Characterizing the heterogeneity in 5-aminolevulinic acid–induced fluorescence in glioblastoma

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OBJECTIVE 5-aminolevulinic acid (5-ALA)–induced protoporphyrin IX (PpIX) fluorescence is an effective surgical adjunct for the intraoperative identification of tumor tissue during resection of high-grade gliomas. The use of 5-ALA-induced PpIX fluorescence in glioblastoma (GBM) has been shown to double the extent of gross-total resection and 6-month progression-free survival. The heterogeneity of 5-ALA-induced PpIX fluorescence observed during surgery presents a technical and diagnostic challenge when utilizing this tool intraoperatively. While some regions show bright fluorescence after 5-ALA administration, other regions do not, despite that both regions of the tumor may be histopathologically indistinguishable. The authors examined the biological basis of this heterogeneity using computational methods.

METHODS The authors collected both fluorescent and nonfluorescent GBM specimens from a total of 14 patients undergoing surgery and examined their gene expression profiles.

RESULTS In this study, the authors found that the gene expression patterns characterizing fluorescent and nonfluorescent GBM surgical specimens were profoundly different and were associated with distinct cellular functions and different biological pathways. Nonfluorescent tumor tissue tended to resemble the neural subtype of GBM; meanwhile, fluorescent tumor tissue did not exhibit a prominent pattern corresponding to known subtypes of GBM. Consistent with this observation, neural GBM samples from The Cancer Genome Atlas database exhibited a significantly lower fluorescence score than nonneural GBM samples as determined by a fluorescence gene signature developed by the authors.

CONCLUSIONS These results provide a greater understanding regarding the biological basis of differential fluorescence observed intraoperatively and can provide a basis to identify novel strategies to maximize the effectiveness of fluorescence agents.

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KEYWORDS glioblastoma; 5-aminolevulinic acid; tumor heterogeneity; fluorescence-guided surgery; oncology

GLIOMASTOMA (GBM) is the most aggressive and common form of primary brain tumor in adults, with a median patient survival of less than 2 years despite aggressive multimodal treatment that includes maximal resection, chemotherapy, radiation therapy, and, most recently, tumor-treating fields.^{15,30,31} Studies have

demonstrated that maximizing the extent of tumor resection is a significant modifiable factor that improves overall survival.⁵ However, the surgeon's ability to achieve a gross-total resection (GTR) is challenged by the lack of discernible tumor margins, given the diffusely infiltrative nature of GBM.⁴⁵ The use of 5-aminolevulinic acid

ABBREVIATIONS GBM = glioblastoma; GSEA = Gene Set Enrichment Analysis; GTR = gross-total resection; IPA = Ingenuity Pathway Analysis; PC = principal component; PCA = PC analysis; PpIX = protoporphyrin IX; RMA = robust multichip analysis; TCGA = The Cancer Genome Atlas; 5-ALA = 5-aminolevulinic acid.

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(5-ALA)-induced protoporphyrin IX (PpIX) fluorescence has been shown to almost double the rates of GTR and 6-month progression-free survival in patients with GBM.^{10,29} 5-ALA is an endogenous precursor in the heme biosynthesis pathway.²¹ When administered to individuals prior to surgery, the nonfluorescent prodrug 5-ALA leads to overproduction and preferential accumulation of the fluorescent molecule PpIX in malignant or neoplastic cells.²¹ The surgeon can use a surgical microscope modified for fluorescence imaging to illuminate the surgical field with violet-blue light, and tumor tissues with sufficient PpIX will demonstrate a visible red-pink fluorescence and normal tissues will not.²¹

The effectiveness of 5-ALA-induced PpIX fluorescence during resection^{10,29} is compromised by the observed heterogeneity of tumor fluorescence, such that tumor tissues with histopathologically indistinguishable features will demonstrate different fluorescence characteristics from bright fluorescence to no fluorescence.^{16,25,36,37} Tissue fluorescence was identified as bright red fluorescence or no fluorescence, in which the latter is categorized as likely not containing tumor. 5-ALA-induced PpIX fluorescence has a high positive predictive value, such that tissue with visible fluorescence is most likely to contain malignant cells.^{36,37} Nevertheless, this technique has demonstrated a low negative predictive value, in that tissue that does not display visible red fluorescence may or may not contain significant amounts of tumor.^{25,36} We have previously demonstrated the utility of quantitative technologies to help improve sensitivity and detection of lower levels of fluorescence by accounting for the varying effects of tissue optical properties.^{35,37,40} Nevertheless, this prior work did not explore, at a biological level, why some histopathologically indistinguishable tumor tissues produce higher levels of PpIX and others do not. In this study, we found that the gene expression profiles characterizing fluorescent and nonfluorescent GBM surgical specimens were profoundly different and were associated with distinct cellular functions and different biological pathways.

Methods

Patient Tumor Sample Collection

Tissue samples were collected from patients who were part of a larger investigational study to correlate 5-ALA-induced fluorescence, neuroimaging, and histopathology in patients undergoing volumetric resection of intracranial tumor (clinical trial registration no. NCT00870779, clinicaltrials.gov). Both the larger study and this study were approved by the Institutional Review Board for the Protection of Human Subjects, and informed consent was obtained from all patients. 5-ALA was used under an FDA Investigational New Drug approval.

Patients received 5-ALA orally (20 mg/kg bodyweight, dissolved in 50 mL water; DUSA Pharmaceuticals) 3 hours prior to the induction of anesthesia. Tissue to be collected as a study specimen was graded *in situ* for visible fluorescence (no, low, moderate, or high fluorescence) by the surgeon (D.W.R.) under violet-blue light illumination using a Zeiss Pentero operating microscope modified for fluorescence guidance (Blue 400 fluorescence imaging

module, Carl Zeiss Surgical GmbH). Specimens in this study had fluorescence grades of either no fluorescence or moderate/high fluorescence. Biopsies of sampled sites were divided into portions placed in formalin for histopathology or flash frozen and stored at -80°C for subsequent microarray analysis (Fig. 1).

Histopathology

Tissues were formalin fixed, paraffin embedded, and stained with H & E. Each H & E-stained tissue section was assessed based on WHO histopathological criteria by a neuropathologist (B.T.H.) as previously described.²⁵

Microarray Analysis

Microarray analysis was conducted by the Dartmouth Genomics Shared Resource. Total RNA was purified using the TRIzol method and labeled using the Illumina Total-Prep kit protocol or Affymetrix WT Plus kit per manufacturer's guidelines, and probed using the HumanHT-12 v4 BeadChip (Illumina) for the discovery study or the Clariom S human assay (Affymetrix) for the validation study. Raw data generated from the HumanHT-12 v4 BeadChip were processed in the BRB Array Tools Software using Variance Stabilizing Transformation followed by Robust Spline Normalization. Raw data generated from Clariom S mouse arrays were processed using Affymetrix Expression Console Software. CEL files containing feature intensity values were converted into summarized expression values by robust multichip analysis (RMA), which consists of a background adjustment, quantile normalization, and summarization across all chips. All samples passed quality control thresholds for hybridization, labeling, and the expression of housekeeping gene controls.

Bioinformatics and Statistical Analysis

To generate the list of differentially expressed genes (Supplementary Tables 1 and 2), we RMA normalized the microarray expression data of fluorescent and nonfluorescent specimens and converted the data to log₂ values. To obtain the log₂ fold change values, we averaged fluorescent and nonfluorescent specimen values for each gene, and we used the Student t-test to determine statistical significance. The volcano plot was generated using R (r-project.org). The fold changes and associated p values of the differential gene expression between fluorescent and nonfluorescent samples were exported using R for further analysis by Ingenuity Pathway Analysis (IPA).²⁷ Further statistical analyses and heat maps were performed with GraphPad Prism version 7.02 for Windows. Gene Set Enrichment Analysis (GSEA) was performed as previously described utilizing the GO and BIOCARTA gene sets indicated in Supplementary Fig. 1A–F.^{3,24,32}

To perform the principal component analysis (PCA), the expression levels of fluorescent and nonfluorescent samples from 10 patients were transformed into PCs using the R package FactoMineR.¹⁹ Centroid values of the genes corresponding to the classical, mesenchymal, neural, and proneural subtypes of GBM were downloaded from https://tcga-data.nci.nih.gov/docs/publications/gbm_exp/ and separately transformed into PCs. We plotted the top 3

FIG. 1. Schematic representation of the experimental workflow to analyze the pathologic grade and mRNA expression in fluorescent and nonfluorescent GBM-derived tissue.

PCs derived from the centroid values and the microarray data from fluorescent and nonfluorescent samples (see Fig. 2). A cutoff ($\log_2$ fold change $p < 0.1$) was set to define differential gene expression given the small sample size of this study. A validation experiment, which was performed using samples from 4 different patients and an independent microarray platform, provided additional confidence in our analysis.²⁰

We generated signature scores of the samples for each of The Cancer Genome Atlas (TCGA) GBM patients utilizing the Cox proportional hazards model as previously described.¹ Scores of less than 0 for either the fluorescent or nonfluorescent signatures were categorized as “low” and scores greater than 0 were categorized as “high.” To generate the fluorescence scores utilized in Fig. 3 and Supplementary Fig. 2, the microarray profiles of fluorescent and nonfluorescent samples were compared to one another by taking the $\log_2$ fold change of all genes in the array. A p value was computed for each gene and $-\log_{10}$ transformed. These values were then used to generate fluorescence and nonfluorescence signatures and scores as previously described.²

Results

Intraoperative 5-ALA-Induced Fluorescence in GBM Specimens Is Associated With a Distinct mRNA Expression Profile

To better understand the biological basis for the het-

erogeneity of 5-ALA-induced PpIX fluorescence in GBM, both fluorescent and nonfluorescent GBM specimens were collected from surgical patients undergoing 5-ALA-induced PpIX fluorescence-guided surgery following our previously published methodology.^{35,37,40} Tissue specimens were examined using microarray technology (Fig. 1). Both fluorescent and nonfluorescent specimens represented viable tissue with similar histological appearance (Fig. 2). Specimens with large areas of necrosis were excluded from analysis. As would be expected, microarray expression profiles of high-grade glioma specimens were associated with poor patient survival regardless of their fluorescence status (Fig. 3). We derived a signature of fluorescence and a signature of nonfluorescence utilizing the most upregulated genes in fluorescent and nonfluorescent samples, respectively, as described in *Methods*. We then used these molecular signatures to derive fluorescence and nonfluorescence “scores” for each of the patients diagnosed with GBM in TCGA database as we have previously reported.¹ We found that both signatures were associated with decreased survival in TCGA patients (Fig. 3A and B) without a significant overall survival difference between fluorescence groups (Fig. 3C). These observations are consistent with the fact that fluorescent and nonfluorescent GBM specimens in this study were extracted from tumor regions of equivalent pathologic grade (Fig. 2).

The gene expression profiles from fluorescent and nonfluorescent GBM specimens were markedly different (Fig. 4, Supplementary Tables 1 and 2). Tissue with

FIG. 2. Microscopy images of fluorescent and nonfluorescent GBM specimens. Representative high-power H & E images from 3 cases with biopsies from fluorescent and nonfluorescent resection areas. Each row represents a different case. Bar = 100 μ m, original magnification $\times 400$.

visible fluorescence expressed higher levels of genes such as *IGFBP2*, *CHI3L1*, *COL4A1*, and *VGF* that are associated with growth, survival, or angiogenesis (Fig. 4A and

B).^{7,14,43,46} In contrast, nonfluorescent samples expressed genes associated with mature cells of the central nervous system such as *MOBP*, *MAL*, and *OPALIN* (Fig. 4A and C).^{4,9,11} Importantly, expression of genes in the PpIX biosynthetic pathway between fluorescent and nonfluorescent GBM samples was not significantly different (Supplementary Table 3).

IPA and GSEA of the gene expression patterns from fluorescent and nonfluorescent GBM specimens revealed that they were associated with distinct cellular functions and biological pathways (Fig. 4D and E, Supplementary Fig. 1A–F).^{18,32} Interestingly, nonfluorescent GBM specimens expressed genes associated with IPA cellular functions characteristic of the neural subtype of GBM such as neural development, neuritogenesis, and neurotransmission (Fig. 4E).⁴² Consistent with this finding, the gene expression profile of nonfluorescent GBM specimens was also enriched for genes associated with GO pathways such as neuron projection development and synaptic transmission (Supplementary Fig. 1D and F).^{3,18} The neural subtype of GBM is the subtype that has a gene expression pattern most similar to the pattern observed in normal brain tissue such as mature neurons and oligodendrocytes.⁴²

The gene expression profile of fluorescent GBM specimens, however, was not enriched for genes associated with any one specific molecular subgroup of GBM⁴² (Fig. 4D, Supplementary Fig. 1A–C). The fluorescent specimens expressed genes associated with cellular functions shared by all subgroups of GBM such as proliferation, survival, and invasion (Fig. 4D). Similarly, GSEA revealed that the gene expression profile of fluorescent specimens was enriched for genes in GO and BIOCARTA pathways characteristic of the proneural, mesenchymal, and classical subtypes of GBM such as NF- κ B signaling, TNFR1 signaling, and Notch signaling (Supplementary Fig. 1A–C).^{3,8,12,24,26,42} To-

FIG. 3. Signatures derived from top upregulated genes in fluorescent and nonfluorescent samples are associated with decreased survival in GBM patients. **A:** Kaplan-Meier survival curves comparing TCGA GBM patients with high fluorescent signature scores (High) and low fluorescent signature scores (Low). Log-rank test, $p = 0.000441$. **B:** Kaplan-Meier survival curves comparing GBM patients with high nonfluorescent signature scores and low nonfluorescent signature scores. Log-rank test, $p = 0.000013$. **C:** Kaplan-Meier survival curves comparing GBM patients with high fluorescent signature scores (High Fluor) and GBM patients with high nonfluorescent signature scores (High Non-Fluor). Log-rank test, $p = 0.454$.

FIG. 4. Intraoperative 5-ALA-induced fluorescence is associated with a distinct mRNA expression profile in human GBM specimens. **A:** Volcano plot of differentially expressed genes in fluorescent GBM specimens in comparison with nonfluorescent GBM specimens expressed as $\log_2$ fold change. Green dots represent genes whose $\log_2$ fold change values are at least 2 with a statistical significance of $p < 0.02$. Red dots represent genes whose $\log_2$ fold change values have a statistical significance of $p < 0.05$. Orange dots represent genes whose $\log_2$ fold change values have a statistical significance of $0.05 < p < 0.1$. Black dots represent genes whose $\log_2$ fold change values have a statistical significance of $p < 0.1$. **B:** The 30 most highly expressed genes in fluorescent human GBM specimens when compared with nonfluorescent human GBM specimens. Data represent the $\log_2$ fold change of fluorescent GBM specimens ($n = 9$) relative to nonfluorescent specimens ($n = 9$) $\pm$ SEM. **C:** The 30 most highly expressed genes in nonfluorescent human GBM specimens when compared with the gene expression found in fluorescent human GBM specimens. Data represent the $\log_2$ fold change of nonfluorescent GBM specimens ($n = 9$) relative to fluorescent specimens ($n = 9$) $\pm$ SEM. **D:** The top 10 cellular functions associated with genes significantly upregulated ($p < 0.05$) in fluorescent GBM specimens when compared with nonfluorescent GBM specimens as determined by IPA.¹⁸ **E:** Top 10 cellular functions associated with genes significantly upregulated ($p < 0.05$) in nonfluorescent GBM specimens when compared with fluorescent GBM specimens as determined by IPA.

gether, these data demonstrate that fluorescent and non-fluorescent specimens have markedly distinct genetic profiles.

The Gene Expression Profiles of Nonfluorescent GBM Specimens Cluster Around the Neural Subtype of Glioma

To further characterize the genetic profile of fluorescent and nonfluorescent GBM specimens, we performed a PCA of the gene expression profile from 20 fluorescent and nonfluorescent GBM surgical specimens along with the molecular signatures of the 4 subtypes of GBM: classical, mesenchymal, neural, and proneural (Fig. 5A). To perform this analysis, the gene expression levels of these GBM specimens and the centroid values of the molecular signatures corresponding to each of the 4 subtypes of GBM were transformed into PCs.⁴² In Fig. 5A, we plotted these data utilizing the top 3 PCs derived from this analysis. We observed that the gene expression profiles of the nonfluorescent GBM specimens clustered around the neural subtype of glioma, while those of the fluorescent GBM specimens did not. To validate this finding, we replicated this experiment with microarray expression data from 4 different patients that were obtained using an independent microarray platform (Fig. 5B). In Fig. 5B, we

confirmed that the expression profile of the nonfluorescent GBM specimens most closely resembles the neural subtype of glioma.

To corroborate our findings in a larger cohort of patients, we developed a fluorescence signature from the differential gene expression profile between the fluorescent and nonfluorescent GBM specimens. We then used this molecular signature to derive a fluorescence score for each of the neural and nonneural GBM samples in TCGA database as indicated in *Methods*. We observed that human GBM samples in TCGA database that belonged to the neural subgroup of GBM exhibited significantly lower fluorescence scores than GBM samples that belonged to the other subgroups (Fig. 5C). These data demonstrate that fluorescent and nonfluorescent GBM tissues express distinct gene patterns.

Interestingly, GBM samples from TCGA database exhibited mutations in specific genes based on their fluorescence score. Tumors with mutations in the ABCC9, a protein of the ABC transporter family responsible for the efflux of 5-ALA,¹³ had significantly higher fluorescence scores ($p = 0.0086$) consistent with the possibility that the ABCC9 transporter could be playing a role in 5-ALA-induced PpIX fluorescence in GBM. Tumors with mutations

FIG. 5. Molecular profile of nonfluorescence GBM tissue most closely resembles the neural subtype of glioma. **A:** PCA plot of GBM molecular subtypes and GBM specimens. GBM subtype centroids corresponding to the classical, mesenchymal, neural, and proneural subtypes of GBM are illustrated as *black dots*. Letters A–J denote the 10 different human tumors analyzed. GBM specimens that fluoresced after 5-ALA administration are illustrated as *green dots*, and GBM specimens that did not fluoresce after 5-ALA administration are illustrated as *gray dots*. **B:** Confirmatory PCA plot of GBM molecular subtypes corresponding to 7 human GBM specimens. GBM subtype centroids corresponding to the classical, mesenchymal, neural, and proneural subtypes of GBM are illustrated as *black dots*. Letters W–Z denote the 4 different human tumors analyzed. GBM specimens that fluoresce after 5-ALA administration are illustrated as *green dots*, and GBM specimens that do not fluoresce after 5-ALA administration are illustrated as *gray dots*. **C:** Fluorescence score of GBM patients from TCGA, as determined by our fluorescence signature, subdivided as either neural or nonneural. Data represent 600 GBM patient samples presented as the mean $\pm$ SD, *** $p < 0.001$.

in IDH1 and ATRX had significantly lower fluorescent scores ($p = 0.00038$ and $p = 0.00076$, respectively) (Supplementary Fig. 2).^{6,17,23} These observations further reveal the distinctions between fluorescent and nonfluorescent GBM tissues.

Discussion

GBM is the most common and aggressive primary brain tumor in adults. Despite optimal surgical and medical therapy, recurrence of disease typically develops adjacent to the resection margins due to residual microscopic infiltrating glioma cells that were not resected or eradicated after radiation or chemotherapy. 5-ALA-induced PpIX fluorescence-guided resection has been shown to improve GTR

and 6-month progression-free survival in patients.^{10,29} However, the efficacy of this technique in GBM has been compromised by the lack of visible 5-ALA-induced PpIX fluorescence in histopathologically confirmed tumor tissue.^{16,25,36,37,44} A better understanding of the biological basis for this heterogeneity in 5-ALA-induced PpIX tumor fluorescence might contribute to improving intraoperative detection of malignant tissue and decreasing the rate of glioma recurrence.

Our findings indicate that tissues taken from different areas of the same GBM tumor exhibit different levels of 5-ALA (fluorescence vs nonfluorescence) and different patterns of gene expressions measured at the transcriptional level. These observations suggest a molecular basis for the

heterogeneity in 5-ALA-induced fluorescence observed in different GBM tumors and within individual tumors. Consistent with this notion, nonfluorescent regions were observed to more closely resemble the neural subgroup of GBM.

Understanding of those factors responsible for false-negative fluorescence imaging and the suboptimal predictive value of tissue that does not fluoresce is incomplete. Surgeons should be aware that a lack of fluorescence in high-grade glioma may result not only from tumor necrosis, an intact blood-brain barrier, low levels of tumor cell invasion, or lower-grade tumor, but also from molecular subtypes of high-grade tumor being present within an individual tumor. PpIX fluorescence is not just a function of PpIX tissue levels, but it also reflects tissue architecture and constituents.^{22,37,39–41} Variable optical properties of tissues may “mask” PpIX fluorescence. These optical properties are themselves determined by factors such as cellularity, necrosis, oxygenation, vascularity, and hemoglobin, among other factors. Our work provides an additional determinant, namely, cellular variability based on different transcriptional patterns that characterize GBM subtypes.

Roberts and colleagues previously showed that using a multiparametric approach that detects not only PpIX but additional biomarkers using a quantitative optical probe significantly improved diagnostic accuracy (e.g., improved sensitivity and negative predictive value) not just in high-grade gliomas but also in other tumor subtypes such as low-grade gliomas.³⁸ Since GBM is very heterogeneous, often containing different glioma molecular subtypes (neural, proneural, mesenchymal, or classical) within the same tumor, an approach using multiple fluorophore and optical biomarkers may be more effective in detecting these highly heterogeneous tumors intraoperatively. Combining 2 or more different fluorescent biomarkers for intraoperative detection methods, such as ALA, fluorescein, anti-EGFR or anti-VEGFR targeted fluorophores, quantum dot nanoparticles, or other agents, one could potentially target different subtypes of GBM simultaneously^{27,39} and thereby improve intraoperative tumor detection and GTR. To this end, an improved molecular understanding will help guide further technological developments to advance the field of fluorescence-guided neurosurgical oncology.

Conclusions

In this study, we found that fluorescent and nonfluorescent GBM surgical specimens expressed distinct genetic expression profiles (Figs. 3–5). The expression profile of nonfluorescent tumor tissue was enriched for genes associated with cellular functions and biological pathways characteristic of the neural subgroup of GBM (Figs. 3 and 4). Furthermore, PCA revealed that the expression profiles of nonfluorescent tumor tissues most closely resemble the neural subgroup of GBM (Fig. 5A). Consistent with these observations, human GBM samples from TCGA database that belonged to the neural subgroup of GBM were identified to have lower fluorescence scores than samples that belonged to other subgroups of GBM (Fig. 5B).

In a manner consistent with the high degree of heterogeneity observed in high-grade gliomas,²⁸ our results dem-

onstrate that sections within glioma tumors that respond differently to 5-ALA are molecularly distinct. These findings are consistent with previous findings demonstrating differences in individual gene expression associated with 5-ALA-induced fluorescence in GBM.^{33,34,47} Furthermore, these findings are consistent with other work using quantitative technologies, which highlights the importance of tissue constituents and their effects on the detected PpIX fluorescence.³⁸ That work provides further rationale to develop novel technologies for improved detection of PpIX fluorescence and intrinsic tissue properties toward a multiparametric approach for fluorescence-guided surgery as well as help explore combinations of fluorescence agents such as fluorescein, quantum dot nanoparticles, anti-EGFR, or anti-VEGFR antibody-bound fluorophores that could potentially target different molecular subtypes of GBM.²⁷

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Supplemental Information

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