAIR mismatch (AFMM), where restricted diffusion presented in conjunction with T2/FLAIR hyperintensity. Contrast-enhancing voxels were excluded from this analysis. AFMM maps were generated using imaging acquired prior to death in 7 patients with high-grade glioma who eventually donated their brains upon death. Histological samples were taken from numerous regions of abnormal FLAIR and AFMM. Each sample was computationally processed to determine cell density. Custom software was then used to downsample coregistered microscopic histology to the more coarse MRI resolution. A voxel-by-voxel evaluation comparing ADC and cellularity was then performed.

Results. An ADC threshold of $0.929 \times 10^{-3}$ mm$^2$/s was calculated from meningioma-induced edema and was used to define AFMM. Regions of AFMM showed significantly greater cell density in 6 of 7 high-grade glioma cases compared with regions of hyperintense FLAIR alone ($P < .0001$). Two patients had small regions of diffusion-restricted necrosis that had significantly lower ADC than nearby hypercellularity.

Conclusions. Regions of AFMM contain hypercellularity except for regions with extremely restricted diffusion, where necrosis is present.

Keywords: ADC, brain tumor, diffusion, FLAIR, glioblastoma.

Glioblastoma multiforme (GBM) is the most common primary brain tumor in adults and is highly lethal. The current standard of care produced a median survival of 14.6 months when first described, with small improvements reported through aggressive protocols for treating recurrence in the ensuing years. Local recurrence following initial treatment is the most common form of relapse due to infiltrating neoplastic cells invisible to current contrast-enhanced imaging techniques. Because of this, fluid attenuated inversion recovery (FLAIR) or T2-weighted images have recently been included in imaging protocols for the evaluation of tumor progression. The criteria of the Response Assessment in Neuro-Oncology deem “significant increase in non-enhancing lesion” grounds for tumor progression. Nonenhancing lesion consists of regions of abnormal FLAIR or T2 hyperintensity, which generally indicates the presence of vasogenic edema or infiltrative glioma. Edema results from tumor breakdown of the endothelial junctions, allowing intravascular proteins and fluid to penetrate the cerebral extracellular space, often spreading down white matter tracts.

One of the troubling aspects of T2/FLAIR hyperintensity is the vast extent to which it often reaches beyond the T1-weighted...
contrast-enhancing tumor. In cases of invasive glioblastoma, malignant tumor cells have been found 4 cm away from contrast-enhancing tumors,9 and 96% of patients recur within 3 cm of their initial lesion following surgery.5,7 Furthermore, patients on the anti-vascular endothelial growth factor (VEGF) drug bevacizumab commonly exhibit disease progression in non-enhancing lesions rather than in areas of traditional enhancement, as the drug “seals” the blood–brain barrier and prevents contrast agent from leaking out of the capillary bed.8 It has also been suggested that the drug changes the tumor phenotype from angiogenic to mesenchymal.9,10 It is therefore imperative to contrast agent from leaking out of the capillary bed.8 It has

ment, as the drug “seals” the blood–brain barrier and prevents contrast agent from leaking out of the capillary bed.8 It has also been suggested that the drug changes the tumor phenotype from angiogenic to mesenchymal.9,10 It is therefore imperative to be able to monitor and detect tumor infiltration beyond the contrast-enhancing boundary.

MRI-based diffusion weighted imaging (DWI) has shown promise in neuro-oncology. DWI-derived apparent diffusion coefficient (ADC) measures the average diffusion of water molecules within each voxel. ADC has been shown to decrease in the presence of hypercellularity.11–19 Some debate remains as to whether or not ADC is predictive of tumor grade, as some studies have found a correlation,11,19–22 while others have found considerable overlap between tumor grades,18,23 or no correlation at all.24 Complicating the issue, ADC is sensitive to other tissue characteristics, including the presence of extracellular fluid due to vasogenic edema or tumor destruction of the extracellular matrix. It is therefore unclear how restricted diffusion need be to indicate “hypercellularity” and how to localize such regions outside of contrast-enhancing tumor.

This study accomplishes 2 objectives. First we examine cases of noninfiltrative meningioma to determine normal ADC values within vasogenic edema known to not contain an infiltrative tumor component. The ADC threshold from these patients is then used to define regions of significantly decreased ADC within FLAIR hyperintensity (ie, ADC-FLAIR mismatch [AFMM]) in 7 cases of high-grade glioma. Six of these patients underwent extensive treatment, while the seventh refused treatment. Following death, each patient was evaluated with ex vivo histology to precisely compare ADC and cell density voxel by voxel. We hypothesized that regions with ADC below the minimum ADC threshold and colocalized with T2/FLAIR hyperintensity would have increased cell density compared with regions with ADC above threshold and colocalized with T2/FLAIR hyperintensity.

### Materials and Methods

#### Study Participants

Eight patients presenting with World Health Organization (WHO) grade I meningioma with MRI scans acquired prior to surgical intervention were included retrospectively for AFMM calibration and ADC threshold definition. They had given written consent to be included in an imaging database approved by our institutional review board (IRB). Four of the meningiomas were confirmed pathologically as WHO grade I, and 4 were diagnosed radiographically and presumed to be WHO grade I lesions based on imaging findings. Seven patients with high-grade gliomas were included for the ex vivo portion of this IRB-approved study. A brief clinical history of each patient is included in Table 1.

#### Imaging

Each patient underwent routine clinical imaging at our institution using a 1.5T GE MRI scanner. The typical imaging sequence consisted of a conventional pre- and postcontrast T1-weighted acquisition with the following parameters: echo time (TE)/repetition time (TR) of 14 ms/500 ms, matrix of 512 × 512, voxel size of 0.43 × 0.43 mm, and flip angle of 90 degrees. Diffusion weighted images were also gathered, with a TE/TR of 102.2 ms/8000 ms, slice thickness of 5 mm, skip 1.5 mm, matrix of 256 × 256, field of view of 240 mm, and 2 diffusion weightings (b-values) of b = 0 and b = 1000 s/mm². ADC maps were then calculated from these images. Axial FLAIR imaging was also gathered, with an inversion time of 2200 ms, TE/TR of 125.2 ms/10 000 ms, slice thickness of 5 mm, skip 1.5 mm, flip angle of 90 degrees, matrix of 512 × 512, and voxel size of 0.43 × 0.43 mm. Finally, a high-resolution spoiled gradient recalled structural scan was typically acquired with a TE/TR of 3.16 ms/8.39 ms and 2 averages (number of excitations).

#### ADC Threshold Definition

ADC measures the physical quantity of water molecule diffusion magnitude in absolute units of square millimeters per second. This allowed us to determine an ADC cutoff of 2 standard deviations below the mean within meningioma-induced, benign vasogenic edema. Hyperintense T2/FLAIR regions of interest (ROIs)

### Table 1. Patient demographics and treatments for the 7 high-grade glioma patients included in this study

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Last MRI to Death, d</th>
<th>Samples Processed</th>
<th>Age at Death, y</th>
<th>Tumor Type</th>
<th>Surgical Resection</th>
<th>Therapy</th>
<th>Temozolomide Days from Final Dose</th>
<th>Bevacizumab</th>
<th>Diffusion-Restricted Necrosis</th>
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<td>5</td>
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<td>+</td>
<td>+/16</td>
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<tr>
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<td>F</td>
<td>10</td>
<td>9</td>
<td>39</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<td>7</td>
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<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
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</tr>
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</table>
were generated using a semiautomated thresholding technique followed by manual removal of non–tumor related hyperintensities. This was done for the 8 patients with meningiomas and the 7 ex vivo patients with gliomas. T1-weighted contrast-enhancing voxels were likewise contoured and excluded from all analyses for all patients. ADC values within the meningioma patients’ hyperintense T2/FLAIR ROIs were then measured and combined across patients. The means and SDs were calculated from the histogram distribution. Diffusion-restricted voxels were defined as those with ADC values more than 2 SDs below the mean.

For purposes of threshold verification, a second receiver operator curve (ROC) method was applied to the meningioma imaging. Regions of normal-appearing white matter were generated for all cases, and the ADC values within were compared with those within the T2/FLAIR hyperintensity. The maximal area under the curve (AUC) differentiating vasogenic edema from normal white matter was calculated from iteratively varying the ADC threshold for each patient. The ADC cutoff was defined as the mean of the optimized ADC threshold across patients.

Ex vivo Histology Processing

Large tissue samples (~4 cm²) were taken from each of the 7 high-grade glioma patients in regions suspicious of tumor and free from MR acquisition artifacts (for example, see Supplementary Fig. S2). Histological samples were paraffin embedded following formalin fixation and were hematoxylin and eosin (H&E) stained. To determine VEGF immunoreactivity of one sample containing diffusion-restricted necrosis, we additionally processed it with VEGF monoclonal antibody immunohistochemistry (IHC). Each slide was then photographed at 10× across the entire sample using a motorized microscope stage and Nikon Instruments software. Each sample typically had upward of 1500–2000 photos taken to cover the entire slide. Each photo was processed individually and then downsampled and stitched together with all other photos using custom software written in Matlab (MathWorks).

The histology segmentation process began with a white background correction, where the average of 20–40 photos taken from regions of empty slide (ie, no tissue in view) was calculated and subtracted from each image. This corrected for subtle edge effects of the microscope optics. The RBG photos were reduced to the red component alone, giving the best black and white contrast among the tissue types for the H&E stained slides. A contrast optimization was applied to best segment the images. A k-means clustering algorithm implemented in Matlab was then used to segment each photo. A representative segmentation is shown in the center of Fig. 1.

Precise Histology to MRI Correlation

Additional custom software (Supplementary Fig. S1, bottom) was developed in-house to precisely relate histology findings to the
MRI acquired prior to tissue excision using MatLab code. The software operates under 2 important assumptions. First, the precise location of the histological sample is known with respect to the MRI. To address this, digital photographs were taken at the time of brain cutting and sampling for anatomical comparison with clinical MRI. Second, there exists a slice that accurately represents the histology within the clinical MRI exam. During the brain slicing, careful attention was paid to the sagittal and coronal orientation of the brain to best slice brain tissue in the same axial plane as the most recent MRI.

Coregistration of histology to MRI was performed using a manually defined linear rotational and translational transformation applied to align each histology slide to the MRI. The location of each sample was matched visually to the MRI slice that best represented the sample’s location. This was determined using photographs of the brain slices acquired before and after each sample was taken. An example of this is shown in Supplementary Fig. S1, where anatomical landmarks (yellow arrows) in the photograph are visible in the corresponding MRI slice. Landmarks considered were chosen based on the relative distance to the samples, weighting those closest highest for aligning.

Regions of interest within each histology slide that were free from tissue-processing artifacts such as tears and folds were sampled for comparison with the MRI (Supplementary Fig. S1, red boxes). These regions were transformed to the MRI resolution using linear scaling based on the microscope field of view and the voxel dimensions. The number of samples coregistered for each patient is shown in Table 1.

Histology from within each ROI drawn using the HISToMRI toolbox was then downsampled to the MRI resolution for a direct 1-to-1 comparison (method shown in Fig. 1 and Supplementary Fig. S2). Histological segmentation values along with the MRI values within each voxel were then extracted and combined across all samples for visualization in scatter plots (Fig. 2). The binarized AFMM image (excluding T1 contrast enhancement) was included as well as a binarized FLAIR ROI. These ROIs were used to classify voxels (Figs 3 and 4).
To compare cell density between categories, a normalized cell density was calculated by dividing the percentage of space occupied by cell nuclei in regions of AFMM and FLAIR hyperintensity by the average percentage of space occupied by cell nuclei in regions of normal MRI. Resulting normalized cell density values from voxels of AFMM and FLAIR hyperintensity were compared with Student’s t-test. A strict P-value of <.003 was considered significant when Bonferroni-corrected for multiple comparisons.

Additional Analysis of Necrotic Regions

Microscopic analysis of the histology of patients 2 and 5 revealed regions of necrosis surrounded by viable tumor. Each case is shown, patient 5 in Fig. 4 and patient 2 in Supplementary Fig. S3. These 2 regions warranted further imaging analyses, as both appeared to be void of cellularity and were extremely diffusion restricted on ADC. Binarized ROIs were generated on the histology samples and upsampled to the MRI resolution (method shown in Supplementary Fig. S3, right). ADC values from voxels consisting of >50% upsampled necrosis were compared with nearby regions of confirmed hypercellularity and to a region of normal-appearing contralateral white matter. Voxel values from these 3 categories were compared with a 1-way ANOVA. For this analysis, Bonferroni-corrected P < .008 was considered significant.

Results

Vasogenic Edema ADC Threshold

Analysis of 8 meningiomas and the ADC values within regions of heightened FLAIR resulted in a mean ADC measure of $1.533 \times 10^{-3}$ mm$^2$/s with SD = $0.308 \times 10^{-3}$ mm$^2$/s. Based on these measures, the lower 95% cutoff was $0.929 \times 10^{-3}$ mm$^2$/s. The ROC analysis differentiating meningioma-induced edema from...
normal white matter produced a similar ADC cutoff of $0.947 \times 10^{-3} \text{ mm}^2/\text{s}$.

Figure 2 shows a scatter plot for each of the 7 ex vivo patients. The color of each dot indicates the normalized cell density in each voxel, while the axes are ADC and FLAIR voxel intensities. Bar charts summarize the findings in Fig. 3 (and Supplementary Fig. S5 for the ROC-derived ADC threshold), where 6 of the 7 patients had significantly greater normalized cell density in regions of AFMM versus regions of hyperintense FLAIR alone ($P < .0001$).

Analysis of the 2 necrotic regions revealed extreme diffusion restriction compared with regions of confirmed hypercellularity and normal white matter in both patients (ANOVA $P < .005$). Necrotic voxels had mean ADC values of 0.593 and 0.637 $\times 10^{-3} \text{ mm}^2/\text{s}$, which were lower than nearby hypercellular regions, which had mean ADC values of 0.643 and 0.786 $\times 10^{-3} \text{ mm}^2/\text{s}$ for patients 2 and 5, respectively (Fig. 5).

Discussion

This analysis precisely correlated MRI-derived ADC values to histology findings on a voxel-by-voxel basis in ex vivo human brain samples. We first found an ADC cutoff of $0.929 \times 10^{-3} \text{ mm}^2/\text{s}$ for defining AFMM (regions of hypercellularity in the presence of T2/FLAIR hyperintensity) in 8 patients with noninvasive brain tumors. This threshold was then applied to ADC images masked by hyperintense FLAIR ROIs excluding contrast enhancement in 7 cases of high-grade glioma. In 6 of the 7 cases, we found hypercellularity in AFMM compared with hyperintense FLAIR voxels alone. Two regions of AFMM were noted to contain necrosis upon microscopic evaluation. We found that these necrotic regions had significantly lower diffusion than regions of confirmed hypercellularity.

There is considerable disagreement in the field regarding diffusion restriction in brain cancer. Several recent studies found hypercellularity,\textsuperscript{11–19,25} while others found necrosis\textsuperscript{26–28} in regions of restricted diffusion measured with ADC. Studies have evaluated bevacizumab as a cause of these regions, and in our analysis, 3 of the 7 patients were undergoing bevacizumab treatment at the time of death, including both patients with diffusion-restricted necrotic regions. Our findings confirm both hypercellularity and necrosis within diffusion-restricted regions but suggest that the level of diffusion restriction is deterministic of histological status.

Gupta and colleagues\textsuperscript{29} found that diffusion restriction preceded the development of enhancing tumor in a subset of glioblastoma patients, independent of bevacizumab usage, while Mong et al\textsuperscript{16} found increased survival in patients showing stable diffusion restriction following bevacizumab. These seemingly conflicting results may be explained by the presence of both hypercellular and necrotic regions, as we found in patients 2 and 5. These studies suggest that bevacizumab therapy is the cause of diffusion-restricted necrotic regions, while diffusion restriction in the absence of bevacizumab indicates tumor hypercellularity, but further research is necessary.

Future studies should seek to characterize the histological makeup of the regions of atypical diffusion-restricted necrosis described in this study. In a previously presented abstract, we showed that diffusion-restricted necrotic regions expressed VEGF,\textsuperscript{30} likely due to extreme hypoxia (Supplementary Fig. S4). Extreme hypoxia has previously been reported in a region of restricted diffusion sampled in a GBM patient.\textsuperscript{28} Two case reports\textsuperscript{31,32} and 2 patients sampled with biopsy from larger studies\textsuperscript{26,28} have shown similar findings of necrosis, sometimes referred to as coagulative. This type of necrosis appears to be linked to bevacizumab, and indeed both of our patients with regions of it present were undergoing bevacizumab treatment at the time of death. These 2 regions of atypical necrosis very closely resemble histologically the tissue shown in each of these previously published reports.\textsuperscript{26,28,31,32} These cases therefore are the fifth and sixth pathologically confirmed reported in the literature. It should be noted that both of our patients had regions of hypercellularity nearby, indicating that the presence of both is possible (and likely in cases studied at autopsy, assuming cancer as the cause of death). This report is the first to show that coagulative necrotic regions have more restricted diffusion (ie, lower ADC values) associated with them compared with viable tumor. These 2 regions were also relatively small compared with the regions of viable tumor, which explains why cell density was still significantly greater across all AFMM voxels compared with T2/FLAIR hyperintensity alone (Fig. 3).

There are several potential confounding factors to this analysis. The orthogonal orientation of the MR acquisition, as well as the sampling thickness of the MRI itself, contributes to potential sources of error. Histology is generally sliced at 5–10 $\mu$m thicknesses, and high-resolution clinical MRI acquisitions are generally limited to 1 mm slice thickness. We calculated normalized cell density to correct for differences in the sampling thickness. Coregistering the histology and imaging was also a potential step that may have introduced errors. For this study, we used all tools available to us, including digital photographs taken at the autopsy, prior imaging studies, and anatomical landmarks, to ensure as precise a colocalization of the 2 modalities as possible. Future studies should evaluate individual variation in coregistration of the histology and MRI to determine whether operator bias is a substantial concern, as well as to determine whether nonlinear coregistration between the 2 modalities improves results.

Finally, the time between imaging and patient death is another factor that may have contributed a significant source of error to the analysis. Rapid tumor growth may have changed the histological features between the 2 time points. To correct for this we sampled at least 5 large regions of brain in regions suspicious of tumor. It is unlikely that all the tissue sampled changed substantially in-between the 2 time points, yet it is possible. We also excluded enhancing tumor, where rapid changes are most likely possible. Future studies, however, should look at imaging brain tissue ex vivo for comparison.

Conclusion

In conclusion, we found that voxels with hyperintense T2/FLAIR signal due to the presence of vasogenic edema or infiltrative glioma with ADC values $<0.929 \times 10^{-3} \text{ mm}^2/\text{s}$ contain significantly greater cellularity than regions of hyperintense T2/FLAIR alone. This ADC threshold was determined in patients with benign, noninvasive meningioma tumors as control subjects and applied to cases of high-grade glioma tumors. Upon closer examination,
we found coagulative necrosis in extremely diffusion-restricted regions, which has been previously reported in the literature. This information should help clinicians determine more accurately whether patients are responding to therapy or have tumors that are progressing.

Supplementary Material

Supplementary material is available at Neuro-Oncology Journal online (http://neuro-oncology.oxfordjournals.org/).

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Conflicts of interest statement. None declared.

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