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The value of visible 5-ALA fluorescence and quantitative protoporphyrin IX analysis for improved surgery of suspected low-grade gliomas

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Abstract

OBJECTIVE—In patients with suspected diffusely infiltrating low-grade gliomas (LGG), the prognosis is dependent especially on extent of resection and precision of tissue sampling. Unfortunately, visible 5-aminolevulinic acid (5-ALA) fluorescence is usually only present in high-grade gliomas (HGGs), and most LGGs cannot be visualized. Recently, spectroscopic probes were introduced allowing in vivo quantitative analysis of intratumoral 5-ALA–induced protoporphyrin IX (PpIX) accumulation. The aim of this study was to intraoperatively investigate the value of visible 5-ALA fluorescence and quantitative PpIX analysis in suspected diffusely infiltrating LGG.

METHODS—Patients with radiologically suspected diffusely infiltrating LGG were prospectively recruited, and 5-ALA was preoperatively administered. During resection, visual fluorescence and absolute tissue PpIX concentration (C_{PpIX}) measured by a spectroscopic handheld probe were determined in different intratumoral areas. Subsequently, corresponding tissue samples were safely collected for histopathological analysis. Tumor diagnosis was established according to the World Health Organization 2016 criteria. Additionally, the tumor grade and percentage of tumor cells were investigated in each sample.

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Author Contributions

Conception and design: Berger, Widhalm, Han, Chang, Roberts. Acquisition of data: Berger, Widhalm, Olson, Weller, Bravo, Han, Phillips, Hervey-Jumper, Roberts. Analysis and interpretation of data: all authors. Drafting the article: Berger, Widhalm, Olson, Bravo, Han, Phillips, Hervey-Jumper, Chang, Roberts. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Berger. Statistical analysis: Widhalm. Administrative/technical/material support: Berger, Olson, Bravo, Roberts. Study supervision: Berger.

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Disclosures

Dr. Roberts reports being a cofounder of and having an ownership interest in InSight Surgical Technologies LLC.

RESULTS—All together, 69 samples were collected from 22 patients with histopathologically confirmed diffusely infiltrating glioma. Visible fluorescence was detected in focal areas in most HGGs (79%), but in none of the 8 LGGs. The mean C_{PpIX} was significantly higher in fluorescing samples than in nonfluorescing samples (0.693 μg/ml and 0.008 μg/ml, respectively; $p < 0.001$). A significantly higher mean percentage of tumor cells was found in samples with visible fluorescence compared to samples with no fluorescence (62% and 34%, respectively; $p = 0.005$), and significant correlation of C_{PpIX} and percentage of tumor cells was found ($r = 0.362$, $p = 0.002$). Moreover, high-grade histology was significantly more common in fluorescing samples than in nonfluorescing samples ($p = 0.001$), whereas no statistically significant difference in mean C_{PpIX} was noted between HGG and LGG samples. Correlation between maximum C_{PpIX} and overall tumor grade was highly significant ($p = 0.005$). Finally, 14 (40%) of 35 tumor samples with no visible fluorescence and 16 (50%) of 32 LGG samples showed significantly increased C_{PpIX} (cutoff value: 0.005 μg/ml).

CONCLUSIONS—Visible 5-ALA fluorescence is able to detect focal intratumoral areas of malignant transformation, and additional quantitative PpIX analysis is especially useful to visualize mainly LGG tissue that usually remains undetected by conventional fluorescence. Thus, both techniques will support the neurosurgeon in achieving maximal safe resection and increased precision of tissue sampling during surgery for suspected LGG.

Keywords

suspected LGG; visible fluorescence; PpIX analysis; tumor visualization; anaplastic foci; oncology

NEUROSURGICAL resection is the primary treatment of diffusely infiltrating low-grade gliomas (LGGs).[26] There exists clear evidence that maximal safe resection of LGG results in a significantly improved prognosis.[9,21,25] However, LGGs constitute a special challenge for the neurosurgeon due to their only minor differences in appearance compared with normal brain, and to their histopathological intratumoral heterogeneity with potential high-grade glioma (HGG) areas (anaplastic foci).[16,19] This insufficient visualization of LGG might result in incomplete resections.[22,24,25] A large study from multiple centers found a complete resection with “no residual signal abnormality on postoperative magnetic resonance imaging (MRI)” in only 80 (12%) of 674 analyzed LGG.[3] Furthermore, incorrect histopathological tumor diagnosis/tumor grading might occur if tissue sampling during surgery is not conducted from a potential anaplastic focus.[13,19] Although molecular markers are nowadays of major importance for postoperative treatment decisions, the tumor grade still plays an important role. Thus, histopathological undergrading of gliomas due to sampling error might influence decision making with respect to postoperative patient management and could delay adjuvant treatments.

Currently, visualization of brain tumors with 5-aminolevulinic acid (5-ALA) fluorescence is widely applied to maximize the resection of HGG.[7,28] After oral 5-ALA administration, this well-tolerated drug results in intratumoral accumulation of fluorescing protoporphyrin IX (PpIX).[7,35] Moreover, 5-ALA was also identified as a powerful marker for intraoperative detection of anaplastic foci in initially suspected LGGs.[5,36,38] However, the actual fluorescence technique is not able to visualize the vast majority of pure LGGs and a

subgroup of WHO grade III gliomas/anaplastic foci.[5,23,36,38] To overcome these current limitations, intraoperative tools are required to optimize identification of PpIX accumulation in suspected LGGs.

Lately, a novel method for intraoperative identification of brain tumors originating from fluorescence detection technology was introduced: the use of handheld spectroscopic probes,[8,32] which are capable of measuring the absolute intratumoral PpIX concentration (C_{PpIX}) during resections after preoperative 5-ALA administration.[32] Quantification of this intratumoral PpIX accumulation allows objective measurement of fluorescence and seems to be a promising tool for intraoperative identification of brain tumors that remain undetected by conventional fluorescence.[32] Recently, Valdes et al. investigated this innovative tool in the first low-grade tumors and detected significant C_{PpIX} in such usually nonfluorescing tumors as well.[30]

The aim of this study is to systematically analyze the value of visible 5-ALA fluorescence and quantitative PpIX analysis by a spectroscopic handheld probe in radiologically suspected diffusely infiltrating LGG. For this purpose, visible fluorescence and C_{PpIX} were investigated in multiple intratumoral areas and corresponding tissue samples were collected for histopathological analyses.

Methods

Patients undergoing resection of a radiologically suspected newly diagnosed or recurrent diffusely infiltrating LGG were prospectively recruited between November 2016 and August 2017 at the Department of Neurological Surgery, University of California, San Francisco (UCSF). This study was approved by the UCSF Committee of Human Research and all included patients gave informed consent. This study was registered with the ClinicalTrials.gov database (http://clinicaltrials.gov; registration number NCT01116661) and is part of that trial. The present study was performed under an investigational new drug application including a protocol specifically for patients with suspected LGG and the spectroscopic probe/analytical methods.

Study Cohort

Inclusion and exclusion criteria of a previously published UCSF clinical phase II trial of 5-ALA fluorescence-guided resection were applied in this study.[14] In brief, adult patients between 18 and 72 years with a Karnofsky Performance Scale score ≥ 60 and normal organ/marrow function were included. Patients with a personal or family history of porphyria, hypotension, uncontrolled concurrent illness, pregnancy, or history of allergy to compounds with composition similar to 5-ALA were excluded.[14] Furthermore, patients enrolled in clinical trials of investigational therapeutic agents were excluded according to the study protocol. Finally, patients with a post-resection histopathological diagnosis other than diffusely infiltrating glioma (WHO grades II–IV) were excluded from the final study cohort.

Preoperative Imaging and 5-ALA Management

A specific MRI protocol for brain tumors including contrast-enhanced sequences and diffusion tensor imaging (DTI) was conducted on a 3-T clinical scanner prior to surgery for

preoperative planning and intraoperative navigation. According to the pattern of contrast enhancement (CE) on T1-weighted images, only suspected diffusely infiltrating LGGs with nonsignificant CE (no CE, patchy/faint CE, or focal CE) were included in this study as described previously.[36–38] Since slight CE on preoperative MRI might occur in a subgroup of LGGs, especially in oligodendrogliomas,[18,34] we also included gliomas with minor CE (patchy/faint or focal CE) in this study to avoid exclusion of low-grade oligodendrogliomas. Approximately 3 hours before anesthesia, patients received a standard oral dose of 5-ALA (20 mg/kg body weight; DUSA Pharmaceuticals/Sun Pharma).[7,29,35] Patients were protected from sunlight and strong indoor light sources for up to 72 hours after 5-ALA administration to minimize the risk of potential drug-related phototoxicity.

Spectroscopic Probe for In Vivo Quantitative Measurement of PpIX Accumulation

In this study, we used a handheld spectroscopic probe previously described by the group of Roberts and Valdés.[2,30,32] The custom system consists of a 4-fiber fiberoptic probe connected to LED light sources and a spectrometer (with wavelength response of 200–1100 nm), controlled by a laptop computer.[30,32] To accomplish absolute fluorophore concentrations, tissue optical properties (absorption and scattering) were determined at the time of each measurement using a light-transport model and the white-light (450–720 nm) reflectance spectra. The spectrum generated by the fluorescent emission from blue-light excitation (405 nm) was spectrally resolved for PpIX, autofluorescence, and the principal PpIX photoproducts, and corrected for optical properties. Quantification of tissue PpIX concentration (C_{PpIX}) was then accomplished using a PpIX basis spectrum with equivalent fluorophore concentration in μg/ml. The measurement of C_{PpIX} required less than 5 seconds.

Glioma Resection With Analysis of Visible and Quantitative Fluorescence

Tumor resection was routinely performed with navigational guidance (Brainlab) including DTI fiber tracts. All neurosurgical procedures were conducted by the senior author (M.S.B.). Dependent on tumor localization, awake or asleep brain mapping/stimulation was used to limit the resection and thus minimize the risk of a new postoperative neurological deficit. In addition to routine tumor resection, we investigated the visual fluorescence status as well as conducted quantitative PpIX analysis in different areas of the suspected tumor (inside the T2-weighted/FLAIR abnormality) and, if feasible, of the suspected tumor margin (border of the T2-weighted/FLAIR abnormality). For this purpose, the modified microscope was switched to violet-blue excitation light repeatedly during surgery, and various fluorescing and/or nonfluorescing areas were analyzed in detail. The visible fluorescence level was classified by the senior author (M.S.B.) as no fluorescence (5-ALA level 0), mild brightness (5-ALA level I), moderate brightness (5-ALA level II), or robust brightness (5-ALA level III) as described previously in each of these areas.[14,20,30,32] Additionally, the C_{PpIX} was measured in each area using the spectroscopic probe. Finally, corresponding tissue samples of all analyzed areas were safely collected for histopathological analysis. The site of each tissue collection within the area of suspected tumor or margin was documented by a navigation-screen snapshot. As a reference, we also analyzed the C_{PpIX} of “normal cortex” areas. “Normal cortex” was defined as an area of normal-appearing cortical surface exposed after craniotomy and dura opening outside the T2-weighted/FLAIR abnormality verified by the navigation and not included in the site of tumor resection. The C_{PpIX} measurements of

the normal cortex were performed in areas as far as possible from the tumor region. In contrast to the above-described collection of specimens from different fluorescing and/or nonfluorescing areas, however, we never collected tissue samples from areas of normal cortex.

Histopathological Assessment

All formalin-fixed and paraffin-embedded tissue samples were processed for routine histopathology. The tumor diagnosis of each patient was established according to the histopathological World Health Organization (WHO) 2016 criteria.[15] We also assessed the isocitrate dehydrogenase (IDH) mutational status by immunohistochemistry, applying the IDH1-R132H mutation-specific antibody (anti–human IDH1-R132H). According to the guideline of the European Association for Neuro-Oncology (EANO), negative immunostaining for IDH1-R132H mutation was followed by sequencing for alternative mutations in *IDH1* or *IDH2* in patients who were 55 years of age or younger at diagnosis or had a history of a pre-existing lower-grade glioma.[33] Additionally, the determination of tumor content, tumor grade, and percentage of tumor cells were analyzed in each of the collected samples by an experienced board-certified neuropathologist (J.P.). The determination of tumor content and grade was based on the criteria used by a neuropathologist for a clinical diagnosis of diffusely infiltrating glioma as defined by the WHO.[15] Distinction between infiltrating neoplastic cells and reactive astrocytes/other stromal cells was performed according to routine clinical practice.

Statistical Analysis

Statistical analyses were performed using SPSS version 24.0 software (IBM Corp.). Dichotomous variables were compared with the fluorescence status using chi-square tests. For analyses of differences in visible fluorescence or C_{PpIX} between 2 features, the nonparametric Mann-Whitney U-test was applied, and for differences between more than 2 features the Kruskal-Wallis test was applied. For correlation of visible fluorescence and the amount of CE, the Kendall tau correlation coefficient was used, and the Spearman rank correlation coefficient was used for assessing correlation between C_{PpIX} and percentage of tumor cells. Receiver operating characteristic (ROC) curve analysis was applied for definition of an optimal C_{PpIX} cutoff value to distinguish between tumor and tumor-free/normal tissue. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for indication of tumor tissue depending on visible fluorescence and quantitative PpIX analysis. A p value of < 0.05 was considered significant.

Results

All together, 23 patients with resection of a suspected diffusely infiltrating LGG were recruited. After histopathological examination, the presence of a diffusely infiltrating glioma (WHO grades II–IV) was confirmed in 22 of these patients. In the remaining patient, the initially suspected LGG was histopathologically classified as “glioneural lesion” and thus this patient was excluded. Therefore, 22 patients with a histopathologically confirmed diffusely infiltrating glioma (8 WHO grade II, 11 WHO grade III, and 3 WHO grade IV

gliomas) formed our final study cohort. In the course of this study, we did not observe any significant side-effects associated with 5-ALA administration.

Patient Characteristics

The group of 22 patients included 16 men and 6 women with a median age of 49 years (range 24–70 years). Twelve of the tumors were newly diagnosed (55%) and 10 were recurrent gliomas (45%). The most common tumor localization was the frontal lobe (9/22 cases; 41%), followed by the temporal lobe (7/22 cases; 32%) and the insular region (4/22 cases; 18%). According to preoperative MRI, no CE was found in 11 cases (50%), focal CE was found in 6 cases (27%), and patchy/faint CE (23%) was found in 5 cases. Further details are shown in Tables 1 and 2.

Visible 5-ALA Fluorescence

Suspected LGG—Visible fluorescence (5-ALA level II in 9 patients and 5-ALA level I in 2 patients) was observed during surgery in 11 patients (50%) and was present only in focal intratumoral regions. In contrast, no fluorescence (5-ALA level 0) was found in 11 patients (50%). We found a significant correlation of visible fluorescence and the amount of CE on MRI ($\tau = 0.577$, $p < 0.001$). In terms of tumor grade, all 3 (100%) WHO grade IV gliomas, 8 (73%) of 11 WHO grade III gliomas, and none of 8 WHO grade II gliomas demonstrated visible fluorescence. We did not find a statistically significant difference in visible fluorescence between IDH-mutated and wildtype tumors or between newly diagnosed and recurrent gliomas.

Tissue Samples—Visible and quantitative fluorescence was investigated in 69 regions (median per patient 3, range 1–6) of different intratumoral areas, and the time period of these fluorescence analyses was in the range of approximately 5–10 hours after 5-ALA administration (Table 2). Visible fluorescence was found in 18 (32%) of 56 suspected tumor regions and none of 13 analyzed suspected margin areas. All together, 51 nonfluorescing samples (5-ALA level 0) and 18 fluorescing samples (5-ALA level II in 10 samples and 5-ALA level I in 8 samples) were safely collected. Tumor cells were present in 50 samples, whereas 19 specimens did not show definite tumor cells. A significantly higher mean percentage of tumor cells (62% vs 34%, $p = 0.005$) and frequency of high-grade histology ($p = 0.001$) were found in fluorescing compared to nonfluorescing samples.

Quantitative PpIX Analysis

Suspected LGG—In the 22 gliomas, the maximum C_{PpIX} investigated by the spectroscopic probe ranged from less than 0.001 μg/ml to 2.824 μg/ml. In the single patient with $C_{PpIX} < 0.001$ μg/ml, only 1 suspected tumor region was available for analysis, and it did not contain tumor tissue according to histopathological assessment. In our study, the mean C_{PpIX} of normal cortex (n = 18) was 0.001 μg/ml (SD 0.001 μg/ml). In the remaining 4 patients, measurement of C_{PpIX} in the normal cortex was not possible because the limited size of the craniotomy did not allow sufficient access to normal brain. The maximum C_{PpIX} was significantly higher in gliomas with high-grade (mean C_{PpIX} 0.785 μg/ml, SD 0.994 μg/ml) compared to low-grade histology (mean C_{PpIX} 0.008 μg/ml, SD 0.008 μg/ml, $p = 0.005$) as well as tumors with visible fluorescence (mean C_{PpIX} 0.987 μg/ml, SD 1.036

μg/ml) compared to no fluorescence (mean C_{PpIX} 0.017 μg/ml, SD 0.035 μg/ml, $p < 0.001$). Furthermore, we found a significant difference in C_{PpIX} and the amount of CE on MRI ($p = 0.019$). We did not find a statistically significant difference in C_{PpIX} between IDH-mutated and wildtype tumors as well as newly diagnosed and recurrent gliomas.

Tissue Samples—In terms of different intratumoral areas, the mean C_{PpIX} was significantly higher in suspected tumor regions (mean C_{PpIX} 0.230 μg/ml, SD 0.592 μg/ml) compared to suspected margin areas (mean C_{PpIX} 0.002 μg/ml, SD 0.002 μg/ml, $p = 0.001$). Furthermore, the mean C_{PpIX} was significantly higher in fluorescing samples (mean C_{PpIX} 0.693 μg/ml, SD 0.893 μg/ml) compared to nonfluorescing samples (mean C_{PpIX} 0.008 μg/ml, SD0.019 μg/ml, $p < 0.001$). Moreover, a significant correlation of C_{PpIX} and percentage of tumor cells was noted ($r = 0.362$, $p = 0.002$). Finally, we did not find a statistically significant difference in the mean C_{PpIX} between LGG and HGG samples. Further details of visible/quantitative fluorescence analyses and illustrative cases are provided in Table 2 and Figs. 1–3.

Diagnostic Performance of Visible and Quantitative Fluorescence

With regard to visible fluorescence, the sensitivity for detection of tumor tissue was 30%, the specificity was 84%, the PPV was 83%, and the NPV was 31%. Prior to the corresponding calculations of quantitative fluorescence, we determined an optimal C_{PpIX} cutoff value (0.005 μg/ml) for our study cohort using ROC curve analyses (see also Fig. 1G) including C_{PpIX} of all normal cortex measurements and tumor-free samples as well as all tumor-containing samples. By application of our predefined C_{PpIX} cutoff value of 0.005 μg/ml, the sensitivity of quantitative fluorescence was 58%, the specificity was 81%, the PPV was 81%, and the NPV was 59%. Moreover, 14 (40%) of 35 tumor samples not visible with conventional fluorescence (27 WHO grade II and 8 WHO grade III samples) showed significantly increased C_{PpIX}. See also Table 3.

Usefulness of Quantitative Fluorescence in Actual LGGs

Focusing only on actual LGGs, quantitative fluorescence found increased C_{PpIX} values (C_{PpIX} cutoff value: 0.005 μg/ml) in 6 (75%) of 8 LGGs and was thus able to identify these tumors that were all not detectable with conventional visible fluorescence. Furthermore, 32 samples with LGG histology were collected in our study. Quantitative fluorescence was capable of detecting 16 (50%) of these 32 LGG samples. It is of note that the majority of these samples (69%; 11 of 16 samples) could not be detected by visible fluorescence. According to the diagnostic performance especially the sensitivity for detection of LGG tissue could be markedly increased by quantitative (50%) compared to visible fluorescence (15%). Furthermore, the specificity was 82% and 92%, the PPV was 70% and 63%, and the NPV was 65% and 56% for quantitative fluorescence and visible fluorescence, respectively.

Discussion

Spectroscopic probes enabling quantitative PpIX analysis are useful for visualizing brain tumors that remain undetected by conventional 5-ALA fluorescence.[8,32] Recently, Valdés et al. described the successful use of such spectroscopic probes during surgery of the first low-

grade tumors.[30] However, this study was limited by its small number of patients and inclusion of different histopathological low-grade tumors such as ganglioglioma, DNET (dysembryoplastic neuroepithelial tumor), ependymoma, and pleomorphic xanthoastrocytoma aside from diffusely infiltrating LGG.[30] Furthermore, this study only included LGGs,[30] but MRI is usually unreliable to predict the presence of low-grade histopathology prior to surgery.[1,6,12] Consequently, we designed the current study to systematically analyze the value of visible 5-ALA fluorescence and quantitative PpIX analysis in a homogeneous cohort of patients with radiologically suspected diffusely infiltrating LGG.

Visible Fluorescence

All 3 WHO grade IV and the majority of WHO grade III gliomas (73%) showed visible fluorescence in circumscribed intratumoral areas during surgery in our study. This is in accordance with the current literature.[5,10,36,38] In contrast, visible fluorescence was absent in all 8 WHO grade II gliomas in our study. Similar rates of visible fluorescence in only 0%–16% of LGGs have been reported.[5,10,36,38] Surprisingly, approximately two-thirds of tumors in our patients were histopathologically classified as HGGs (11 WHO grade III and even 3 WHO grade IV gliomas) although only radiologically suspected LGG were included. This represents a very high rate of HGG in tumors suspicious for LGG, a finding that is commonly observed in 32%–45% of cases.[1,6,12] We assume that our 5-ALA tissue sampling technique, with the ability to identify even small fluorescing intratumoral areas (Fig. 3I) during surgery suspicious for focal malignant transformation, is one of the main reasons for such a high portion of HGGs in our study. In this sense, a significantly higher percentage of tumor cells and frequency of high-grade histology were found in fluorescing compared to nonfluorescing samples. Similarly, previous studies found increased proliferation and histopathological parameters of anaplasia in fluorescing areas of tumors initially suspected to be LGGs.[10,31,36,38] In gliomas with focal CE, neuronavigation is of value to crudely localize such enhancing areas, while the additional use of fluorescence supports the neurosurgeon in identifying its exact localization unaffected by brain-shift. This is especially useful for precise identification of small and deep-seated areas of focal CE. In gliomas with no or patchy/faint CE, no definite target is available for tissue sampling, and fluorescence is especially useful for identification of potential anaplastic foci in such cases. Indeed, we were able to identify focal areas of visible fluorescence in 6 (38%) of 16 gliomas with no or patchy/faint CE that were all classified as WHO grade III/IV tumors. Therefore, these data indicate that visible 5-ALA fluorescence is a powerful tool for visualization of most HGG/anaplastic foci to optimize tissue sampling and thus improve further patient management.

Quantitative PpIX Analysis

In this study, we analyzed for the first time the C_{PpIX} in different intratumoral areas in addition to visible 5-ALA fluorescence in a cohort only including patients with suspected diffusely infiltrating LGG. We found significantly higher C_{PpIX} in suspected tumor compared to margin areas and fluorescing compared to nonfluorescing regions. Furthermore, 40% of tumor samples not visible with conventional fluorescence showed significantly increased C_{PpIX}. Thus, quantitative PpIX analysis was capable of visualizing samples with low-grade histology and WHO grade III glioma tissue/anaplastic foci not detectable with

visual fluorescence alone. Moreover, quantitative PpIX analysis was able to markedly increase the sensitivity (58% vs 30%) for detection of tumor tissue and the NPV (59% vs 31%) compared to visible fluorescence. Similarly, in an ex vivo study of various gliomas and an in vivo study of a small cohort of different low-grade tumors, Valdés and colleagues found significantly higher C_{PpIX} in fluorescing compared to nonfluorescing samples.[30,31] Thus, our current data in a homogenous patient cohort comprising only suspected diffusely infiltrating LGG are in line with the first ex vivo and in vivo observations in various glioma types.[30,31] Objective techniques such as quantitative fluorescence analysis therefore allow for more effective use of 5-ALA compared to subjective methods (surgeon's impression) alone while maintaining an acceptable margin of safety.

Additionally, we observed a significant correlation of C_{PpIX} and percentage of tumor cells in our study. To our knowledge, this is the first report describing a significant correlation of C_{PpIX} and percentage of tumor cells in an in vivo study comprising only suspected diffusely infiltrating LGG. In their ex vivo study, Valdés et al. similarly found a strong correlation between C_{PpIX} and proliferation/total number of proliferating cells in various gliomas.[31] These data highlight the value of quantitative PpIX analysis to visualize histopathological intratumoral heterogeneity during glioma surgery. It is of note that although the maximum C_{PpIX} was significantly higher in patients with HGG than in those with LGG, we did not find a statistically significant difference in the mean C_{PpIX} between LGG and HGG samples. This finding is in contrast to the ex vivo observations of Valdés et al. describing a strong correlation between C_{PpIX} and a histopathologic score based on the WHO grading.[31] Most likely, this lack of significant difference in C between LGG and HGG samples might be explained by the relatively small number of samples in our study.

Interestingly, in some of our PpIX measurements, we observed an additional shifted peak at 627 nm aside from the typical main PpIX peak at 635 nm (Fig. 2J). According to our hypothesis, such a shifted peak seems to be present especially in tissues with very low C_{PpIX}. A previous report described a similar "two-peaked 5-ALA induced PpIX fluorescence emission spectrum" at 620 nm and 634 nm predominately in LGG and infiltrative tissue of glioblastomas.[17] The significance of this finding and the added value of this shifted peak for improved glioma visualization has to be further investigated.

5-ALA Fluorescence and Potential Influencing Factors

Prior treatments, scar tissue, and changes in blood supply in recurrent gliomas might potentially influence PpIX metabolism/visualization.[4,11] In this study, approximately half of the suspected LGGs were recurrent cases. However, we did not detect a statistically significant difference in C_{PpIX} or visible fluorescence between newly diagnosed and recurrent gliomas in this study.

The intensity of 5-ALA fluorescence is time-dependent,[27,29] and the maximal fluorescence effect was found approximately 6 hours after 5-ALA administration in a rat C6 malignant glioma model, followed by a decrease of fluorescence after 9 hours.[27] In human HGG, undisturbed fluorescence is present even 12–16 hours after 5-ALA administration.[29] In our study, the fluorescence analyses were in the range of approximately 5–10 hours after 5-ALA administration, whereas most of the fluorescence investigations were conducted in the time

frame of 6–9 hours. In LGG, the optimal time point of 5-ALA administration to achieve maximal fluorescence is not fully clarified so far.

Study Limitations

Limitations of this study include: 1) The number of patients with final histopathological diagnosis of LGG (n = 8) was relatively low in this study. However, we assume that the main reason for such a low rate of pure LGG was our 5-ALA tissue sampling technique. Thus, larger studies are needed to collect sufficient numbers of samples of different WHO grades in suspected LGG to compare C_{PpIX} between WHO grades II, III, and IV gliomas. 2) We did not systematically collect tumor-containing samples from the glioma margin as well as suspected tumor-free samples from subsequent regions for PpIX analysis and histopathological correlations. However, this issue was not the scope of the current study. In a further study, we intend to use the data generated in this study to determine whether this approach can help to more reliably detect also the tumor margin. 3) Moreover, we did not systematically perform tissue sampling and analysis of visible fluorescence/C_{PpIX} from a metabolic “hotspot” visualized by multivoxel MRI spectroscopy and/or positron emission tomography. This important issue should be further analyzed. 4) Finally, the semiquantitative classification with regard to the different visible fluorescence levels applied in our study is subjective. Furthermore, we did not independently determine a kappa statistic for interrater reliability in this study. However, the semiquantitative fluorescence classification was performed by 1 neurosurgeon (M.S.B.) with large experience with 5-ALA fluorescence-guided procedures. The interobserver variability in the semiquantitative fluorescence classification should be investigated in future studies.

Conclusions

In this study, we intraoperatively investigated the value of visible 5-ALA fluorescence and quantitative PpIX analysis in a cohort including only patients with radiologically suspected diffusely infiltrating LGG. According to our data, visible fluorescence was able to visualize focal intratumoral areas in the majority of gliomas finally classified as HGG and thus minimizes the risk of histopathological undergrading. However, visible 5-ALA fluorescence has a very limited role in the resection of pure LGG. The additional use of quantitative PpIX analysis supports the neurosurgeon in enhanced detection of LGG tissue that usually remains undetected by conventional fluorescence.

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ABBREVIATIONS

5-ALA	5-aminolevulinic acid
CE	contrast enhancement
C_{PpIX}	PpIX concentration

DTI	diffusion tensor imaging
HGG	high-grade glioma
IDH	isocitrate dehydrogenase
LGG	low-grade glioma
MRI	magnetic resonance imaging
NPV	negative predictive value
PpIX	protoporphyrin IX
PPV	positive predictive value
ROC	receiver operating characteristic
UCSF	University of California, San Francisco
WHO	World Health Organization

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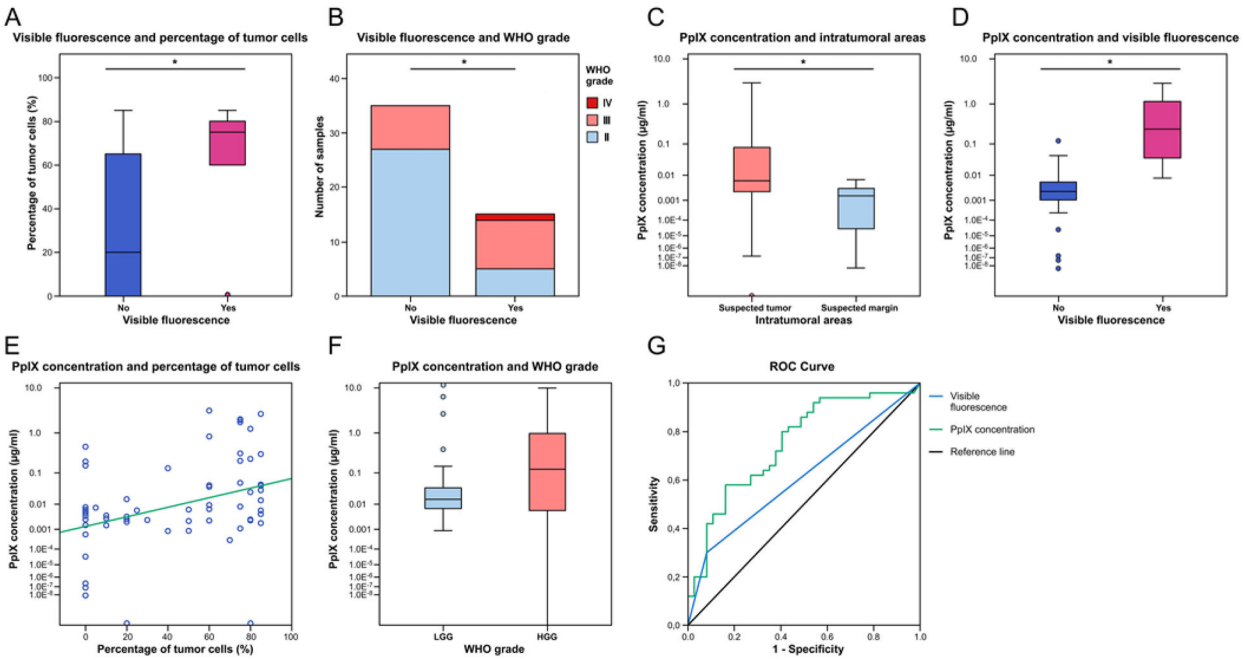

FIG. 1.

Visible 5-ALA fluorescence and quantitative PpIX analysis in tissue samples of suspected LGGs. **A:** The median percentage of tumor cells was significantly higher (*asterisks*) in tissue samples with visible fluorescence than in those with no fluorescence. **B:** Glioma tissue with high-grade histology (WHO grades III and IV) was significantly more common in fluorescing samples than in nonfluorescing samples. **C:** The median C_{PpIX} was significantly higher in suspected tumor regions than in suspected margin areas. **D:** The median C_{PpIX} was significantly higher in fluorescing samples than in nonfluorescing samples. **E:** Percentage of tumor cells and C_{PpIX} showed a significant correlation. **F:** No statistically significant difference in the median C_{PpIX} between LGG and HGG tissue samples was noted. **G:** With the assistance of ROC curve analysis an optimal C_{PpIX} cutoff value (0.005 µg/ml) was defined to distinguish between tumor and tumor-free/normal tissue in our study. The *boxplots* indicate interquartile ranges (*boxes*) with *whiskers* extending to 1.5 times the height of the box or to the minimum or maximum values (SPSS default).

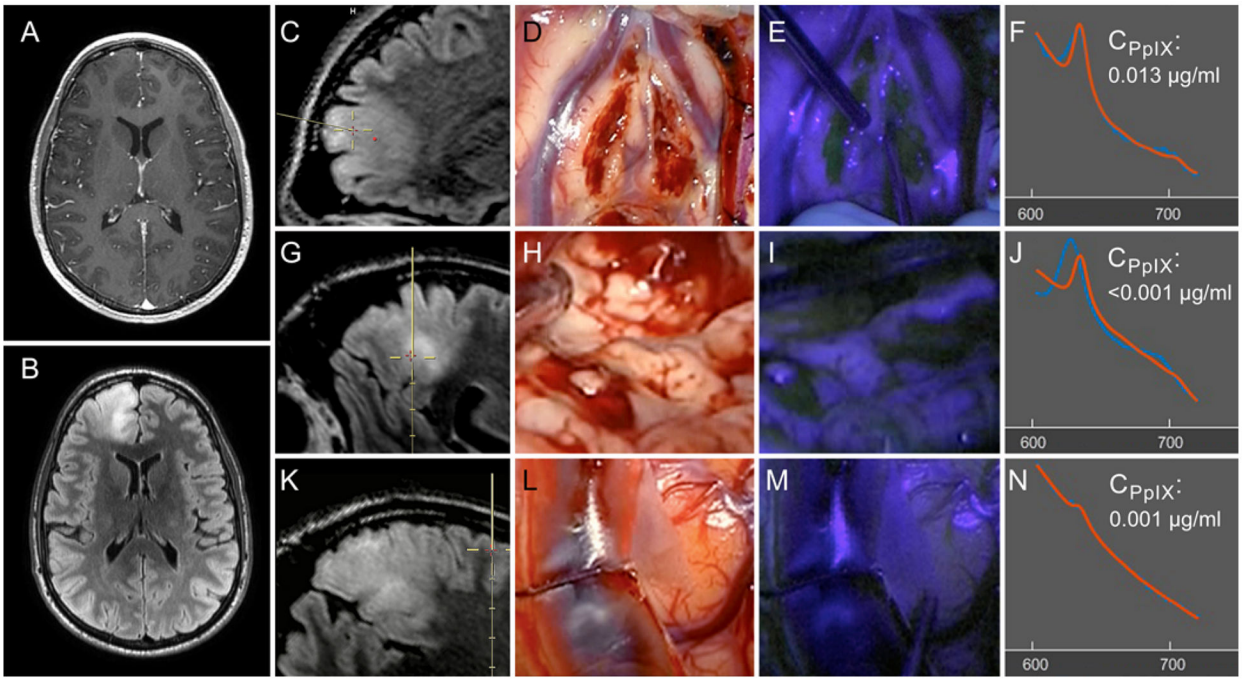

FIG. 2.

Illustrative case of a patient with a newly diagnosed suspected LGG in the right frontal lobe with final histology of an oligodendroglioma WHO grade II (case 6). **A and B:** No CE is detected within the tumor by T1-weighted MRI sequences (A) and the lesion is hyperintense on FLAIR images (B). **C–E:** The first analyzed area inside the tumor verified by neuronavigation (C) shows only slight macroscopic abnormalities under white-light microscopy (D) and this tissue cannot be visualized by visible fluorescence using violet-blue excitation light (E). **F:** Quantitative PpIX analysis reveals significantly increased levels of C_{PpIX} of 0.013 µg/ml in this region and histology of this sample corresponds to LGG tissue. **G–I:** The second area at the suspected tumor margin (G) appears to be “normal” under white-light microscopy (H) and no visible fluorescence is detected (I). **J:** Quantitative PpIX analysis shows a very low C_{PpIX} of < 0.001 µg/ml in this region and histology does not reveal tumor tissue in the corresponding tissue sample. Interestingly, an additional shifted peak at 627 nm is present aside from the typical main PpIX peak at 635 nm in this PpIX analysis. **K–M:** In the area of normal cortex (K), no abnormalities under white-light (L) and no visible fluorescence (M) are present. **N:** Quantitative PpIX analysis shows a very low C_{PpIX} of 0.001 µg/ml in the region of normal cortex.

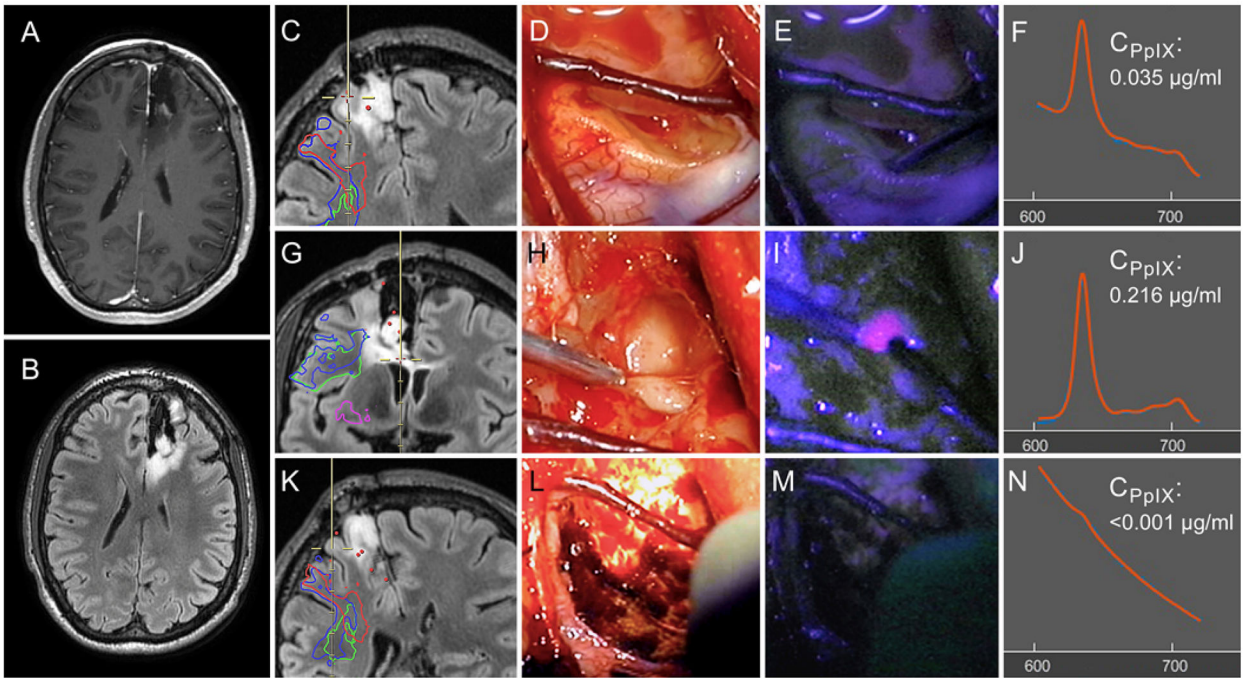

FIG. 3.

Illustrative case of a patient with a recurrent suspected LGG in the left frontal lobe with final histology of an astrocytoma WHO grade III (case 3). **A and B:** Focal CE is found within the tumor by T1-weighted MRI sequences (A) and the lesion is hyperintense on FLAIR images (B). **C–E:** The first area inside the nonenhancing tumor (C) shows only slight macroscopic abnormalities under white-light microscopy (D) and this tissue cannot be visualized by visible fluorescence with assistance of violet-blue excitation light (E). **F:** Quantitative PpIX analysis reveals significantly increased levels of C_{PpIX} of 0.035 µg/ml in this region and histology of this sample corresponds to LGG tissue. **G–I:** The second area inside the tumor near the region of focal CE shows macroscopic abnormalities comparable to the first analyzed area under white-light microscopy (H) but shows a small region of visible fluorescence (I). **J:** Quantitative PpIX analysis of this small fluorescing region shows a very high C_{PpIX} of 0.216 µg/ml, and histology reveals tissue of an HGG in the corresponding sample. **K–M:** In the area of normal cortex (K), no abnormalities are seen under white-light (L), and no visible fluorescence is present (M). **N:** Quantitative PpIX analysis shows a very low C_{PpIX} of < 0.001 µg/ml in the region of normal cortex.

TABLE 1.

Patient characteristics

Characteristic	Value
No. of patients	22 (100)
Sex, F/M	1:2.7
Age in yrs, median (range)	49 (24–70)
Recurrent tumor	
No	12 (55)
Yes	10 (45)
Intraoperative mapping	
Awake	13 (59)
Asleep	6 (27)
No mapping	3 (14)
Tumor localization	
Frontal	9(41)
Temporal	7 (32)
Insular	4 (18)
Central	2 (9)
Tumor side	
Right	11 (50)
Left	11 (50)
CE on MRI	
No CE	11 (50)
Focal	6 (27)
Patchy/faint	5 (23)
Visible fluorescence	
No fluorescence	11 (50)
Moderate brightness	9 (41)
Mild brightness	2 (9)
Histology	
Astrocytoma WHO gr II	4 (18)
Oligodendroglioma WHO gr II	4 (18)
Astrocytoma WHO grill	8 (36)
Oligodendroglioma WHO gr III	3 (14)
Glioblastoma WHO gr IV	3 (14)
IDH mutational status	
IDH mutated	16 (73)
IDH wildtype	6 (27)

gr = grade.

Data are n (%) unless otherwise indicated.

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TABLE 2.

Detailed patient data

Case No.	Age (yrs), Sex	Tumor Localization	Side	Recurrent Tumor	MRICE	Intraop Mapping	Histopathology	WHO Grade	IDH Mutational Status	Visible Fluorescence*	c_{PpIX} Max	Time Period After 5-ALA Dose (hrs:mins)
1	42, M	Temporal	Right	Yes	None	Awake	Astrocytoma	2	Mutated	None	0.005	6:21†
2	59, M	Frontal	Left	No	None	Asleep	Oligodendroglioma	3	Mutated	Mod	0.008	7:31–8:39
3	44, M	Frontal	Left	Yes	Focal	Asleep	Astrocytoma	3	Mutated	Mild	0.216	6:38–7:24
4	43, M	Central	Right	Yes	Patchy/ faint	Awake	Astrocytoma	3	Mutated	Mod	0.036	5:24–6:06
5	43, F	Frontal	Right	No	Focal	Awake	Oligodendroglioma	2	Mutated	None	0.005	4:52–6:23
6	30, M	Frontal	Right	No	None	No	Oligodendroglioma	2	Mutated	None	0.013	5:54–7:12
7	55, M	Temporal	Right	No	None	Asleep	Glioblastoma	4	Wildtype	Mod	0.287	5:01–6:48
8	65, M	Insular	Left	No	Patchy/ faint	Awake	Glioblastoma	4	Wildtype	Mod	2.824	6:27–8:53
9	70, M	Temporal	Left	No	Focal	Awake	Astrocytoma	3	Wildtype	Mod	1.152	5:16–7:20
10	44, M	Frontal	Right	Yes	Focal	No	Oligodendroglioma	3	Mutated	Mod	0.437	5:56–7:20
11	53, M	Temporal	Right	Yes	Patchy/ faint	Asleep	Oligodendroglioma	3	Mutated	Mod	0.023	5:55–6:30
12	55, F	Central	Left	Yes	Focal	Awake	Astrocytoma	3	Mutated	Mod	1.675	5:42–6:28
13	29, M	Temporal	Left	No	Focal	Awake	Glioblastoma	4	Mutated	Mod	1.793	6:09–10:04
14	55, M	Temporal	Left	No	None	No	Astrocytoma	2	Wildtype	None	<0.001‡	6:02–8:09
15	59, F	Insular	Right	Yes	None	Awake	Astrocytoma	2	Mutated	None	0.026	5:57–8:17
16	24, M	Frontal	Left	Yes	None	Asleep	Astrocytoma	3	Mutated	None	0.121	5:25–6:17
17	26, M	Frontal	Right	No	None	Awake	Oligodendroglioma	2	Mutated	None	0.002	5:54–7:12
18	53, M	Temporal	Right	Yes	Patchy/ faint	Asleep	Oligodendroglioma	2	Mutated	None	0.005	5:20–8:45
19	42, F	Frontal	Right	Yes	None	Awake	Astrocytoma	3	Mutated	None	0.003	6:32–7:08
20	38, F	Frontal	Left	No	None	Awake	Astrocytoma	2	Mutated	None	0.006	6:27–8:45
21	59, M	Insular	Left	No	None	Awake	Astrocytoma	3	Wildtype	None	0.004	7:00–9:22
22	54, F	Insular	Left	No	Patchy/ faint	Awake	Astrocytoma	3	Wildtype	Mild	2.410	7:03–9:39

Hrs = hours; max = maximal; mins = minutes; mod = moderate.

[*] Moderate and mild refer to the degree of brightness.

[†] Only 1 analysis of visible and quantitative fluorescence available.

[‡] The only available tissue specimen did not contain tumor tissue.

TABLE 3.

Diagnostic performance in detecting tumor tissue using visible and quantitative fluorescence

Factor	Visible Fluorescence	Quantitative Fluorescence at 0.005 μg/ml C_{Ppix} Cutoff
Sensitivity	30%	58%
Specificity	84%	81%
PPV	83%	81%
NPV	31%	59%