A Data-Driven Approach to Predicting 5-Aminolevulinic Acid–Induced Fluorescence and World Health Organization Grade in Newly Diagnosed Diffuse Gliomas

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BACKGROUND: A growing body of evidence has revealed the potential utility of 5-aminolevulinic acid (5-ALA) as a surgical adjunct in selected lower-grade gliomas. However, a reliable means of identifying which lower-grade gliomas will fluoresce has not been established.

OBJECTIVE: To identify clinical and radiological factors predictive of intraoperative fluorescence in intermediate-grade gliomas. In addition, given that higher-grade gliomas are more likely to fluoresce than lower-grade gliomas, we also sought to develop a means of predicting glioma grade.

METHODS: We investigated a cohort of patients with grade II and grade III gliomas who received 5-ALA before resection at a single institution. Using a logistic regression-based model, we evaluated 14 clinical and molecular variables considered plausible determinants of fluorescence. We then distilled the most predictive features to develop a model for predicting both fluorescence and tumor grade. We also explored the relationship between intraoperative fluorescence and diagnostic molecular markers.

RESULTS: One hundred seventy-nine subjects were eligible for inclusion. Our logistic regression classifier accurately predicted intraoperative fluorescence in our cohort with 91.9% accuracy and revealed enhancement as the singular variable in determining intraoperative fluorescence. There was a direct relationship between enhancement on MRI and the likelihood of observed fluorescence. Observed fluorescence correlated with MIB-1 index but not with isocitrate dehydrogenase (IDH) status, 1p19q codeletion, or methylation of the O6-methylguanine DNA methyltransferase promoter.

CONCLUSION: We demonstrate a strong correlation between enhancement on preoperative MRI and the likelihood of visible fluorescence during surgery in patients with intermediate-grade glioma. Our analysis provides a robust method for predicting 5-ALA–induced fluorescence in patients with grade II and grade III gliomas.

KEY WORDS: Aminolevulinic acid, Fluorescence, Glioma, Statistical models, Magnetic resonance imaging

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RESEARCH—HUMAN—CLINICAL STUDIES

B y allowing surgeons to visualize otherwise undetectable tumor, 5-aminolevulinic acid (5-ALA) has had a major impact in malignant glioma surgery and safely doubles the likelihood of gross total resection compared with white light. Food and Drug Administration (FDA) approval of 5-ALA for use in patients with glioma (suspected grades III and IV) as an adjunct for visualization of malignant tissue during surgery was granted in 2017.

Visible fluorescence is observed in 98% to 100% of patients with glioblastoma. In addition, fluorescence is also observed in 75% to 85% of all grade III and 16% to 20% of grade II diffuse gliomas of patients. The factors that are associated with visible fluorescence in intermediate-grade gliomas (World Health Organization [WHO] II and III) have not been determined.

ABBREVIATIONS: 5-ALA, 5-aminolevulinic acid; BBB, blood brain barrier; CE, contrast enhancement; IDH, isocitrate dehydrogenase; IDH, Karnofsky performance status; LOOCV, leave-one-out at a time cross-validation; MGMT, methylguanine DNA methyltransferase; PpIX, protoporphyrin IX; WHO, World Health Organization.
Here, we sought to develop a systematic preoperative approach to identify intermediate-grade gliomas most likely to exhibit visible fluorescence. We hypothesized that a statistical model could be developed to predict the likelihood of visible fluorescence based on clinical and radiographic factors. We also hypothesized that higher-grade gliomas would be more likely to fluoresce than lower-grade gliomas and, therefore, aimed to develop a predictive model to predict grade as well.

To develop our models, we used a cohort of patients with grade II and grade III glioma who received 5-ALA. We used detailed imaging data to quantitatively examine how MRI features predict fluorescence and tumor grade. We then distilled the most predictive features to develop straightforward models for predicting both fluorescence and grade. Finally, we explored the correlation between observed fluorescence and key molecular markers (MIB-1 index, isocitrate dehydrogenase (IDH) status, and 1p/19q codeletion).\(^7\,8\)

**METHODS**

**Study Design and Patients**

We used a database derived from patients with glioma receiving 5-ALA at a single academic center from 2011 to 2019 for our retrospective analysis (institutional board approval was obtained; individual patient consent was not required). We identified 179 patients with newly diagnosed grade II and grade III gliomas who received 5-ALA for resection from 2011 to 2019.

**Preoperative MRI Analyses**

Fluid-attenuated inversion recovery (FLAIR)-positive tumor volume on MRI was calculated as the product of the 3 largest diameters on 3-dimensional FLAIR imaging divided by 2 as previously described.\(^9\,10\) The enhancement patterns were defined as either: none (CE 0), patchy (multiple smaller areas of enhancement covering less than 50% of any nonenhancing tumor cross section, CE 1), focal (1 single area of enhancement covering less than 50% of any nonenhancing tumor cross section, CE 2), or abundant (enhancing tumor volume greater than 50% of nonenhancing tumor, CE 3, Figure 1).

**Fluorescence-Guided Resection**

All patients received 20 mg/kg 5-ALA (Medac) orally 3 to 4 hours before surgery. Operations were performed either with a Zeiss Meditech Pentero or Pentro 900 equipped with the Zeiss Meditech BLUE400 option (Zeiss Meditec). Three surgeons performed all operations following a strict protocol for fluorescence observation. Specifically, the working distance of the microscope was maintained at 25 cm, blue light was set to 100%, and room lights were diminished. Surgeons toggled between bright and darkfield conditions as needed and recorded the presence of fluorescence in the operative notes. In tumors without homogenous fluorescence, care was taken specifically to biopsy regions with fluorescence, when encountered.

**Neuropathology**

Neuropathologists were blinded to observed fluorescence. Histopathology was rendered based on the 2016 WHO classification of central nervous system tumors’ with immunohistochemistry for Ki-67/MIB-1 proliferation index and IDH1 mutations.\(^11\) Further molecular analyses were also performed. IDH1 wild-type tumors, diagnosed by immunohistochemistry, were sequenced for noncanonical mutations.\(^12\) 1p/19q codeletions were determined by multiplex ligation-dependent probe amplification using probes (SALSA MLPA P088 Oligodendrogliona 1p-19q probemix) using manufacturer’s protocols (MRC Holland). O6-methylguanine DNA methyltransferase (MGMT) promoter methylation status was determined by methylation-specific polymerase chain reaction of bisulfite-converted DNA (EZ DNA Methylation-Gold Kit; Zymo Research).\(^13\)

**Data Collection**

Continuous and discrete variables were collected on each patient. Continuous variables were age, Karnofsky performance status, highest MIB index, and FLAIR-positive tumor volume. Discrete variables included sex, contrast enhancement (CE) on preoperative MRI, fluorescence, fluorescence pattern, presenting symptoms, steroid administration before resection, tumor grade (determined postsurgery), glioma subtype, the presence or absence of midline shift, predominant tumor location on preoperative MRI, tumor enhancement pattern on preoperative MRI, presence of IDH mutation, and MGMT promoter methylation status. Of the 179 eligible patients in the database, 157 had complete data for all variables.

**Statistical Analyses**

Analyses were performed using the R statistical software package version 3.6.2 (https://www.R-project.org/) or SPSS version 26 (IBM Corp.). To determine factors predicting fluorescence, a logistic regression model was built using the aforementioned variables as predictors,
followed by a forward and backward stepwise regression procedure using Akaike information criterion (AIC) as the selection criterion. Let \( P_i \) denote the probability of fluorescence for patient \( i \). The selected logistic regression model is

\[
\log \left( \frac{P_i}{1 - P_i} \right) = \alpha + \beta_1 \text{Enhance}_i + \beta_2 \text{Pattern}_i,
\]

where \( \alpha \) is the intercept and the \( \beta \)'s are regression coefficients for MRI enhancement and MRI enhancement pattern. “Enhance,” is a binary variable equaling 1 if enhancement is present and 0 otherwise, and “pattern,” is an ordinal variable at 4 levels of observed MRI CE: none (CE 0), patchy (CE 1), focal (CE 2), and abundant (CE 3). Pattern is treated as a linear ordinal variable. The 157 patients with complete data records for all variables were used in this analysis (84 without fluorescence and 73 with fluorescence).

Next, leave-one-out at a time cross-validation (LOOCV) was performed to determine the predictive performance of this model. For predictive purposes, a probability threshold of 0.5 was used to classify fluorescence of the patient left out. A probability of 0.5 or greater was classified as fluorescent and less than 0.5 as nonfluorescent.

Subset analysis on fluorescence was then performed using the above model and methods. The 2 subsets were patients with grade II tumors and patients with grade III tumors.

To determine factors that are predictive of tumor grade (II vs III), we used a stepwise model selection procedure on a logistic regression model. Let \( Q_i \) denote the probability of grade III tumor for subject \( i \). The final logistic regression model is

\[
\log \left( \frac{Q_i}{1 - Q_i} \right) = \alpha + \beta_1 \text{age}_i + \beta_2 \text{Pattern}_i + \beta_3 \text{Shift}_i + \beta_4 \text{Vol}_i,
\]

where \( \alpha \) is the intercept and \( \beta \)'s are the regression coefficients for age, MRI CE pattern, midline shift, and volume (FLAIR). MRI enhancement pattern is treated as a linear ordinal variable, and midline shift is a binary variable.

### TABLE 1. Baseline Patient Characteristics (n = 179)

| Characteristic                     | Study population | Missing data |
|-----------------------------------|------------------|--------------|
| Median age (IQR)                  | 45.0 (35.0-56.5) | 0            |
| Sex female (male)                 | 73 (106)         | 0            |
| Median KPS (IQR)                  | 100 (100.0-100.0) | 0          |
| Median FLAIR tumor volume         | 9.4 (4.0-22.5)   | 2            |
| Midline shift yes (no)            | 24 (154)         | 1            |
| Side left (right)                 | 79 (95)          | 1            |
| Bilateral yes (no)                | 7 (172)          | 0            |
| Contrast enhancement yes (no)     | 96 (82)          | 1            |
| CE 0—no contrast enhancement (n)  | 82 (46%)         | 0            |
| CE 1—patchy contrast enhancement (n) | 50 (28%)        | 0            |
| CE 2—focal contrast enhancement (n) | 23 (13%)        | 0            |
| CE 3—abundant contrast enhancement (n) | 23 (13%)      | 0            |
| Steroid use, yes (no)             | 80 (92)          | 7            |
| Intraoperative fluorescence, yes (no) | 81 (92)        | 6            |
| WHO grade II (III)                | 113 (66)         | 0            |
| IDH mutation, yes (no)            | 125 (39)         | 15           |
| 1p/19q codeletion, yes (no)       | 40 (138)         | 1            |
| MGMT promoter methylation, yes (no) | 58 (119)       | 2            |

### TABLE 2. Logistic Regression Classifier for Determining the Preoperative Value Most Predictive of Visible Fluorescence

| Parameter                          | Estimate | \( P \) value |
|------------------------------------|----------|---------------|
| **Full logistic model**            |          |               |
| Age                                | -0.010   | .640          |
| Sex (male)                         | 0.331    | .536          |
| KPS                                | -0.063   | .589          |
| Onset of symptoms                  | 1.364    | .328          |
| Duration of symptoms               | 0.452    | .182          |
| Steroid use yes/no                 | -0.649   | .298          |
| Left-sided lesion                  | 0.124    | .828          |
| Right-sided lesion                 | -0.235   | >.999         |
| Frontal lesion                     | -14.612  | .997          |
| Insular lesion                     | -14.463  | .997          |
| Occipital lesion                   | -14.149  | .997          |
| Parietal lesion                    | -16.608  | .997          |
| Temporal lesion                    | -14.323  | .997          |
| Thalamic lesion                    | -13.878  | .997          |
| Gliomatosis                        | -17.442  | .998          |
| Bihemispheric lesion               | 17.621   | .996          |
| Midline shift                      | -0.228   | .799          |
| FLAIR volume                       | 0.002    | .916          |
| Enhancement yes/no                 | 2.680    | .005          |
| Enhancement pattern                | 2.692    | .019          |
| Astro vs oligo                     | 0.240    | .743          |
| **Final logistic model**           |          |               |
| Enhancement yes/no                 | 2.450    | .002          |
| Enhancement pattern                | 2.171    | .027          |

KPS, Karnofsky performance scale.

Parameter estimates for the full logistic model and for the final logistic model. Bolding indicates statistically significant.

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CE, contrast enhancement; IDH, isocitrate dehydrogenase; IQR, interquartile range; KPS, Karnofsky performance status; MGMT, methylguanine DNA methyltransferase; NOS, not otherwise specified; WHO, World Health Organization.
(1 = shift, 0 = no-shift). A total of 157 patients were included in this analysis (100 patients with grade II tumor and 57 patients with grade III tumor).

Next, leave-one-out cross-validation was performed to determine the predictive performance of this model. For predictive purposes, a probability threshold of 0.5 was used to classify the tumor grade of the patient left out. A probability of 0.5 or greater was classified as grade III and less than 0.5 as grade II.

Additional analyses were performed for interrater reliability for parameters at risk for subjective interpretation on imaging (tumor size and enhancement pattern). To do so, 43 patients were randomly selected from the database (approximately 25% of patients for which all data were available) comprising both grade II and grade III gliomas. Three experienced tumor neurosurgeons were asked to independently assess enhancement patterns and tumor sizes on MRI from the preselected patients. Fleiss Kappa coefficients and intraclass correlation coefficients using a two-way mixed effects model, respectively, were calculated from the resulting data.

### RESULTS

A total of 179 patients with grade II or III glioma were identified in our study population (Table 1). One hundred thirteen were diagnosed with grade II and 66 with grade III gliomas.

#### Identification of Preoperative Factors Predictive of Fluorescence and Development of a Visible Tumor Fluorescence Classifier

A logistic regression classifier was developed to determine the preoperative variables most predictive of visible tumor fluorescence during surgery. By stepwise variable selection, we determined that enhancement was singularly predictive of visible fluorescence (Table 2). The classifier for predicting fluorescence was validated using LOOCV, demonstrating that the presence of fluorescence was correctly predicted 91.9% of the time while the absence of fluorescence was predicted correctly 78.6% of the time. Subsequently, we separated out patients with grade II and grade III gliomas to determine how well enhancement status predicted grade in each subpopulation. Overall accuracy for predicting the presence of fluorescence based on enhancement status was 81.0% in grade II tumors and 93% in grade III tumors.

#### Enhancement Pattern as a Predictor of Fluorescence

We further evaluated the relationship between enhancement and observed fluorescence. We noted that the degree of enhancement correlated with the likelihood of fluorescence among grade II and grade III tumors. In addition, the presence of abundant enhancement (CE 3) was correlated with a 91.3% probability of visible fluorescence, and the absence of enhancement (CE 0) was associated with a 91.2% probability of absence of fluorescence.

#### Fluorescence as a Predictor of Tumor Grade, Molecular Subtype, and Proliferation Index

Subsequently, we investigated whether fluorescence might predict tumor grade or molecular subtype. We used a logistic regression model to fit tumor grade as a function of fluorescence (Table 3) and performed LOOCV. We noted that grade II tumors were accurately predicted 66.4% of the time while grade III tumors were correctly predicted 74.2% of the time. Presence or absence of fluorescence did not correlate with IDH status, 1p19q codeletion, or MGMT promoter methylation. However, fluorescence directly correlated with the MIB index.

#### Identification of Factors Predictive of Grade and Development of a Tumor Grade Classifier

A logistic regression classifier was also used to identify the preoperative variables most predictive of tumor grade. Using all the variables collected, both a full logistic regression model and a stepwise variable selection were performed. The final classifier included age, pattern of enhancement, midline shift, and FLAIR tumor volume (Table 4). To validate the logistic regression model, we performed LOOCV, demonstrating an accuracy of 86% for predicting grade II and 53.4% for predicting grade III tumors (Table 5).
Interrater Reliability for Enhancement Patterns and Tumor Size

Using the definition chosen for this study the Fleiss Kappa coefficients (κ) for tumor enhancement pattern indicated excellent reliability (κ = 0.868), especially for the categories CE 0 (“none”, κ = 0.903) and CE 4 (“abundant”, κ = 0.906) (Table 6). The interclass correlation coefficient r was calculated for FLAIR tumor volumes and found equally to reflect excellent interrater reliability at a value of 0.943 (Table 6).

DISCUSSION

5-ALA has been approved for surgery in suspected high-grade gliomas in part because most high-grade gliomas fluoresce. Growing experience indicates that some but not all grade II and grade III gliomas fluoresce, indicating that grade alone does not predict fluorescence status.²,⁵,⁶,¹⁴ Therefore, we undertook this study to identify factors most predictive of observed fluorescence.

We evaluated an array of clinical variables that we considered as plausible determinants of fluorescence in patients with intermediate-grade glioma. Interestingly, our logistic regression classifier, which accurately predicted fluorescence with 91.9% accuracy, revealed enhancement as the singular predictive variable in determining fluorescence in grade II and grade III gliomas. This was unexpected, especially given incorporation of 14 candidate variables in the full logistic regression model. Importantly, the observation linking enhancement to fluorescence was supported in a quantitative manner: The degree of MRI CE directly correlated with observed fluorescence (Figure 2). Our data, however, suggest that gross blood brain barrier (BBB) disruption is neither necessary nor sufficient for fluorescence in gliomas because 19 patients with contrast enhancing tumor did not demonstrate visible fluorescence, and 7 patients lacking enhancement were observed to have visible fluorescence. In patients with enhancement but no fluorescence, we presume that the blood brain barrier was open, but tumor cells did not generate visually detectable levels of protoporphyrin IX (PpIX), the fluorescent metabolite of 5-ALA. Patients lacking enhancement while demonstrating fluorescence reveal that BBB disruption alone does not guarantee accumulation of visible quantities of PpIX. Vascular permeability for a small compound such as 5-ALA (131 Daltons) is only 1 aspect. In addition, given a sufficient metabolic potential of tumor cells, such cells will accumulate PpIX, even in regions in which the integrity of the BBB is great enough to still exclude larger molecules such as gadolinium chelates or fluorescein. To this

| TABLE 6. Fleiss Kappa Coefficients for Enhancement Patterns and Intraclass Correlation Coefficient for FLAIR Tumor Volume |
|---------------------------------------------------------------|
|                                 | Kappa | Standard error | Significance  | Lower 95% CI | Upper 95% CI |
| Overall agreement             | 0.868 | 0.054          | <0.001        | 0.865        | 0.872        |
| CE 0                          | 0.903 | 0.088          | <0.001        | 0.898        | 0.909        |
| CE 1                          | 0.882 | 0.088          | <0.001        | 0.877        | 0.888        |
| CE 2                          | 0.698 | 0.088          | <0.001        | 0.693        | 0.704        |
| CE 3                          | 0.906 | 0.088          | <0.001        | 0.901        | 0.912        |

| Intraclass correlation coefficient | Standard error | Significance  | Lower 95% CI | Upper 95% CI |
|-----------------------------------|----------------|---------------|--------------|--------------|
| FLAIR tumor volume               | 0.943          | n.a.          | <0.001       | 0.905        | 0.967        |

CE, contrast enhancement.
None (CE 0), patchy (multiple smaller areas of enhancement covering less than 50% of any nonenhancing tumor cross section) (CE 1), focal (1 single area of enhancement covering less than 50% of any nonenhancing tumor cross section) (CE 2), or abundant (enhancing tumor volume greater than 50% of nonenhancing tumor) (CE 3).
regard, 5-ALA has been repetitively shown to enable tissue fluorescence beyond the contrast enhancing tumor.\textsuperscript{15,16} To-gether, it is unclear why some nonenhancing gliomas accumulate PpIX and vice versa. These observations support the notion that multiple factors apart from mere BBB disruption and malignancy are responsible for PpIX accumulation. Ferrochelatase deficiency and other molecular changes have also been discussed,\textsuperscript{17} and although BBB permeability may play a permissive role in allowing ALA to pass into the brain, cellular accumulation of downstream PpIX depends on such additional changes.\textsuperscript{18} The correlation of fluorescence and contrast enhancement has previously been reported.\textsuperscript{19,20} However, it is unclear whether BBB disruption is an epiphenomenon related to tumor biology rather than the sole factor governing PpIX accumulation.

The clinical utility for using 5-ALA in apparently lower-grade gliomas is 2-fold. First, 5-ALA has been shown to help finding anaplastic foci, thus establishing an adequate treatment plan according to the accurate histopathological diagnosis.\textsuperscript{21} Second, 5-ALA–induced fluorescence has proven to correlate with oncological prognosis in histologically verified low-grade gliomas.\textsuperscript{5,2,22} Further confirmatory studies will need to show if these observations justify the expanding of FDA approval.

Recognizing the increasingly central role for molecular classification in the modern diagnosis of primary brain tumors,\textsuperscript{8} we examined whether fluorescence might be associated with the major genetic factors used in the WHO grading system. Consistent with a previous publication, we found no correlation between fluorescence and IDH and 1p19q or alpha thalassemia X-linked intellectual disability syndrome (ATRX) status in our cohort.\textsuperscript{2} Given the genetic nuances and heterogeneity of primary brain tumors, it is possible that a more granular analysis evaluating a broader array of genetic alterations in a larger cohort might reveal correlations between molecular factors and fluorescence in the future.

Although molecular factors serve as a better proxy for glioma classification than the concept of tumor grade, predicting grade is of practical importance, especially in the United States where the use of 5-ALA is restricted to use in the context of "suspected" WHO grades III and IV. Grade IV tumors are generally easy to recognize with the classic findings of rim enhancement and central necrosis. On the other hand, using structural MRI features for predicting grade has been challenging with sophisticated novel computational approaches not generally available.\textsuperscript{23-25} To address this challenge, we hypothesized that a systematic approach incorporating preoperative clinical data might yield a more reliable method for predicting grade.

Our systematic approach, incorporating age, pattern of enhancement, midline shift and tumor volume, was effective for predicting grade 74% of the time with better accuracy in grade II (86%) than grade III tumors (53.4%). Although generally useful in the study cohort, the performance of our grade classifier confirmed previous reports that have highlighted the challenge of predicting glioma grade from preoperative clinical data most likely because of overlap of magnetic resonance imaging features found in low-grade and high-grade gliomas.\textsuperscript{26-29}

**CONCLUSION**

Using a logistic regression-based approach to analyze a large cohort for analyzing grade II and III gliomas treated with 5-ALA, we uncovered a strong correlation between enhancement on MRI and the likelihood of fluorescence during surgery in patients with intermediate-grade glioma. Our analysis provides a robust method of assigning a probability of 5-ALA–induced fluorescence based on the pattern of enhancement on preoperative MRI. A prospective evaluation of the statistical models developed in this cohort would provide further evidence of their clinical utility.

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Dr. Johnson and Orringer serve as consultants to NXDC (Lexington, Kentucky, USA). Dr. Orringer is a shareholder in Invenio Imaging, an entity that manufactures microscopes that assist with visualization of microscopic brain tumor infiltration, a topic that is peripherally related to this study. Walter Stummer reports consultant and lecture activities for medac (Wedel, Germany), Zeiss Meditec (Oberkochen, Germany), SBIAlaPharma (Tokyo, Japan), and NXDC (Lexington, Kentucky, USA). The other authors have no personal, financial, or institutional interest in any of the drugs, materials, or devices described in this article. All authors had full access to the data, take full responsibility for the integrity of the data and the accuracy of the data analysis, and had complete freedom and independence in the decision to publish the results of the study.

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