Preoperative dynamic contrast-enhanced MRI correlates with molecular markers of hypoxia and vascularity in specific areas of intratumoral microenvironment and is predictive of patient outcome


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Background. Measures of tumor vascularity and hypoxia have been correlated with glioma grade and outcome. Dynamic contrast-enhanced (DCE) MRI can noninvasively map tumor blood flow, vascularity, and permeability. In this prospective observational cohort pilot study, preoperative imaging was correlated with molecular markers of hypoxia, vascularity, proliferation, and progression-free and overall patient survival.

Methods. Pharmacokinetic modeling methods were used to generate maps of tumor blood flow, extraction fraction, permeability-surface area product, transfer constant, washout rate, interstitial volume, blood volume, capillary transit time, and capillary heterogeneity from preoperative DCE-MRI data in human glioma patients. Tissue was obtained from areas of peritumoral edema, active tumor, hypoxic penumbra, and necrotic core and evaluated for vascularity, proliferation, and expression of hypoxia-regulated molecules. DCE-MRI parameter values were correlated with hypoxia-regulated protein expression at tissue sample sites.

Results. Patient survival correlated with DCE parameters in 2 cases: capillary heterogeneity in active tumor and interstitial volume in areas of peritumoral edema. Statistically significant correlations were observed between several DCE parameters and tissue markers. In addition, MIB-1 index was predictive of overall survival ($P = .044$) and correlated with vascular endothelial growth factor expression in hypoxic penumbra ($r = 0.7933$, $P = .0071$) and peritumoral edema ($r = 0.4546$). Increased microvessel density correlated with worse patient outcome ($P = .026$).

Conclusions. Our findings suggest that DCE-MRI may facilitate noninvasive preoperative predictions of areas of tumor with increased hypoxia and proliferation. Both imaging and hypoxia biomarkers are predictive of patient outcome. This has the potential to allow unprecedented prognostic decisions and to guide therapies to specific tumor areas.

Keywords: DCE-MRI, HIF-1, hypoxia, vascularity, VEGF..
grade of a glioma and provides important anatomical and diagnostic information, postoperative histological grade or tumor type is often different from that predicted by imaging alone. Dynamic contrast-enhanced (DCE) MRI is capable of quantitatively measuring tissue blood flow, vascularity, and parenchymal contrast uptake via kinetic modeling methods and has been used in the assessment of gliomas, especially GBM. These techniques have also been used to evaluate the oxygenation status of tumors. Molecular markers of hypoxia include the transcription factor hypoxia-inducible factor-1α (HIF-1α) as well as other hypoxia-regulated proteins such as vascular endothelial growth factor (VEGF), carbonic anhydrase IX (CA-IX), and glucose transporter-1 (GLUT-1). We and others have shown that there are correlations between brain tumor grade, vascularity, and HIF-1α expression based on a small series of brain tumors. Although others have reported hypoxia markers predictive of patient outcome in a number of tumor types, we have been unable to find any association of hypoxia biomarkers and patient outcome. It is possible that there is differential expression of these biomarkers in specific microenvironments within a given tumor. Thus, random sampling of a tumor might not reflect expression of these markers in a meaningful manner. We hypothesize that measurement of these hypoxia markers in specific, well-defined areas of the tumor may be more predictive of patient outcome. Furthermore, we hypothesize that preoperative imaging from these areas might also prove useful for predicting patient outcome noninvasively.

In this pilot study, presurgical imaging studies were directly correlated with tissue taken from specific areas of a given tumor. Using an intraoperative navigation system, tumor tissue was taken from 4 distinct tumor regions for analysis. These areas included presumed necrotic areas (NC, heterogeneous nonenhancing tumor core), presumed hypoxic penumbra (HP, enhancing area immediately surrounding areas of necrosis), active tumor (AT, nodular enhancing tissue at the outer edge of the tumor), and peritumoral edema (PE, nonenhancing area surrounding the tumor, which appears bright on T2-weighted imaging) (Fig. 1). Each of these areas was evaluated for the expression of hypoxia-regulated molecules as well as tumor vascularity and tumor proliferation (MIB-1 labeling index). Expression levels of the various markers in these samples were correlated with imaging biomarkers derived from DCE-MRI data from spatially colocated regions of interest, as well as with tumor behavior and patient outcome.

**Methods**

**Patient Selection and Enrollment**

Patients with a newly suspected malignant glioma, which was identified by MRI as having a single focus at least 2 cm in cross-sectional diameter and considered to be surgically resectable at the time of presentation, were approached about the study. After a thorough discussion, written consent was obtained from each patient for this University of Utah Institutional Review Board-approved protocol.

**Measures of Patient Outcome**

Complete resection was indicated by removal of all gadolinium-enhancing tumor tissue (or all tissue showing T2 signal abnormality for nonenhancing tumors). Progression was defined as the time at which management was first altered from that initially begun for the patient. For most cases, this
was when there was either unequivocal increase in fluid-attenuated inver-
sion recovery (FLAIR)/T2 signal abnormality or newly detected areas of con-
trast enhancement on follow-up MRI requiring further surgery, radiation, or
chemotherapy. Progression-free survival (PFS) was calculated from the
date of initial imaging to documented progression. Overall survival (OS)
was calculated from the date of initial imaging to documented death from
the Social Security Death Index. There was no central review of
imaging.

Preoperative Patient Imaging
Patients who satisfied all of the eligibility criteria underwent standard pre-
operative contrast-enhanced anatomic MRI and DCE-MRI. Up to 4 distinct
sites were identified for specific imaging analysis to provide resection targets: (i) presumed NC, (ii) presumed HP, (iii) AT, and (iv) PE. In some
tumors, such as homogeneously enhancing lesions or minimally enhancing
lesions, it was not possible to identify all 4 regions. All images were obtained
such that they could be loaded into the intraoperative navigation system, as
per protocol for any patient undergoing surgical resection (Fig. 2).

DCE-MRI Data Acquisition
T1-weighted DCE-MRI data were acquired as described previously. After
standard noncontrast imaging for preoperative planning was completed,
the lesion of interest was identified (Fig. 2), and maps of precontrast longitudi-
nal relaxation time (T10) were measured using the variable flip angle
spoiled gradient echo (SPGR) method with flip angles of 3° and 20°. DCE-MRI measurements were then performed using a fast 3-dimensional
SPGR sequence on a 1.5T Siemens TIM Avanto scanner or a 3T Siemens
TIM Trio and Verio scanners with the same field of view used for the variable
flip angle measurements. Pulse sequence parameters were chosen to
maximize sampling rate within constraints of adequate signal-to-noise
ratio and coverage of the lesion of interest. Because DCE-MRI data were
acquired as add-on measurements to clinically indicated stereotactic imaging prior to brain surgery, imaging protocols varied slightly to accom-
modate different scanner hardware and specific absorption rate limits. A
standard (0.1 mmol/kg) dose of low-molecular-weight gadolinium
chelate contrast agent (Omniscan, GE Healthcare or Multihance, Bracco
Diagnostics) was injected over 4 seconds through an 18–22 gauge intra-
venous catheter into the antecubital vein, followed by a 20 mL saline
flush, injected at 2 mL/s using a power injector (Medrad Spectris Solaris).
Contrast injection was timed to coincide with the end of the 10th frame
of dynamic data. Data were acquired at an isotropic spatial resolution of
2 mm for a total imaging time of at least 6 minutes with temporal reso-
lation of 5 seconds per frame or better. A 12-channel transmit/receive
head coil was used. Flip angle variation along the slab-encoding direction
was estimated using a homogeneous phantom of known T10, and, in 3
study patients, by assuming a constant T10 value of 240 milliseconds for
subcutaneous fat. These experiments indicated that the impact of flip
angle variation near the slab boundaries was small, except for roughly
the outer 10% of slices at each boundary. To minimize the potential for
bias in conversion of signal to contrast concentration, these slices were
excluded from our analysis. Precontrast signal intensity, S0(t), was deter-
moved by averaging the 10 baseline images, and signal-to-noise ratio
was computed from the ratio of the baseline signal to its standard
deviation. Time curves of relative enhancement were generated from the
time-dependent DCE-MRI signal, S(t), as S(t) = S(t) − S0(t)/S0(t). Tissue
concentration-time curves, C(t), were then computed by numerical solu-
tion of the full nonlinear concentration dependence of the SPGR signal in
the fast exchange limit, with voxelwise tissue T10 values and flip angle cor-
corrected for slab profile effects. Literature values for the r1 and r2 relaxivities,
appropriate to the administered contrast agent at the imaging field
strength, were used. Concentration measurement uncertainties were
computed as described by Schabel and Parker. The direct effect of T10
on concentration measurements was eliminated by the use of relative en-
hancement; the very short echo times used in imaging should result in
minimal susceptibility-induced signal loss. A T10 value of 50 milliseconds
was assumed in calculations of contrast concentration uncertainty.

DCE-MRI Pharmacokinetic Modeling
Measured concentration-time curves, representing tissue uptake of contrast
tag as determined from the DCE-MRI data, were fit using nonlinear
regression modeling to the Gasmic Capillary Transit Time (GCTT) model,
which is a distributed parameter model that incorporates both contrast
capillary transit through the tumor microvasculature and uptake by the tumor par-
enchyma. In addition to our prior studies in gliomas, this model has shown
utility in analysis of tumors in a rat model. Applying the GCTT model to
our data provided spatial maps of imaging biomarkers including
tumor blood flow (F), extraction fraction (E), permeability–surface area
product (PS), transfer constant (ktrans), washout rate (kout), interstitial
volume (Vd), blood volume (Vb), capillary transit time (tc), and capillary het-
erogeneity (α−1). Mean parameter values were calculated by averaging
these maps over spherical regions of interest 5 mm in diameter (roughly
corresponding to the volume of sampled tissue) that were manually core-
gistered with sampling sites reported by the MRI-guided intraoperative
navigation system.

Tumor Resection, Tissue Procurement, and Patient
Treatment
Surgery was performed within 24 hours of the imaging studies. Stereotactic
post-contrast MRI images were loaded on the intraoperative navigation system
and used to select the desired regions of tumor (Fig. 2). Tumor was
resected by the senior author with the intention of gross total resection
before any “brain shift” could occur from cerebrospinal fluid removal or
brain relaxation. Only tumors 2 cm in diameter or larger were included in
this study to ensure that the small amount of tumor used as part of this
study would not interfere with the ability of the neuropathologist to make
an accurate diagnosis. The majority of the tumor was sent for standard pathologi-
al examination and tumor grade determination according to the
WHO classification system. Remaining tissue was immediately snap
frozen and stored in liquid nitrogen until time of analysis.

All patients were treated with radiation and chemotherapy based on their
specific diagnosis under the guidance of neuro-oncologists. Patients under-
went routine blood work and MRI every 2 months per standard-of-care
guidelines and as needed. The specific changes between scans were quanti-
ﬁed and recorded. If a subsequent operation was necessary at any time
during the study, further tissue analysis was conducted in a manner
similar to the initial procedure.

HIF-1α, VEGF, CA-IX, and GLUT-1
HIF-1α immunohistochemistry was performed as previously described
using the Catalyzed Signal Amplification System (DAKO) according to the
manufacturer’s recommended protocol and primary antibody H1a67 (Novus
Biologica) at a dilution of 1:1000. VEGF, CA-IX, and GLUT-1 immu-
nohistochemistry was done using anti-VEGF Ab-1 polyclonal antibody (1:50
dilution; Calbiochem), anti-CA-IX goat polyclonal antibody (1:200; Santa
Cruz Biotechnology), or rabbit anti-GLUT-1 (1:100, Santa Cruz Biotechnol-
ogy), and the Vectastain ABC kit (Vector Laboratories) as previously
described. Slides were counterstained with toluidine blue. Negative con-
trast replaced the primary antibody with nonimmune serum, with all
other steps performed as above. Positive controls for HIF-1α, VEGF, CA-IX, and GLUT-1 were developed on paraffin-fixed sections of tumors grown in mice using human U251 cell lines that were immunohistochemically positive for these proteins using the same steps as above.9

All slides were examined under 200× magnification using an Olympus BX41 microscope and scored by an investigator blinded to the patient information and tumor grade. The immunohistochemical staining results of HIF-1α, VEGF, CA-IX, and GLUT-1 were scored from zero to 4 (0, 0% to

Fig. 2. Coronal (top row), sagittal (second row), and axial (third row) gadolinium-enhanced images from a patient with GBM representing regions of interest assayed for hypoxia biomarkers. (AT) active tumor; (HP) hypoxic penumbra; (NC) necrotic core; (PE) peritumoral edema. Plots in the bottom row show the measured median DCE-MRI relative enhancement corresponding to each sampled tumor site, the GCTT model regression to these data, and the curve fit residuals.
<25%; 1, 25% to <50%; 2, 50% to <75%; 3, 75% to <100%; and 4, 100%) based on the number of cells stained in a given field.

**Proliferation Index**

The proliferation index (PI) was calculated using Ki-67 (clone MIB-1, dilution 1:300) on the Ventana ES (Ventana Medical Systems) as previously described. Positive controls were performed on human thymus, which has >90% cell staining. Negative controls replaced the primary antibody with nonimmune serum. PI was calculated as previously described.

Briefly, pictures were taken at 400× (10 ocular × 40 objective) magnification using an Olympus Microfire camera and analyzed using Image-Pro Plus 5.0. PI was calculated as the number of MIB-1-stained cells divided by the total number of cells in the field repeated 3 times for each picture and averaged, as done in our prior studies.

**Microvascular Density Index**

The slides for the microvascular density (MVD) index analysis were prepared using the same steps as described above for the MIB-1 analysis, except that they were pretreated with Factor VIII (rabbit polyclonal, dilution 1:100) Protease 2 (Ventana Medical Systems). Negative controls replaced the primary antibody with nonimmune serum, with all other steps performed as above.

The MVD index was calculated based on a previously published method. Briefly, 3 pictures of the most vascular area of the slide were taken at 200× magnification using an Olympus Microfire camera and transferred to Photoshop CS 7 (Adobe Systems Incorporated). Any positive cell that was separate from other stained cells and not contiguous or branching from other vessels was counted. The results for each slide were averaged for the resulting MVD and divided by 0.26 mm² to normalize the size of the picture field.

**Tumor Protein Isolation**

Tumor samples were chopped, and tissue was homogenized with a Polytron homogenizer in 3 mL of digest buffer containing 10 mM HEPES (pH 7.6), 0.1 mM ethylene glycol tetraacetic acid, 2 mM dithiobis (DTT), 0.4 mM phenylmethysulfonyl fluoride, 1 mM NaVO₃, 1× protease inhibitor cocktail (Sigma), 100 mM NaF, and 10 mM Na₃P₂O₇. The homogenate was centrifuged at 580 relative centrifugal force (RCF) for 5 minutes at 4 °C. The supernatant was transferred to a new tube with glycerol added for final concentration of 5%, then vortexed and centrifuged at 15 000 RCF for 15 minutes. The pellet was resuspended in 300 μL of lysis buffer (400 mM NaCl, 20 mM HEPES at pH 7.5, 10 mM NaF, 10 mM p-nitrophenyolphosphate, 1 mM NaVO₃, 0.1 mM EDTA, 10 μM Na₃P₂O₇, 10 mM β-glycerophosphate, 20% glycerol, 1× protease inhibitor cocktail, 1 mM DTT) and shaken gently at 4 °C for 30 minutes, then centrifuged at 15 000 RCF at 4 °C for 30 minutes and stored at ~80 °C until ready for analysis.

**HIF and VEGF Immunoassay**

The Total HIF-1 Whole Cell Assay and Multi-Array Human VEGF assay kits from MSD were used according to provided instructions. These antibody-based assays use electrochemiluminescent detection for increased sensitivity over chemical-based ELISAs, allowing us to analyze minimal amounts of patient samples. In an MSD assay, specific capture antibodies for the analytes are coated in arrays in each well of a 96-well carbon electrode plate surface. The detection system uses patented SULFO-TAG labels (Meso Scale Discovery) that emit light upon electrochemical stimulation initiated at the electrode surfaces of the plates. This stimulation is decoupled from the output signal, which is light, to generate assays with minimal background. When possible, samples were analyzed in triplicate at 5, 10, and 20 μg total protein. For VEGF, a standard curve was used to calculate total VEGF concentration. VEGF values are expressed as μg of VEGF per μg of total protein. Total HIF-1 was determined on a relative scale based on a curve of CoCl₂-treated whole cell extracts. HIF-1 values are expressed as relative units without absolute values. Total protein was determined for normalizing samples between experiments.

**Statistical Methods and Data Analysis**

Descriptive statistics (means, medians, range) were used to quantify the imaging results, molecular markers, tumor characteristics, and tumor grade. Kaplan–Meier survival curves were plotted and stratified by levels of the putative prognostic factors to determine whether the molecular markers and imaging test results were associated with progression-free and overall survival. Log-rank tests were used to determine differences between groups. Relationships between molecular and imaging parameters were assessed using Spearman correlations (termed r) calculated to 3 decimal points. Because of the exploratory nature of this study, we did not adjust for multiple comparisons. Statistical analyses were done using Prism 6.0 software (Graph Pad Software). Two-sided P-values were calculated with the significance level set at .05.

**Results**

**Patient Data and Tumor Characteristics**

All of the 18 participants had received a preliminary diagnosis of malignant glioma at the time of enrollment in the study. After surgery, histological diagnosis revealed that 12 of the participants’ tumors were GBM, 2 were anaplastic oligodendrogliomas (AO), and 1 each was anaplastic astrocytoma, low-grade glioma, meningioma, and pleomorphic xanthoastrocytoma (PXA) (Table 1). Biomarker and imaging parameters were analyzed for all participants; however, imaging and biomarker correlation with PFS and OS were

**Table 1. Patient characteristics**

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age (yrs. sex)</th>
<th>Diagnosis</th>
<th>Patient Status</th>
<th>Overall Survival (weeks)</th>
<th>Progression-free Survival (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>44, M</td>
<td>AA</td>
<td>Dead</td>
<td>232</td>
<td>133</td>
</tr>
<tr>
<td>2</td>
<td>70, F</td>
<td>AO</td>
<td>Alive</td>
<td>274</td>
<td>274</td>
</tr>
<tr>
<td>3</td>
<td>60, M</td>
<td>AO</td>
<td>Alive</td>
<td>183</td>
<td>167</td>
</tr>
<tr>
<td>4</td>
<td>69, M</td>
<td>GBM</td>
<td>Dead</td>
<td>40</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>79, F</td>
<td>GBM</td>
<td>Dead</td>
<td>22</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>75, M</td>
<td>GBM</td>
<td>Dead</td>
<td>82</td>
<td>51</td>
</tr>
<tr>
<td>7</td>
<td>51, M</td>
<td>GBM</td>
<td>Dead</td>
<td>45</td>
<td>17</td>
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<tr>
<td>8</td>
<td>73, M</td>
<td>GBM</td>
<td>Dead</td>
<td>3</td>
<td>3</td>
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<tr>
<td>9</td>
<td>19, M</td>
<td>GBM</td>
<td>Dead</td>
<td>101</td>
<td>20</td>
</tr>
<tr>
<td>10</td>
<td>65, M</td>
<td>GBM</td>
<td>Dead</td>
<td>43</td>
<td>15</td>
</tr>
<tr>
<td>11</td>
<td>43, F</td>
<td>GBM</td>
<td>Dead</td>
<td>83</td>
<td>20</td>
</tr>
<tr>
<td>12</td>
<td>49, F</td>
<td>GBM</td>
<td>Dead</td>
<td>126</td>
<td>16</td>
</tr>
<tr>
<td>13</td>
<td>52, M</td>
<td>GBM</td>
<td>Dead</td>
<td>111</td>
<td>26</td>
</tr>
<tr>
<td>14</td>
<td>63, M</td>
<td>GBM</td>
<td>Alive</td>
<td>162</td>
<td>84</td>
</tr>
<tr>
<td>15</td>
<td>59, F</td>
<td>GBM</td>
<td>Dead</td>
<td>77</td>
<td>12</td>
</tr>
<tr>
<td>16</td>
<td>27, M</td>
<td>LGA</td>
<td>Dead</td>
<td>279</td>
<td>90</td>
</tr>
<tr>
<td>17</td>
<td>70, F</td>
<td>Men</td>
<td>Alive</td>
<td>324</td>
<td>324</td>
</tr>
<tr>
<td>18</td>
<td>22, F</td>
<td>PXA</td>
<td>Alive</td>
<td>261</td>
<td>261</td>
</tr>
</tbody>
</table>

Abbreviations: GBM, glioblastoma multiforme; LGA, low-grade astrocytoma; Men, meningioma; PXA, pleomorphic xanthoastrocytoma.
only performed for the malignant gliomas (GBM, AO, and AA). The mean age of the participants was 55 ± 18 years (range, 19–79 years) with mean of 58 ± 16 years (range, 19–79 years) for high-grade tumors and 40 ± 26 years (range, 22–70 years) for low-grade tumors. Gross total resection was the surgical goal and was achieved in most participants. Only 3 of the participants with malignant gliomas were alive at the end of this study. The 2 AO participants were 274 and 183 weeks from diagnosis, and 1 participant with GBM was 162 weeks from diagnosis at the end of the study. One AO participant has not experienced tumor progression, while the other 2 have both required further treatment after tumor growth. No participants were lost to follow up.

The median length of follow-up was 83 weeks (range, 3–274 weeks) for high-grade tumors, 270 weeks for low-grade tumors (range, 90–324 weeks), and 106 weeks (range, 3–324 weeks) for the entire study population. The median PFS for all high-grade tumor participants was 20 weeks (16 weeks for GBM; 167 weeks for AA/AO). The median OS for the entire study population was 106 weeks (80 weeks for GBM, 232 weeks for AA/AO, 83 weeks for all high-grade tumors, 279 for low-grade tumors). For the survival analysis and comparison with imaging parameters, only the malignant glioma data were used.

**Tissue Biomarkers and Overall Survival**

Immunohistological examination of VEGF, CA-IX, HIF-1, and GLUT-1 on paraffin-embedded tumor tissue, taken from participants enrolled in this study, did not yield any predictive survival data. This was similar to our prior experience.9 This was one of the motivations of this study because we had hypothesized that specific tumor areas might yield more accurate expression data for these hypoxia biomarkers and overall survival.

We found that gross tumor immunohistological staining results for VEGF, CA-IX, HIF-1 and GLUT-1 were not associated with predictions of PFS or OS, which was similar to our prior studies. We did, however, find that regional expression of VEGF and HIF-1 in the specific area of AT was predictive of OS (Table 2). Measurement of VEGF expression <1000 μg/μg total protein was associated with longer survival (P = .045, Fig. 3A) compared with tumors with >1000 μg/μg total protein of VEGF within AT regions. HIF-1 expression had a similar association in areas of AT, with lower expression of HIF-1 being predictive of better outcome than higher expression, HIF-1 relative units greater or lesser than 10 000 (P = .0215, Fig. 3B). VEGF and HIF expression in areas of PE, NC, and HP were not predictive of survival. Not all tumors had defined necrotic areas, and areas of HP were also difficult to determine in some participants. Not surprisingly, expression of HIF-1 and VEGF was correlated in AT (r = 0.764, P = .003), HP (r = 0.860, P = .013), and PE (r = 0.622, P = .041) but not in areas of NC (r = 0.085, P = .92).

MIB-1 index was calculated from random areas on paraffin-fixed tissue taken from the whole tumor and not tumor-specific areas. This index was found to be predictive of OS, as MIB-1 index >10 was correlated with worse outcome (P = .044, Supplemental Figure 1A, Table 3). MIB-1 index is correlated with VEGF expression in HP (r = 0.964, P = .0028) but not NC or AT. MIB-1 index was not associated with HIF-1 expression in any of the areas studied. Microvascular density (MVD) was measured on paraffin sections of the whole cross-sectional area of tumor and not specific tumor regions. We found that higher MVD was also correlated with worse participant outcome (P = .026, Supplemental Figure 1B, Table 3). We did not find any correlations between MVD and expression of either HIF-1 or VEGF in any tumor areas studied.

**Tissue Biomarkers and Progression-free Survival**

Immunohistological examination of VEGF, CA-IX, HIF-1, and GLUT-1 on paraffin-embedded tumor tissue also did not yield any predictive PFS data. In contrast, we found that expression of VEGF in PE and HIF-1 expression in AT areas was predictive of PFS (Table 2). Lower VEGF expression was found to be associated with longer PFS (P = .034, Fig. 3C) in areas of PE. In a similar fashion, lower HIF-1 expression in both AT (P = .044, Fig. 3D) and PE (P = .044, Fig. 3E) regions was associated with longer PFS. VEGF expression was not predictive in areas of AT (P = .17). Expression levels of these biomarkers in areas of NC and HP were also not helpful in predicting outcome in the smaller number of samples from these regions. MIB-1 index and MVD were calculated on paraffin-fixed tissue as described above and found not to be predictive of PFS (Table 3).

**Imaging Biomarkers and Participant Outcomes**

Tumor blood flow (F), extraction fraction (E), permeability-surface area product (PS), transfer constant (Ktrans), washout rate (kexp), and extraction fraction (E) were measured in tumor regions of interest in both AT and PE. Ktrans and kexp were calculated using the Tofts model. Perfusion-weighted imaging (PWI) abnormalities were measured in areas of PE and AT. MIB-1 index was correlated with VEGF expression in HP (r = 0.964, P = .0028) but not NC or AT. MIB-1 index was not associated with HIF-1 expression in any of the areas studied. Microvascular density (MVD) was measured on paraffin sections of the whole cross-sectional area of tumor and not specific tumor regions. We found that higher MVD was also correlated with worse participant outcome (P = .026, Supplemental Figure 1B, Table 3). We did not find any correlations between MVD and expression of either HIF-1 or VEGF in any tumor areas studied.

**Table 2. Overall survival and progression-free survival in relationship to HIF and VEGF expression in active tumor and peritumoral edema**

<table>
<thead>
<tr>
<th></th>
<th>HIF-1 &lt;10 000</th>
<th>HIF-1 &gt;10 000</th>
<th>VEGF &lt;1 000</th>
<th>VEGF &gt;1 000</th>
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</thead>
<tbody>
<tr>
<td><strong>Active tumor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median overall survival (weeks)</td>
<td>145.4</td>
<td>81.8</td>
<td>145.4</td>
<td>82.4</td>
</tr>
<tr>
<td>Hazard ratio, 95% CI; P value</td>
<td>4.99, 1.07–23.21; P = .041</td>
<td>90.43</td>
<td>24.29</td>
<td>90.43</td>
</tr>
<tr>
<td>Median PFS (weeks)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hazard ratio, 95% CI; P value</td>
<td>0.271, 0.076–0.965; P = .044</td>
<td>0.271, 0.076–0.965; P = .044</td>
<td>0.46, 0.160–1.373; P = .1671</td>
<td></td>
</tr>
<tr>
<td><strong>Peritumoral edema</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median OS (weeks)</td>
<td>145.4</td>
<td>91.9</td>
<td>145.4</td>
<td>81.6</td>
</tr>
<tr>
<td>Hazard ratio, 95% CI; P value</td>
<td>2.18, 0.546–8.68; P = .041</td>
<td>90.43</td>
<td>24.29</td>
<td>100.2</td>
</tr>
<tr>
<td>Median PFS (weeks)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Hazard ratio, 95% CI; P value</td>
<td>0.271, 0.076–0.965; P = .044</td>
<td>0.271, 0.076–0.965; P = .044</td>
<td>0.287, 0.090–0.9123; P = .034</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: HIF-1, hypoxia-inducible factor-1; OS, overall survival; PFS, progression-free survival; VEGF, vascular endothelial growth factor.
interstitial volume ($V_e$), blood volume ($V_b$), capillary transit time ($t_c$), and capillary heterogeneity ($a_2^1$) were calculated for each of the tumor areas using preoperative DCE-MRI data from the human glioma participants. We found that 2 of the imaging parameters were correlated with participant OS: interstitial volume ($V_e$) in areas of PE ($P = .0073$, Fig. 4A) and capillary heterogeneity ($a_2^1$) in areas of AT ($P = .014$, Fig. 4B, Table 4). The other imaging markers were not helpful for predicting OS. When these same parameters were used to predict tumor progression (PFS), we found that capillary transit time ($t_c$) in areas of PE ($P = .020$, Fig. 4C), blood volume ($V_b$) ($P = .011$, Fig. 4D), and capillary heterogeneity ($a_2^1$) ($P = .019$, Fig. 4E) in areas of AT were correlated with increased PFS. Similarly, in areas of AT, blood volume ($V_b$) correlated with HIF-1 ($r = .747$, $P = .043$) and VEGF ($r = .604$, $P = .032$) expression. Capillary heterogeneity ($a_2^1$) correlated with VEGF ($r = .599$, $P = .034$) expression in AT, and PE ($r = .973$, $P < .0001$). $k_{\text{es}}$, which measures washout rate, correlated with VEGF expression in PE ($r = .761$, $P = .0065$) and NC ($P = .931$, $P = .007$). Capillary transit time ($t_c$) correlated with expression of VEGF ($r = .100$, $P = .003$) and HIF-1 ($r = .761$, $P = .023$) in HP and HIF-1 in PE ($r = .697$, $P = .031$). MIB-1 index also correlated with $V_b$ ($r = .654$, $P = .018$).

**Discussion**

In this study, we obtained preoperative anatomic, contrast-enhanced, and DCE-MRI data on participants with suspected gliomas in a prospective clinical trial. Participants then underwent frameless stereotactic image-guided resection of the tumor with sampling of specific areas of the tumor. These tumor regions were selected based on the preoperative imaging. Areas selected were the nonenhancing central tumor areas thought to be consistent with necrosis and the enhancing tumor immediately adjacent to the nonenhancing areas.
to the presumed necrotic areas that we presumed were hypoxic areas. Furthermore, we sampled the enhancing tumor on the growing edge of tumor adjacent to “normal” brain that we termed “active tumor.” Finally, we sampled the increased T2 and FLAIR signal abnormality surrounding the glioma, which we thought represented areas of PE.

One limitation of this study was the difficulty of identifying these different areas with certainty given the close proximity of these regions within a given tumor. This was even more problematic in tumors with little necrosis or limited areas of edema and in non-enhancing tumors. All efforts were made to sample the tumor areas of interest before any “brain shift” could occur from cerebrospinal fluid removal or brain relaxation. Nevertheless, we do acknowledge that some shift is possible, and we have plans to perform similar studies using our intraoperative magnetic resonance (MR) system to reduce sampling errors. The tissue was then analyzed histologically and measured for the hypoxia-regulated proteins HIF-1 and VEGF, microvascularity, and proliferation. Recently, another group of investigators has taken a similar strategy of using image guidance to sample nonenhancing and enhancing portions of tumors with the intent of correlating the preoperative imaging with histopathological features. They found that diffusion-weighted MRI correlated with histological features of GBM within the noncontrast-enhancing regions, whereas analysis of empirical parameters derived from DSC-MRI measurements was correlated with contrast-enhanced areas. In contrast to our study, they did not examine the correlation of preoperative imaging or histological features and participant outcomes. Furthermore, this prior study used qualitative parameters derived from DSC-MRI in contrast to the quantitative parameters derived from pharmacokinetic modeling of DCE-MRI measurements that we report here.

**Table 4.** Overall survival and progression free survival in relation to DCE-MRI parameters in active tumor and peritumoral edema

<table>
<thead>
<tr>
<th>PE Ve &lt; 0.02</th>
<th>PE Ve &gt; 0.02</th>
<th>PE t&lt; 2</th>
<th>PE t&gt; 2</th>
<th>AT Vb &lt; 0.02</th>
<th>AT Vb &gt; 0.02</th>
<th>AT α&lt; 1</th>
<th>AT α&lt; 1 &gt; 0.85</th>
<th>AT α&lt; 1 &lt; 0.85</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median OS (weeks)</td>
<td>63.14</td>
<td>133.3</td>
<td></td>
<td></td>
<td></td>
<td>133.3</td>
<td>63.14</td>
<td></td>
</tr>
<tr>
<td>Hazard ratio, 95% CI, p value</td>
<td>4.04, 2.57 - 42.80</td>
<td>P = .0073</td>
<td></td>
<td></td>
<td></td>
<td>0.280, 0.037 - 0.563, P = .014</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median PFS (weeks)</td>
<td>83.57</td>
<td>17.14</td>
<td>37.86</td>
<td>111.6</td>
<td>34.29</td>
<td>111.6</td>
<td>34.29</td>
<td>111.6</td>
</tr>
<tr>
<td>Hazard ratio, 95% CI, p value</td>
<td>0.142, 0.027 - 0.733; P = .020</td>
<td>0.307, 0.0348 - 0.502; P = .011</td>
<td>0.316, 0.0461 - 0.653; P = .19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: AT, active tumor; OS, overall survival; PE, peritumoral edema; PFS, progression-free survival; Tc, capillary transit time; Vb, blood volume; Ve, interstitial volume; α< 1 , capillary heterogeneity.

**Fig. 4.** Measures of DCE correlated with patient survival. (A) Kaplan-Meier analysis of interstitial volume (Ve) in areas of PE demonstrates significant survival advantages for patients with Ve parameters > 0.02 (P = .0073). (B) Similar analysis of capillary heterogeneity (α< 1 ) in areas of AT shows that values < 0.85 were correlated with better patient OS (P = .014). When these same parameters were used to predict tumor progression (PFS), we found that capillary transit time (Tc) in areas of PE > 2 (P = .020) and blood volume (Vb) < 0.02 (P = .011) (D) and capillary heterogeneity (α< 1 ) < 0.85 (P = .019) (E) in areas of AT correlated with increased PFS.

**Tissue Biomarker and Participant Outcome**

We found that lower expression of VEGF and HIF-1 in areas we termed “AT” was predictive of longer OS. HIF-1 expression was also predictive of PFS in AT, but VEGF expression was not helpful.
Table 5. Correlation of significant dynamic contrast-enhanced parameters and hypoxia-inducible factor-1/vascular endothelial growth factor expression

<table>
<thead>
<tr>
<th>DCE Parameter</th>
<th>Active Tumor</th>
<th>Hypoxic Penumbra</th>
<th>Necrotic Core</th>
<th>Peritumoral Edema</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIF-1</td>
<td>VEGF</td>
<td>HIF-1</td>
<td>VEGF</td>
</tr>
<tr>
<td>$t_c$</td>
<td>$r = 0.659$</td>
<td>$r = 0.588$</td>
<td>$r = 0.761$</td>
<td>$r = 1.00$</td>
</tr>
<tr>
<td>P</td>
<td>$P = .017$</td>
<td>$P = .034$</td>
<td>$P = .023$</td>
<td>$P = .003$</td>
</tr>
<tr>
<td>$V_0$</td>
<td>$r = 0.747$</td>
<td>$r = 0.604$</td>
<td>$r = 0.500$</td>
<td>$r = 0.543$</td>
</tr>
<tr>
<td>P</td>
<td>$P = .043$</td>
<td>$P = .032$</td>
<td>$P = .27$</td>
<td>$P = .30$</td>
</tr>
<tr>
<td>$\alpha^{-1}$</td>
<td>$r = 0.520$</td>
<td>$r = 0.599$</td>
<td>$r = 0.152$</td>
<td>$r = 0.698$</td>
</tr>
<tr>
<td>P</td>
<td>$P = .072$</td>
<td>$P = .034$</td>
<td>$P = .83$</td>
<td>$P = .17$</td>
</tr>
<tr>
<td>$k_{ep}$</td>
<td>$r = 0.176$</td>
<td>$r = 0.192$</td>
<td>$r = 0.029$</td>
<td>$r = 0.771$</td>
</tr>
<tr>
<td>P</td>
<td>$P = .566$</td>
<td>$P = .53$</td>
<td>$P = .99$</td>
<td>$P = 1.03$</td>
</tr>
<tr>
<td>PS</td>
<td>$r = 0.105$</td>
<td>$r = 0.033$</td>
<td>$r = 0.029$</td>
<td>$r = 0.058$</td>
</tr>
<tr>
<td>P</td>
<td>$P = .73$</td>
<td>$P = .92$</td>
<td>$P = .98$</td>
<td>$P = .861$</td>
</tr>
<tr>
<td>$k_{trans}$</td>
<td>$r = 0.085$</td>
<td>$r = 0.035$</td>
<td>$r = 0.200$</td>
<td>$r = 0.100$</td>
</tr>
<tr>
<td>P</td>
<td>$P = .78$</td>
<td>$P = .90$</td>
<td>$P = .78$</td>
<td>$P = .95$</td>
</tr>
<tr>
<td>$V_e$</td>
<td>$r = 0.170$</td>
<td>$r = 0.071$</td>
<td>$r = 0.314$</td>
<td>$r = 0.771$</td>
</tr>
<tr>
<td>P</td>
<td>$P = .58$</td>
<td>$P = .82$</td>
<td>$P = .56$</td>
<td>$P = .10$</td>
</tr>
<tr>
<td>$E$</td>
<td>$r = 0.280$</td>
<td>$r = 0.412$</td>
<td>$r = 0.143$</td>
<td>$r = 0.029$</td>
</tr>
<tr>
<td>P</td>
<td>$P = .35$</td>
<td>$P = .16$</td>
<td>$P = .803$</td>
<td>$P = .99$</td>
</tr>
<tr>
<td>$F$</td>
<td>$r = 0.324$</td>
<td>$r = 0.291$</td>
<td>$r = 0.257$</td>
<td>$r = 0.200$</td>
</tr>
<tr>
<td>P</td>
<td>$P = .28$</td>
<td>$P = .33$</td>
<td>$P = .658$</td>
<td>$P = .71$</td>
</tr>
</tbody>
</table>

Abbreviations: DCE, dynamic contrast-enhanced; HIF-1, hypoxia-inducible factor-1; VEGF, vascular endothelial growth factor.

Instead, expression of VEGF and HIF-1 in PE was predictive of PFS. Since VEGF is a secreted protein under the transcriptional control of HIF-1 and is thought to be the major protein responsible for the development of PE, this is not completely unexpected.\(^\text{39,40}\)

We were not able to demonstrate utility of these biomarkers in the other tumor areas studied. Whether this is a result of sampling errors or the tumor microenvironment is not definitively demonstrated in this work. In our previous attempts to find a correlation of hypoxia-regulated protein expression and high-grade glioma patient outcome, we were unsuccessful.\(^\text{9}\) This prior study involved a larger patient population but was done on archived tissue in nonspecific tumor areas. We felt that the failure of that study was the nontargeted, nonspecific sampling that could be overcome by this prospective tumor regional sampling technique. The fact that only the AT and, to a more limited extent, the PE regions were predictive most likely reflects that these areas, especially AT, are most representative of the tumor’s true nature and future behavior. Furthermore, since areas of PE may contain infiltrative tumor cells, the measurements in PE regions may be questionable.

MIB-1 index has been proven many times to correlate with tumor grade.\(^\text{41,42}\) That MIB-1 index was found to be predictive of OS is no surprise; however, it was not predictive of PFS. Others have not found MIB-1 useful for predicting GBM patient survival.\(^\text{43}\) MIB-1 index is correlated with VEGF expression in HP and PE. Similar findings have been demonstrated in a more general sense with MIB-1 index correlating with diffusion tensor and DSC-MRI in patients with GBM.\(^\text{44,45}\)

MVD was also found to correlate with patient OS but not PFS. Similar findings have also been reported by Leon et al, who found that MVD had a significant inverse correlation with postoperative survival in patients with astrocytic supratentorial brain tumors.\(^\text{46}\) Their results supported the importance of MVD as a prognostic indicator of postoperative survival in patients with astroglial brain tumors. This is further maintained by work of Yao et al, in which both low-grade astrocytomas and GBMs had lower mean survival times when correlated with increased MVDs.\(^\text{47}\)

**Imaging Biomarker and Patient Outcome**

MRI techniques have the potential to preoperatively image tumor necrosis, hypoxia, blood flow, and vascular perfusion. There are early reports of the use of MR with diffusion tensor imaging\(^\text{48}\) and apparent diffusion coefficient\(^\text{49}\) to predict tumor grade and outcome in patients with malignant gliomas. DCE-MRI has been used to evaluate the oxygenation status of tumors.\(^\text{20–22}\) In addition, these imaging techniques have been used extensively to evaluate tumor grade and tumor progression as well as to differentiate tumor progression from treatment effects.\(^\text{52,53–56}\) Both DSC-MRI and DCE-MRI have been used in the assessment of glial neoplasms\(^\text{16,17}\) including more recently in response to bevacizumab therapy.\(^\text{53}\) Both of these perfusion imaging techniques have their merits and demerits, but DCE seems to offer better results than DSC in imaging those lesions that have a significant disruption of the blood-brain barrier (ie, a significant leakage of contrast material).

In this study, we quantitatively estimated pharmacokinetic parameters from DCE-MRI measurements using a novel modeling methodology. We have previously demonstrated that this approach was able to estimate tumor blood flow (F), extraction fraction (E), permeability-surface area product (PS), transfer constant ($k_{trans}$), washout rate ($k_{ep}$), interstitial volume ($V_i$), blood volume ($V_b$), capillary transit time ($t_c$), and capillary heterogeneity ($\alpha^{-1}$).
for each of the tumor areas. We found that 2 of the imaging parameters were correlated with patient OS: interstitial volume ($V_i$) in areas of PE and capillary heterogeneity ($\alpha^{-1}$) in areas of AT. $V_i$ represents the interstitial volume that is “accessible” to contrast molecules, so the observation that patients with measurable $V_i$ in PE have better outcomes suggests that these patients may have some early disruption of the blood-brain barrier of the peritumoral region that potentially allows elements of the immune system or chemotherapeutic agents to reach infiltrating tumor outside the contrast-enhancing regions. Within regions of active tumor, substantial disruption of the blood-brain barrier is associated with elevated accessible interstitial volume, but other mechanisms could be at play (eg, elevated interstitial fluid pressure, resistance of tumor cells to cytotoxic therapy), potentially explaining why this imaging biomarker is only significant in areas of PE. In fact, there are studies that have correlated imaging characteristics of PE and patient outcome.

The perfusion MR parameter capillary heterogeneity ($\alpha^{-1}$) represents heterogeneity of the distribution of vascular paths within a measured volume. An $\alpha^{-1}$ of zero represents the case where all paths are of uniform length as manifested by a well-ordered vasculature. On the other hand, an $\alpha^{-1}$ of 1 represents the case where there is in some sense maximal vascular disorder with many short transits such as an arteriovenous malformation and other architectural features of disordered vasculature that are prevalent in the tumor vasculature in malignant glioma. The other imaging markers were not helpful for predicting OS, most likely for the same reasons mentioned above when considering the lack of biomarker reliability in areas of NC and HP.

We also found that capillary transit time ($t_c$) in areas of PE and heterogeneity ($\alpha^{-1}$) and blood volume ($V_b$) in areas of AT were predictive of OS and PFS. It is interesting that none of the blood flow and permeability parameters ($K^{trans}$, $F$, $E$, $PS$) commonly used show any correlation with patient outcome in our study, which supports our use of a model that attempts to treat the blood pool contribution with more fidelity. There have been successful attempts to use pretreatment fractional blood volume and $K^{trans}$ for prognosis of overall patient survival. In fact, $K^{trans}$ is the most extensively studied imaging parameter, but it does not have a clear association with well-defined characteristics of tissue pathology as it includes contributions from both blood flow and capillary permeability.

### Tissue and Imaging Biomarker Correlation

We observed that measures of capillary heterogeneity ($\alpha^{-1}$), capillary transit time ($t_c$), washout time ($k_{w}$), and blood volume ($V_b$) were correlated with HIF-1 and VEGF expression in areas of AT, HP, and PE. As described above, both $t_c$ and $\alpha^{-1}$ are parameters characterizing the morphology of the microvascular bed, being dependent on time to transit through the tumor bed, and how heterogeneous the distribution of vascular paths is. Others have demonstrated correlation with cerebral blood flow measured by DCE-MRI and VEGF and HIF-1$\alpha$ expression. Although the investigators were looking at the tumor as a whole, they found that VEGF and HIF-1$\alpha$ expression correlated with regional cerebral blood volume and regional cerebral blood flow. Regional variation of histological features of GBM tumor specimens has been correlated with DCE-MRI. Tissue samples from contrast-enhanced regions had increased tumor score, cellular density, and proliferation.

Contrast-enhanced perfusion imaging variables such as relative cerebral blood volume, peak height, and recovery factor were significantly higher, and the percentage of signal intensity recovery was significantly lower in the contrast-enhanced regions compared with the nonenhanced regions. In our study, MB-1 index was correlated with blood volume ($V_b$). This is not surprising because DCE-MRI has been used to differentiate between high-grade and low-grade brain tumors, while MB-1 index has been proven many times to correlate with tumor grade. We recognize that there is a risk of statistical inaccuracies secondary to the high number of multiple correlations, and this is another limitation of this study. We were not able to control for multiple comparisons because of the small sample size and exploratory nature of this study. In our future definitive studies, we will perform these functions to eliminate such errors.

### Potential Clinical Applications

There have been attempts to use MR spectroscopic imaging for image-guided biopsies that have allowed for semiquantitative and qualitative histopathological analysis of patients with untreated glioma. The results of this study would suggest that perfusion MR might be used in a similar fashion to obtain stereotactic biopsies of areas of tumor most predictive of patient outcome. Areas of AT and PE seem to be the most reliable targets both for imaging and hypoxia biomarkers. Areas containing necrosis, NC, or areas difficult to define such as HP are less desirable targets for biopsy.

A mouse model of radiation treatment demonstrated quantitative changes in diffusion and perfusion soon after starting treatment. Regional cerebral blood volume measured by DSC-MRI has been used to help predict pseudoprogression in patients with GBM. This prior work demonstrated a significant difference between the mean regional cerebral blood volume between pseudoprogression and real tumor progression. Our group has found this technique to be less useful for predicting treatment response and tumor growth; however, one might consider imaging specific areas of a given tumor, as described in our report, with the potential that it might make differentiating these 2 clinical situations more apparent by perfusion imaging techniques. This study is preliminary and somewhat limited by its small sample size and limited analysis, thus it may not be accurate to extrapolate these findings to a larger scale. Despite these limitations, the significant correlations between imaging and histological biomarkers that have emerged from this study are novel and show potential for clinical utility. Validation in a larger patient population is justified based on this work.

### Conclusions

Our study is limited by small patient numbers and its preliminary nature but hints that it may be possible, with further work, to use DCE-MRI to make noninvasive preoperative predictions of areas of tumor with increased hypoxia and proliferation. This strategy has the potential to enhance prognostic decisions and to guide therapies to specific tumor areas.

### Supplementary Material

Supplementary material is available online at Neuro-Oncology (http://neuro-oncology.oxfordjournals.org/).
Jensen et al.: DCE-MRI and hypoxia biomarkers predict outcome

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Conflict of interest statement. None declared.

References