Hypoxia-Regulated Protein Expression, Patient Characteristics, and Preoperative Imaging as Predictors of Survival in Adults With Glioblastoma Multiforme

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BACKGROUND. Regions of hypoxia within glioblastoma multiforme (GBM) are common and may influence a tumor’s aggressiveness, response to treatment, and the patient’s overall survival. In this study, the authors examined 4 markers of hypoxia (hypoxia-inducible factor 1 [HIF-1α], glucose transporter 1 [GLUT-1], vascular endothelial growth factor [VEGF], and carbonic anhydrase 9 [CA IX]), cellular proliferation and microvascular density (MVD) indices, extent of surgical resection, and preoperative imaging characteristics and compared them with the overall survival rates of adults with GBM.

METHODS. In this retrospective cohort study, patients who had lower grade astrocytomas were compared with patients who had GBM to verify that the methods used could establish differences between tumor grades. By using preoperative imaging, the amount of necrosis was established versus the overall tumor area. The authors also compared preoperative images with postoperative images to define the amount of tumor resected; and they compared molecular markers, proliferation, MVD, and imaging studies with survival among patients who had GBM.

RESULTS. The hypoxia-regulated molecules (HRMs) and indices for MVD and cellular proliferation were associated significantly with tumor grade. Survival was improved when ≥95% of the tumor was resected. Although the total tumor area was associated with overall survival, no differences were observed when the amount of necrosis or a tumor necrosis index (area of necrosis/area of tumor) was compared with survival. The findings indicated that GLUT-1 and VEGF were correlated with survival after controlling for age.

CONCLUSIONS. Tumor grade was differentiated with HRMs, MVD, and proliferation, but only GLUT-1 predicted survival in this group of patients with GBM. The results suggested that GLUT-1 may be an important independent prognostic indicator.

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KEYWORDS: hypoxia-inducible factor 1α, vascular endothelial growth factor, glucose transporter 1, microvascular density, glioma, survival.

Glioblastoma multiforme (GBM) is the most common and most malignant brain tumor in humans. Despite resection and adjuvant treatment, the survival rates of patients who have GBM are only 3% and 1% at 5 years and 10 years, respectively, according to the 2001 annual report of the Central Brain Tumor Registry of the United States. Various hypoxia-regulated molecules (HRMs) and other factors, such as cellular proliferation, angiogenesis, tumor necrosis, and extent of resection, have been compared with survival
rates to identify variables that, either individually or in combination, would aid in maximizing response to treatment or act as prognostic indicators of improved survival.2-4

Regions of hypoxia within GBM are common and may be a critical influence on tumor aggressiveness, response to treatment, and the patient’s overall survival.5-7 Molecular markers of hypoxia, including hypoxia-inducible factor 1α (HIF-1α) and its downstream regulated targets vascular endothelial growth factor (VEGF), glucose transporter 1 (GLUT-1), and carbonic anhydrase 9 (CA IX), have been evaluated, often separately or in limited combinations, for their ability to correlate with astrocytoma tumor grades and overall survival.8-10 HIF-1 is a heterodimeric transcription factor that is thought to be the predominant regulator of oxygen homeostasis in cells.11,12 Overexpression of HIF-1α has been described in numerous cancers, including gliomas.13 The specific role of HIF-1α in tumor growth still is not clear, but previous results suggest this transcription factor is necessary for proliferation and angiogenesis.14 Overexpression has been observed specifically in the perinecrotic zones that mark areas of avascularity and hypoxia.15 We previously demonstrated that HIF-1α inhibition using small interfering RNA attenuates glioma growth in xenograph models, suggesting that it may play an important role in tumor growth.16

Intratumoral necrosis, angiogenesis, and cellular proliferation are histologic features of GBM that have been shown to distinguish low-grade gliomas from high-grade gliomas.5,17-19 Conflicting results have been reported regarding the efficacy of the use of cellular proliferation and microvascular density (MVD), which are used as measures of the degree of angiogenesis, in predicting survival.20-27

Surgical management of GBM is important for tissue diagnosis, but the role of radical resection has been less clear. GBM is a very infiltrative tumor, and ‘complete’ resection often is not feasible. The degree of resection of enhancing tumor, however, has been associated with survival. In a very large series of patients with GBM, Lacroix et al28 observed that the amount of tumor resected was associated with a survival advantage if ≥98% of the tumor was removed.

Although the correlation between survival and numerous factors has been investigated, it has been done using individual factors separately or in combination with 1 or 2 others. In this study, we examined whether 4 cellular markers of hypoxia, the proliferation and MVD indices, and the extent of resection and tumor necrosis are predictors of overall survival in adults with GBM.

MATERIALS AND METHODS

Study Participants

After institutional review board approval, we identified adults who had a GBM from the tumor database of R.L.J. Adult patients who had a de novo GBM tumor, underwent resection/biopsy at the University of Utah, had tumor specimens available, and had died were included in the study. Patients with recurrent tumors, those who still were living or had no death documentation, and those for whom tissue samples had not been retained were excluded. Medical records were reviewed retrospectively for information regarding age, sex, the location of the tumor, the extent of resection, treatment (radiotherapy and chemotherapy) received, specific imaging completed before and after resection, the length of progression-free survival (time from the date of initial imaging to imaging documenting progression or recurrence), and length of overall survival (calculated from the date of initial imaging to documented death from the Social Security Death Index). Patients with available preoperative and postoperative magnetic resonance (MR) imaging studies with and without contrast were identified, and their imaging studies were evaluated for tumor and necrotic area and for the extent of resection, as described below.

To document that the methods for evaluating HRMs, MVD, and proliferation would be valid, 10 or 12 randomly chosen patients within 2 additional tumor grades (low-grade gliomas [LGG], World Health Organization [WHO] grade II; anaplastic astrocytomas [AA], WHO grade III) were identified as control groups for comparison with our GBM study group (WHO grade IV). No survival or treatment information was obtained on these patients with lower grade tumors.

Immunohistochemistry

Immunohistochemistry was performed for HIF-1α, VEGF, GLUT-1, and CA IX using commercially available reagents according to manufacturer’s recommendations. For HIF-1α, 4-μm-thick sections of formalin-fixed, paraffin-embedded sections were dehydrated and dewaxed in graded alcohols. The Catalyzed Signal Amplification (CSA) Ancillary system (Dako, Carpinteria, Calif) was used according to the manufacturer’s recommended protocol. The primary antibody was anti-HIF-1α (NB 100-123; Novus Biologicals, Littleton, Colo; 1:1000 dilution). Finally, 3,3’-diaminobenzidine (DAB) chromogen solution (Vector Laboratories, Burlingame, Calif) was applied, and slides were counterstained in 0.1% Toluidine
blue. The slides were dehydrated in graded alcohols, dipped in 3 changes of xylene, and coverslipped.

For VEGF, GLUT-1, and CA IX immunohistochemical analysis, 4-μm-thick sections of formalin-fixed, paraffin-embedded sections were deparaffinized in graded alcohols, heated with steam in citrate buffer (Unmasking Solution; Vector Laboratories) for 30 minutes, and treated with 3% H₂O₂. By using the Vectastain Rabbit Kit (Vector Laboratories) for VEGF and GLUT-1, and the Vectastain Goat Kit (Vector Laboratories) for CA IX, sections were incubated in blocking serum for 20 minutes and then overnight in a moist chamber with the respective primary antibodies. Then, after incubating for 30 minutes with diluted biotinylated antibody solution, the sections were incubated for 30 minutes in Vectastain ABC complex and processed using the DAB kit developing solution. Negative controls were performed by replacing the primary antibody with nonimmune serum, and all other steps were performed as described above. Positive controls for HIF-1, VEGF, GLUT-1, and CA IX were performed on paraffin-fixed sections of tumors that were grown in mice using human U251 cell lines that were positive immunohistochemically for these proteins. Then, the slides were dehydrated in graded alcohols, dipped in 3 changes of xylene, and coverslipped.

All slides were examined by using an Olympus BX41 Microscope. Under ×200 magnification (10 ocular × 20 objective), they were scored by a single investigator (R.L.J.) who was blinded to the specimen tumor grade or patient information. A score from 0 to 4 (0 = 0%-25%; 1 = 25%-50%; 2 = 50%-75%; 3 = 75%-100%; and 4 = 100%) was given based on the number of cells stained in a given field.

Proliferation Index
To determine the proliferation index (PI), 4-μm-thick slices of the slides were cut, melted at 55°C to 60°C for 30 minutes, deparaffinized in xylene for 5 minutes, and rehydrated in graded alcohols for 1 minute each. Ki-67 (clone MIB-1) heat-induced epitope retrieval was applied in citrate buffer, pH 6.0, in an electric pressure cooker for 4 minutes. The following steps were performed on the Ventana ES (Ventana Medical Systems, Tucson, Ariz) at 40°C. The primary antibody (1:160 dilution) was soaked for 28 minutes, and secondary antibody (1:300 dilution) was soaked for 8 minutes (Mouse Fab; Dako). Detection was done by using the IView DAB detection kit (Ventana Medical Systems), and the counterstain was done with hematoxylin (Ventana Medical Systems) for 4 minutes. Positive controls were performed on human thymus, which produces >90% cell staining. Negative controls were performed by replacing the primary antibody with nonimmune serum, and all other steps were performed as described above. Then, slides were dehydrated through graded alcohols for 30 seconds each, dipped in 4 changes of xylene, and coverslipped.

The PI was calculated by taking 6 random photomicrographs representative of each slide at ×400 magnification (10 ocular × 40 objective) by using an Olympus Microfire camera. The photomicrographs with the most and the least staining were discarded, and the remaining 4 were analyzed. The images were transferred to Image-Pro Plus 5.0, and a LoPass large-spectral filter was applied. By using the count/size measurement feature, the MIB-1-stained cells (brown) were counted with the manual intensity range selection tool by histogram-based segmentation. The background-stained cells (blue) were counted in the same manner. The PI was calculated as the number of MIB-1-stained cells divided by the total number of cells in the field ([number of brown-stained cells]/[number of brown-stained cells + number of blue-stained cells]). This calculation was repeated 3 times for each photomicrograph and averaged. The results from all 4 photomicrographs for each slide were averaged for the final PI (Fig. 1a,b). The analysis was duplicated by a separate researcher using a random subset of 20 slides. This method was very reproducible, as demonstrated by good interrater reliability (ρ = 0.99; 95% confidence interval [95% CI], 0.99-1.00) and intrarater reliability (ρ = 0.96; 95% CI, 0.92-0.99).

MVD Index
The slides for the 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) Protease 2 (Ventana Medical Systems) for 4 minutes, and the primary antibody (1:100 dilution) was soaked for 32 minutes. Then, the slides were soaked in a secondary antibody (1:300 dilution) for 8 minutes (Mouse Fab). Positive controls were performed on human tonsil. Negative controls were performed by replacing the primary antibody with nonimmune serum, and all other steps were performed as described above.

The MVD index was calculated based on a previously published method. Briefly, 3 photomicrographs of the most vascular area of the slide, the ‘hotspot,’ were taken at ×200 magnification using an Olympus Microfire camera. Then, the photomicrographs were transferred to Photoshop CS7 (Adobe Systems Incorporated, San Jose, Calif), and any posi-
tive cell that was separate from other stained cells and not contiguous or branching from other vessels was counted. The results from all 3 photomicrographs for each slide were averaged for the resulting MVD and were divided by 0.26 mm$^2$ to normalize the size of the photomicrographic field.

**Image Analysis**

Contrast-enhanced, T1-weighted, preoperative scans on patients with available MR images were uploaded using DicomWorks version 1.3.5 and were evaluated using a computer program that was developed by J.K.R. (MRI_2D). This Java program runs as a plug-in.
to the NIH Imaging program, Image J, and is available upon request.

Each pixel in an MR image has an inherent value between 0 (black) and 255 (white). After the user clicks on an enhancing region of the tumor, the MRI_2D program performs an initial selection by moving from the clicked pixel outward to include all pixels that are within the initial default bounds (138-255; these initial default bounds were chosen during the set-up phase in selecting the majority of enhancing tumors on numerous patient samples). After MRI_2D performs the initial selection, the bounds are adjusted to include the entire region in question. Then, these same numerical bounds are used for all images from that patient (lower bound range, 94-236; average, 144). Necrotic regions are defined as regions within the tumor that have a value of 0 to the lower bound number for the enhancing region of that patient (Fig. 1c,d). These regions of interest are used to calculate the total size of the tumor and area of necrosis in pixels. The ratio of necrosis area to tumor area also is calculated.

**Statistical Analyses**

Our sample size of patients with GBM (n = 62) provided 80% power to detect a hazard ratio (HR) of 2.1 in our Cox regression models, which was close to the effect size we anticipated. No formal sample size determination was undertaken for the 3 tumor groups, because the intention simply was to observe whether our methods could result in observable, but not necessarily statistically significant, differences.

The associations of tumor grade with the HRMs, which were measured as quartiles and treated as ordered categorical variables, were tested for significance using a categorical linear trend test. For pairwise comparisons of these markers between the tumor grade groups, a Wilcoxon Mann-Whitney U test was used with adjustment for multiple comparisons using the Holm P-value adjustment procedure. An identical approach was taken with the continuous-scale molecular markers of MVD and proliferation using these nonparametric tests, because there was a concern about meeting the normality assumption of a parametric approach given the small sample sizes.

Multivariable Cox regression was used to model time to death adjusting for age as a potential confounder. Although linear regression could have been used, because all patients died, the Cox regression approach still is recommended, because it provides more information and sensitivity. The proportional hazards assumption was tested for each predictor variable, both separately and globally, using a formal significance test based on unscaled and scaled Schoenfeld residuals. The covariate age consistently violated the assumption and, thus, was included as a time-dependent covariate to eliminate the need for proportional hazards on that covariate.

Age-adjusted Kaplan-Meier survival curves are reported. For these analyses, the predictor variables HRMs, PI, and MVD were divided at the median, because the HRMs were ordered categorical variables with too few points in each category to provide reliable estimates. The other variables were continuous, but dividing at the median allowed the calculation of the HR that matched the Kaplan-Meier curves, in which dividing at the median permitted 2 lines for visual comparison. Statistical analyses were done using Stata 9.2 software (StataCorp, College Station, Tex) using 2-sided comparisons with significance set at .05.

**RESULTS**

**Patient population**

Initially, 101 patients with GBM were identified, and 39 patients subsequently were excluded (25 had recurrent tumors, 9 were still living, 2 had no death documentation available, and 3 had no available tissue) (Table 1). A subset of 42 patients with available MR studies was identified (Table 1).

The randomly chosen control groups of patients with LGG and AA included 12 patients and 10 patients, respectively. Demographic characteristics for these 2 groups were compared with a randomly selected subset of patients who had GBM (Table 2) and did not differ significantly.

**Tumor Markers and Tumor Grade**

To evaluate our ability to establish differences in HRMs, PI, and MVD between tumor grades, patient samples from the LGG, AA, and GBM groups were compared. All of the HRMs except CA IX increased significantly as the tumor grade increased (Fig. 2) (trend: \( P = .023, P < .001, P = .002, \) and \( P = .18 \) for HIF-1, GLUT-1, VEGF, and CA IX, respectively). Although the trend across tumor grades was significant, no pairwise comparisons of HIF-1α staining between tumor grades were significant after adjusting for multiple comparisons (LGG vs AA, \( P = .16; \) LGG vs GBM, \( P = .15; \) AA vs GBM, \( P = .27 \)). However, when GLUT-1 and VEGF staining in LGG were compared with those in AA (\( P = .006 \) and \( P = .015 \), respectively) and in GBM (\( P < .001 \) and \( P = .011 \), respectively), the differences were highly significant. With CA IX, the trend across tumor grade was not significant, and no differences were observed (LGG vs
AA, P = .10; LGG vs GBM, P = .41; AA vs GBM, P = .37). AA and GBM frequently are grouped into a single 'high-grade' group; therefore, it was not surprising that there were not significant differences observed between these 2 groups for any of the HRMs (P = .27, P = .24, P = .60, and P = .37 for HIF-1, GLUT-1, VEGF, and CA IX, respectively) (Fig. 2e).

In our evaluation of MVD, significant differences were observed between LGG and both AA and GBM (P = .027 and P = .003, respectively) (Fig. 3a). No difference between AA and GBM was observed (P = .09). When comparing the PI across tumor grades, significant differences were observed when GBM was compared with LGG and AA (P = .001 and P = .038, respectively) (Fig. 3b). A significant difference was not observed when LGG were compared with AA (P = .19). The trends for both MVD and PI

### TABLE 1
Characteristics of Patients With Glioblastoma Multiforme

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>Total Cohort</th>
<th>Imaging Subset</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>62</td>
<td>42 (67.75)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>42 (67.74)</td>
<td>30 (71.43)</td>
</tr>
<tr>
<td>Women</td>
<td>20 (32.26)</td>
<td>12 (28.57)</td>
</tr>
<tr>
<td>Age, y</td>
<td>Mean±SD</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>62.47±11.26</td>
<td>63.5±11.38</td>
</tr>
<tr>
<td>GBM</td>
<td>40.7-81.2</td>
<td>40.7-81.2</td>
</tr>
<tr>
<td>Extent of surgery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biopsy</td>
<td>6 (9.68)</td>
<td>0</td>
</tr>
<tr>
<td>Subtotal resection</td>
<td>40 (64.52)</td>
<td>16 (38.10)</td>
</tr>
<tr>
<td>Gross total resection (≥95%)</td>
<td>16 (25.81)</td>
<td>26 (61.90)</td>
</tr>
<tr>
<td>Tumor side</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>29 (46.77)</td>
<td>20 (47.62)</td>
</tr>
<tr>
<td>Left</td>
<td>30 (48.39)</td>
<td>20 (47.62)</td>
</tr>
<tr>
<td>Bilateral</td>
<td>3 (4.84)</td>
<td>2 (4.76)</td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal</td>
<td>19 (30.65)</td>
<td>12 (28.57)</td>
</tr>
<tr>
<td>Occipital</td>
<td>5 (8.06)</td>
<td>3 (7.14)</td>
</tr>
<tr>
<td>Temporal</td>
<td>24 (38.71)</td>
<td>18 (42.86)</td>
</tr>
<tr>
<td>Parietal</td>
<td>12 (19.35)</td>
<td>8 (19.05)</td>
</tr>
<tr>
<td>Thalamus</td>
<td>2 (3.23)</td>
<td>1 (2.38)</td>
</tr>
<tr>
<td>Awake cortical mapping</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11 (17.74)</td>
<td>11 (26.19)</td>
</tr>
<tr>
<td>No</td>
<td>51 (82.26)</td>
<td>31 (73.81)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>44 (70.97)</td>
<td>31 (73.81)</td>
</tr>
<tr>
<td>No</td>
<td>15 (24.19)</td>
<td>8 (19.05)</td>
</tr>
<tr>
<td>Unknown</td>
<td>3 (4.84)</td>
<td>3 (7.14)</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>50 (80.65)</td>
<td>34 (80.95)</td>
</tr>
<tr>
<td>No</td>
<td>11 (17.74)</td>
<td>7 (16.67)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (1.61)</td>
<td>1 (2.38)</td>
</tr>
<tr>
<td>PFS, mo</td>
<td>Mean±SD</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>5.56±3.49</td>
<td>5.84±5.50</td>
</tr>
<tr>
<td>GBM</td>
<td>0.56-24.3</td>
<td>0.56-24.3</td>
</tr>
<tr>
<td>OS, mo</td>
<td>Mean±SD</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>10.77±7.58</td>
<td>11.26±7.87</td>
</tr>
<tr>
<td>GBM</td>
<td>0.07-32.65</td>
<td>0.26-32.65</td>
</tr>
</tbody>
</table>

SD indicates standard deviation; PFS, progression-free survival; OS, overall survival.

### TABLE 2
Characteristics of Patients With 3 Grades of Gliomas

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total</th>
<th>LGG (Grade II)</th>
<th>AA (Grade III)</th>
<th>GBM (Grade IV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>32</td>
<td>12</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mean±SD age, y</td>
<td>50.99±18.29</td>
<td>37.92±16.92</td>
<td>54±16.37*</td>
<td>63.68±10.84*</td>
</tr>
<tr>
<td>Sex, no. [%]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>22 [68.75]</td>
<td>9 [73]</td>
<td>5 [50]</td>
<td>8 [80]</td>
</tr>
</tbody>
</table>

*P = .04.
1P < .001.

WHO indicates World Health Organization; LGG, low-grade glioma; AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; SD, standard deviation.

### FIGURE 2
Hypoxia-regulated molecules (HRMs) compared across tumor grades. (a) No significant differences were observed when hypoxia-inducible factor 1 (HIF-1) staining was compared across tumor grades. LGG indicates low-grade glioma; AA, anaplastic astrocytoma; GBM, glioblastoma multiforme. (b) Glucose transporter 1 (GLUT-1) staining was significantly different when LGG was compared with AA and GBM. (c) Vascular endothelial growth factor (VEGF) staining was significantly different when LGG was compared with AA and GBM. (d) No significant differences were observed when carbonic anhydrase 9 (CA IX) staining was compared across tumor grades. (e) Table of P values for HRMs compared across tumor grades and the P values for trends (light gray, P < .05; dark gray, P < .01).
were significant ($P \text{ for trend} < .001$ for both MVD and PI) (Fig. 3c).

**Preoperative Imaging of GBM**

We observed that the extent of tumor resection was associated with increased patient overall survival ($P \leq .005$). In particular, tumors that were 100% resected, which meant that no residual was observed on postoperative scans, and tumors that were $\geq 95\%$ resected were associated with significantly better survival ($P = .001$ [HR, 0.25] and $P = .01$ [HR, 0.36], respectively) (Fig. 4). The significance disappeared when the total resection was $\geq 85\%$ (Table 3). There was a significant correlation with biopsy and survival ($P = .01$), but the HR was 4.25, indicating a worse outcome for the few patients who underwent biopsy. This may represent a selection bias, because patients often underwent biopsy if they were very ill or if they were not stable enough for surgical resection.

A correlation was observed between overall tumor area and the survival rate ($P = .04$). When the size of the area of necrosis or the necrosis/tumor index was compared with HRM levels, indices, or survival, only the MIB index was correlated significantly with necrosis or the necrosis/tumor index ($P = .03$ and $P = .02$, respectively) (Table 4).

**GBM Tumor Markers**

GLUT-1 and VEGF staining were associated significantly with survival after controlling for age alone (data not shown). When controlling for both age and the amount of resection, a shorter survival was observed in the higher staining group for GLUT-1 ($P = .006$; HR, 2.11; 95% CI, 1.24-3.59) and VEGF ($P = .031$; HR, 1.84; 95% CI, 1.06-3.21). Survival in the higher staining HIF-1 group was shorter, but the difference was not quite significant ($P = .061$; HR, 1.66; 95% CI, 0.98-2.82) (Fig. 5). CA IX was not associated significantly with the length of survival ($P = .52$; HR, 1.19; 95% CI, 0.70-2.03) (Fig. 5). GLUT-1 and VEGF were highly collinearly related (Pearson $r$, 0.54; $P < .001$) and, thus, were not used in this analysis. There was no significant survival difference in the Cox regression models that were controlled for age alone or for age and the amount of resection to compare the high and low groups, as defined by a median cutoff point, for MVD and PI.

**DISCUSSION**

Previous investigators have compared various HRMs, MVD, proliferation, necrosis, and extent of resection with astrocytoma tumor grade and overall survival and have produced conflicting results.20-27 It is difficult to compare the results directly, because different HRMs and methods were used. To our knowledge, the current study is the first to combine 4 well known HRMs and indices of MVD and proliferation compared across 3 tumor grades and with the length of survival within the GBM tumor group. Our results indicate that GLUT-1, VEGF, and indices for MVD and proliferation can differentiate tumor grade. Furthermore, in this study, GLUT-1 and VEGF were associated significantly with overall survival within the group of patients with GBM.

**HRMs, Tumor Grade, and Survival**

Various HRMs have been evaluated to review their level of expression in different regions of tumors, across tumor grades, and in association with survival. Abdulrauf et al32 reported that increased levels of
VEGF staining in individuals with LGG correlated with a shorter survival and an increased chance of malignant transformation, whereas Korkolopoulou et al observed that increased VEGF and HIF-1α staining was correlated with shortened survival but also noted that the significant difference disappeared when the grades were separated and re-evaluated. Increased CA IX expression also has been associated with histologic grade and decreased overall survival across tumor grades.

Although we did not assess the association between the HRMs and overall survival in patients with LGG or AA, the results from the patients with GBM combined with our recent xenograph model of glioma growth suggest that GLUT-1 and the glycolytic pathway may play an important role. The adaptive process observed when hypoxia causes a metabolic shift from oxidative metabolism to glycolysis was proposed by Warburg in 1956, and recent correlations between glycolysis and HIF-1 have been reported in various cell types. Thus, we believe that a focus on the glycolytic pathway may be more beneficial. Recent treatment options that have focused on antiangiogenic or anti-VEGF therapy have yielded mixed results. It is possible that GLUT-1 may play an increased role in the transformation between tumor grades and the aggressiveness of GBM compared with VEGF.

### MVD, Proliferation, Tumor Grade, and Survival

Reported results are divided regarding whether markers of MVD and proliferation can predict transformation from LGG to GBM and correlate with overall survival. Different markers for evaluating MVD, including Factor 8, CD105, and CD31, and various ways of evaluating vascular morphology have been reported. Leon et al reported that microvessel counts ≥70 were correlated with significantly shorter survival across tumor grades. After age and grade were controlled, branching counts of the vessels have been correlated with survival, but the quantification of MVD has not. Sallinen et al demonstrated that the PI may be a better predictor of outcome than histologic grade. McKeever et al observed that an increased MIB index was correlated with shorter survival in individuals with LGG; however, others have not observed this correlation between proliferation and survival.
We observed that, as tumor grade increased, to AA and GBM from LGG, so did MVD. It is noteworthy that there was a difference in the final differentiation into high-grade glioma when evaluating proliferation. No correlation between MVD or proliferation and survival in the GBM group was observed. This suggests that increased vascularity is a step toward the development of higher grade tumors but does not play a role in the biologic behavior of a given GBM. Although GBMs are more vascular than lower grade tumors, it has been demonstrated that low-grade astrocytomas incorporate pre-existing vessels, whereas GBMs develop new vessels. 

In tumors, as they increase in size, chaotic microvasculature develops that is inefficient functionally compared with normal brain tissue and leads to hypoxic regions within the tumor. In fact, it is possible to imagine that an ‘angiogenic switch’ is one of the defining features of the transformation from LGG to AA and that proliferation comes subsequently.

Preoperative Imaging, Tumor Markers, and Survival in GBM

The program MRI_2D was created to increase the speed, accuracy, and precision of brain tumor analysis. MRI_2D provides the researcher with a more standardized approach to image analysis. It allows for the reliable delineation of the enhancing and necrotic regions of tumors when the tumor borders may be unclear to the human eye. MRI_2D also facilitates consistency among the set of images and has hot-keys and layout options that allow the program to perform 5 times faster than previous methods. The use of this program increased our precision, because the researcher evaluating the MR images could not arbitrarily include darker/lighter regions on subsequent slides that may be more ambiguous. Accuracy of this program still relies on the researcher's initial limit settings, but the program maintains its accuracy throughout the rest of the images for each patient.

Increased intratumoral necrosis and peritumoral edema visible on preoperative imaging studies have been correlated with poorer overall patient survival. Other authors have reported that several preoperative imaging features of GBMs are meaningful predictors of survival. Pope et al observed that the combination of noncontrast-enhancing tumor, absent edema, and no satellite or multifocal lesions corresponded with a doubling of median survival compared with other combinations. These imaging features have the potential to help determine the prognosis of patients in routine clinical practice. Our current results demonstrated a correlation of the MIB index and both the area of necrosis and the necrosis/tumor index. The significance of this is unclear, however, because we were unable to demonstrate a relation between either the PI or the area of...
necrosis and survival. Nevertheless, further work on developing preoperative imaging to predict outcome for patients with GBM is warranted.

The prognosis for individuals who are diagnosed with GBM remains poor. Identifying means to improve initial prognosis or to recognize individuals for whom augmented therapy would be beneficial has proven difficult. The current results agree with reports by other authors that the extent of resection can improve survival only up to a point (≥95% resection). Our results also suggest that MVD and proliferation may play an important role in the transformation from LGG to GBM but do not have as much effect on survival as the effects of the resulting hypoxia and its downstream effects. Our next step will be to knock down GLUT-1 in a xenographic model of GBM to evaluate its independent effects on tumor growth as well as its effects in concert with HIF-1α and VEGF. Our current results suggest that more research targeting the glycolytic pathway through GLUT-1 may result in a target for effective treatment with a resulting increase in the overall survival of patients with GBM.

REFERENCES


