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CLINICAL STUDY

Objective assessment of intraoperative tumor fluorescence reveals biological heterogeneity within glioblastomas: a biometric study

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Abstract

Purpose Heterogeneity within GBMs and variability of visualized fluorescence combine to confer practical limitations to the technique of optical imaging. A biometric analysis was planned to objectively ascertain and analyse this phenomenon

Methods 25 adult glioblastoma subjects undergoing resection were prospectively accrued. Biopsies were taken from various parts of the tumor and safe peritumoral zones. White light (WL) and visualized fluorescence was subjectively recorded. Corresponding histopathology [coalescent (C) or infiltrating (I) tumor] and protoporphyrin-IX (PPIX) levels were assayed.

Results WL was very sensitive for detecting tumor. SF was more specific and had high positive predictive value for detecting tumor. WF on the other hand had a poor discriminatory efficacy. Mean PPIX levels were 3.0, 2.01 and 0.16 for SF, WF, and NF respectively. WF had a wide variable range of PPIX levels. Within the coalescent tumor areas, there was a variable distribution of fluorescence (both subjective as well as objective PPIX levels) with only 54% samples showing SF and high PPIX. In seven cases this discordance was noted within the same tumor (biological heterogeneity).

Conclusions Fluorescence may miss important tumor areas even if objective assessment is used. Histologically similar tumor areas may exhibit contrasting fluorescence properties, a phenomenon which needs further investigation and elucidation of underlying mechanisms which could potentially be manipulated to optimize the utility of fluorescence guidance.

Keywords Fluorescence · Intratumoral heterogeneity · Biological heterogeneity · Diagnostic accuracy · Protoporphyrin · Glioblastoma

Background

Aminolevulinic acid (ALA) induced Fluorescence guided resections (FGR) have become standard of care for malignant glioma surgery across the world [1]. The technique is regarded to be very useful for identifying coalescent tumor areas which fluoresce strongly. This coalescent tumor mass which generally corresponds to the enhancing tumor bulk in most malignant gliomas remains the target of most local therapies including surgical resection. The type of fluorescence correlates with the degree of malignancy [2, 3]. However, intratumoral heterogeneity is well known in glioblastomas. This is reflected in the fluorescence patterns too. This could lead to variable areas of fluorescence in the same tumor (higher grade areas showing stronger fluorescence and vice versa) as well as occasionally variable fluorescence properties within histologically seemingly identical tumor regions as we have reported earlier [4]. Most reported studies use visualised fluorescence in assessing the diagnostic

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accuracy. However this can be subjective and that has been our experience too [5]. Objective evaluation using in situ spectroscopy or ex vivo PPIX assays are much more accurate. Their application intraoperatively is limited but could be extremely useful to validate the utility of the more subjective interpretation using visualized fluorescence. For such biometric studies, it is imperative that the degree of fluorescence be reported in an objective manner. Here, we attempted to analyse the diagnostic accuracy of visualized fluorescence with objective PPIX levels as well as to interrogate the heterogeneity using objective assessment.

Methodology

This was a prospective study approved by our institutional review board. Written informed consent was obtained from all patients enrolled in this study. All radiologically suspected malignant gliomas planned for surgical resection but not amenable to gross total resections were screened as has been used in earlier reported studies [6]. In order to capture the heterogeneity, we included only those with variable contrast enhancement.

Sample collection and biometric analysis

Patients underwent surgery using conventional microsurgical principles. All were premedicated with 5-ALA (Gliolan, Medac GmBh, Germany; @ 20 mg/kg body) per oral 4–5 h prior to incision. The OPMI Pentero microscope (Carl Zeiss, Oberkochen, Germany) fitted with the BLUE 400 filter was used for detecting tumor fluorescence. 2 senior neurosurgeons (AM, PS) experienced in fluorescence guided resection performed all the surgeries. Intraoperatively, multiple biopsies were taken from various parts of the tumor and safe peritumoral zones (as well as resection cavity) and graded as per the visualized white light appearance and fluorescence intensity. White light appearance was subjectively classified as normal and abnormal. Visualized fluorescence was subjectively graded as strong (intense red; annotated as SF), weak (pink; annotated as WF), or none (annotated as N). In order to capture the heterogeneity, a diligent and conscious attempt was made to avoid necrosis and collect strongly fluorescing and non-fluorescing abnormal as well as normal (safe areas only) appearing tissue, however additional random sampling was also done. There were no predefined criteria for the number of samples to be collected and it was completely at the surgeon's discretion depending on the intraoperative conditions. Once collected, each sample was further divided into 3 parts. One part was sent for routine histopathological assessment in formalin, another was snap frozen in amber colored tubes and sent for quantification of protoporphyrin-IX (PPIX) levels, and the third was snap frozen for other biological studies. In order to have uniformity in data, only regions where adequate samples could be collected (to enable all the subpart analyses) were included eventually in the results. A neuropathologist (ES) analyzed each sample. Besides routine histo-morphological analysis, the neuropathologist specifically interpreted the tumor pattern as coalescent tumor (sheets of tumor cells with no intervening normal tissue, annotated C), infiltrating tumor (dispersed tumor cells admixed with normal tissue, annotated I), and normal (included normal brain and reactive non-neoplastic tissue) (Figs. 1, 2, 3).

Assay of PPIX

PPIX was objectively quantified in each sample as per a previously published protocol [7]. Initially, standardization was performed on rat brains and normal human brain samples (from non-ALA treated surgical cases). These control experiments were used to establish the calibration curves. Long term stability of PPIX in brain tissue was also ascertained by analyzing the concentration of PPIX in a tumor sample, one month apart. Thus we were reassured that measurements at various time periods after collection of samples would be reliable. The procedure for determination of PPIX in brain tissue is provided in supplementary materials.

Statistical analysis

For each specimen the fluorescence pattern and histological category were tabulated. Since weak fluorescence can have a very subjective interpretation leading to a high interobserver variability, we separately assessed the accuracy of strong fluorescence (SF) alone and then clubbed all the fluorescing tissue together (AF). Similarly the pathological observations were categorized as coalescent tumor versus non-coalescent tumor (this included infiltrating tumor and normal) and then as all tumor (coalescent plus infiltrating) versus no tumor. Sensitivity (Sn), specificity (Sp), positive (PPV), negative predictive values (NPV) as well as the positive and negative likelihood ratios (PLR/NLR) were calculated for both white light appearances as well as the fluorescence pattern. ROC curves were used to evaluate the correlation between the visualized fluorescence qualities and the PPIX levels. This was done for pooled samples as well as for individual patients. For the former, all samples were pooled together and ROC curve was plotted. For the patient-wise analysis, the mean PPIX value for each fluorescence quality (SF, WF, NF) was calculated in each patient and subsequently a ROC curve was plotted. Cutoff values with the best sensitivity and specificity were selected. Statistical significance was set at 0.05. Statistical analysis was performed using SPSS (The Statistical Package For Social Sciences, IBM Corp. 2017. Version 25.0. Armonk, NY).

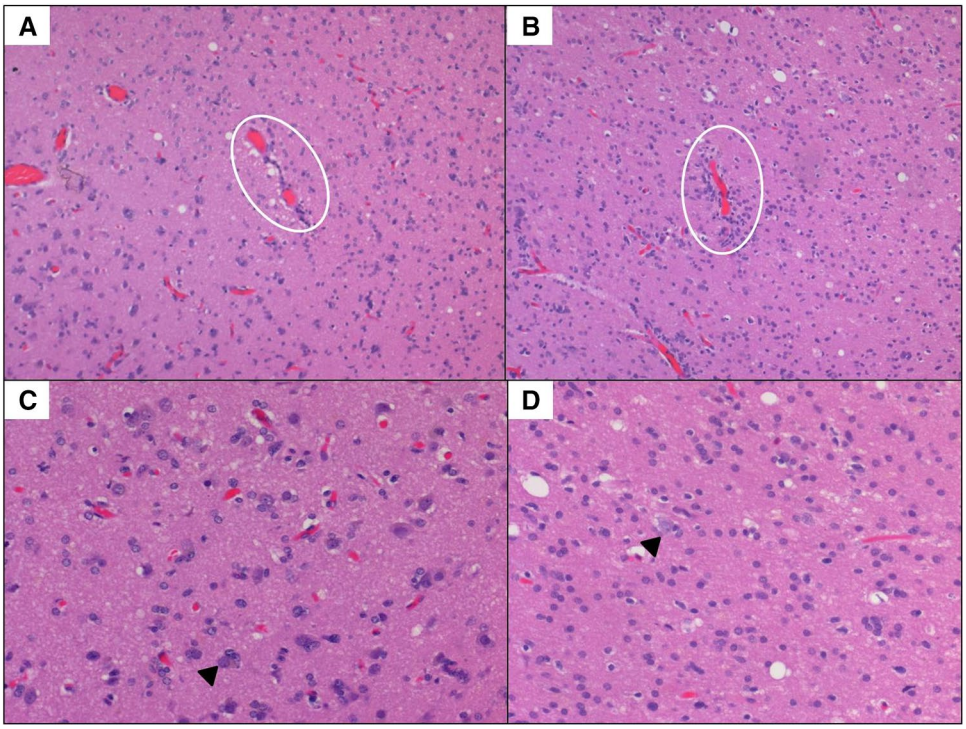

Fig. 1 (HE. **a**, **b** × 100, **c**, **d** × 200) Representative photomicrographs of infiltrative portion of the tumour show low cellularity with spread along the blood vessels (as shown by the white coloured circle in **a** and **b**) and native neurons (as shown by the arrow heads in **c** and **d**)

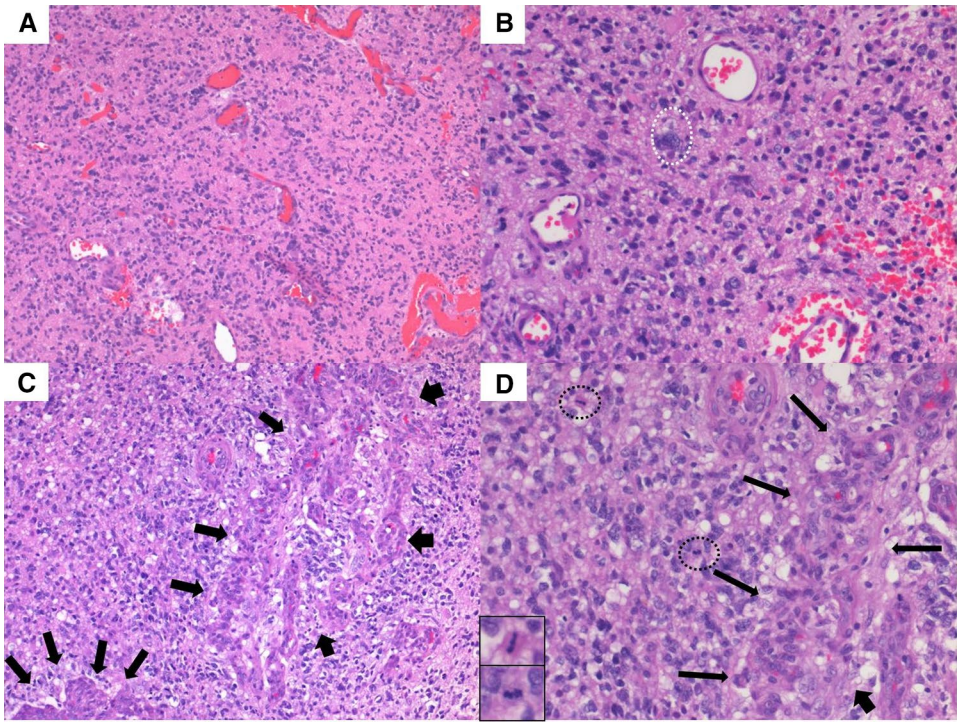

Fig. 2 (HE. **a**, **c** × 100; **b**, **d** × 200) Representative photomicrographs of coalescent tumour portion depicting high grade morphology of high cellularity, nuclear pleomorphism (pleomorphic cells enclosed by a dotted white circle in **b**), mitotic activity (enclosed by a dotted black circle in **d** and shown in the inset) and microvascular proliferation (as shown by the black arrows in **c** and **d**)

Results

A total of 28 cases were recruited. In 3 cases, samples could not be collected due to logistical issues (2 cases had excessive bleeding intraoperatively and in1 case the intraoperative decision was to only do a biopsy). These were therefore excluded from the biometric analysis and only 25 cases were finally included. Histologically all were Glioblastoma except one case which was an anaplastic

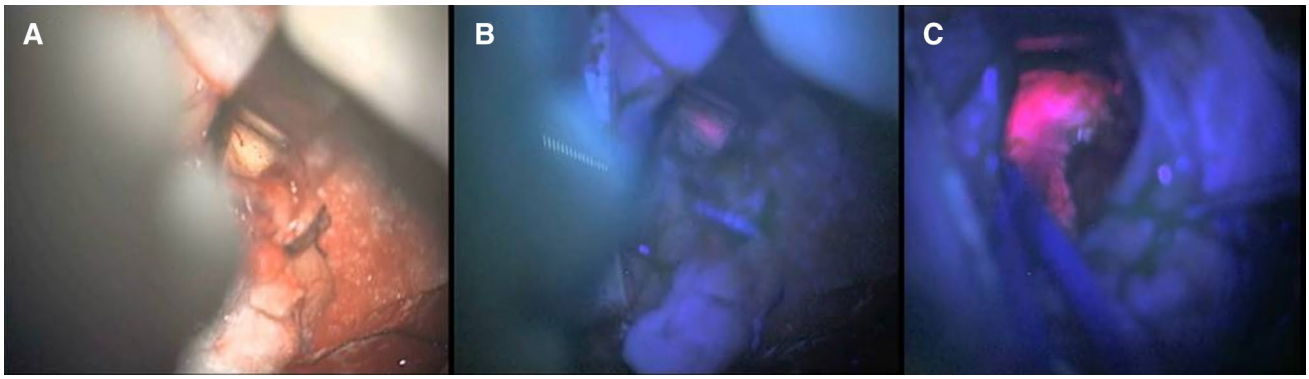

Fig. 3 Intraoperative images showing white light appearance (**a**), weak fluorescence of the same area (**b**) and strong fluorescence in the central tumor (**c**)

oligodendroglioma (which showed negligible fluorescence intraoperatively). Overall 9 tumors showed IDH positivity (confirmed using sequencing), including the solitary oligodendroglioma.

Diagnostic accuracy of visualized white light and fluorescence appearances

Totally 74 tissue samples were analysed from the 25 patients (range 1–5). One each of SF and NF samples showed necrosis and were excluded from the analysis. 72 samples were finally included. The diagnostic accuracy of white light (WL) appearance as well as the visualized fluorescence (VF) quality were analysed [Table 1]. This was calculated in two ways—for predicting coalescent tumor as well as for predicting any tumor. For detecting coalescent tumor areas, WL had the highest sensitivity whereas SF had the highest specificity. PPV and PLR of both WL and SF were similar. AF was not as accurate in detecting coalescent tumor, mainly because it picked up many infiltrating tumor samples also. For detecting any tumor (coalescent or infiltrating), WL and SF remained most sensitive and specific respectively. SF had very high PPV and PLR primarily because there was no normal (non neoplastic or reactive) tissue sample that showed SF. WF on the other hand had a poor discriminatory efficacy, being distributed across coalescent tumor areas, infiltrative edge as well as reactive tissue samples.

Correlation between visualized fluorescence and objective PPIX levels

We correlated the visualized fluorescence qualities with objective assays of PPIX in each sample. (Table 2).

Table 1 Diagnostic accuracy of the visualized white light and fluorescence appearances of the samples

Intraoperative assessment	Sn (95% CI)	Sp (95% CI)	Accuracy (95% CI)	PLR (95% CI)	NLR (95% CI)	PPV (95% CI)	NPV (95% CI)
Detecting coalescent tumor							
WL abnormal	91.67 (80.02–97.68)	63.64 (40.66–82.80)	82.86 (71.97–90.82)	2.52 (1.44–4.41)	0.13 (0.05–0.35)	0.85 (0.76–0.91)	0.78 (0.57–0.90)
Strong fluorescence	54.2 (40.1–68.3)	79.2 (62.9–95.4)	62.5 (61.9–63.1)	2.6 (1.14–5.92)	0.58 (0.4–0.84)	0.84 (0.71–0.97)	0.46 (0.31–0.62)
Any Fluorescence	66.7 (53.3–80.0)	58.3 (38.6–78.1)	63.9 (63.3–64.5)	1.6 (0.96–2.68)	0.57 (0.34–0.96)	0.76 (0.63–0.89)	0.47 (0.29–0.64)
Detecting any tumor							
WL abnormal	77.61 (65.78–86.89)	60.00 (14.66–94.73)	76.39 (64.91–85.60)	1.94 (0.66–5.72)	0.37 (0.16–0.87)	0.96 (0.89–0.99)	0.17 (0.08–0.32)
Strong fluorescence	46.3 (34.3–58.2)	100 (47.8–100)	50.0 (49.3–50.7)	Infinite	0.54 (0.43–0.67)	1.00	0.122 (0.1–0.148)
Any fluorescence	59.7 (48.0–71.4)	60.0 (14.7–94.7)	59.7 (59.1–60.4)	1.49 (0.50–4.45)	0.67 (0.31–1.45)	0.95 (0.87–0.98)	0.1 (0.04–0.19)

WL white light appearance, *Sn* Sensitivity, *Sp* Specificity, *PLR* positive likelihood ratio, *NLR* negative likelihood ratio, *PPV* positive predictive value, *NPV* negative predictive value

Table 2 Mean PPIX levels across different groups of fluorescence properties (pooled)

	Tissue type	Mean PPIX (μg/gm)	Range	Standard deviation
1	Non-fluorescent (NF)	0.16	0–0.35	0.08
2	Weakly fluorescent (WF)	2.01	0.13–3.97	1.27
3	Strongly fluorescent (SF)	3.00	0.80–5.81	1.34
4	All fluorescent (SF + WF)	2.75	0.13–5.81	1.38

PPIX protoporphyrin IX

NF samples showed negligible PPIX levels whereas SF had high PPIX levels. Interestingly, there was a very wide range of values in the WF group. Further, there was a significant overlap in PPIX values between WF samples and SF and NF samples at the extremes of the range. This underlines the subjectivity in interpreting weak fluorescence and the lack of reliability. PPIX levels were always below (0.35 μg/g) in all NF samples. All fluorescing samples had values above 0.9 except 2 cases where the values were 0.13 and 0.16 in the WF samples.

Pooled sample analysis (Table 3, Fig. 4a, b)

A Receiver Operating Characteristics (ROC) curve analysis was done pooling all the samples to look for the discriminatory ability of visualized fluorescence. First, we plotted PPIX levels for any fluorescence (regardless of the intensity of the fluorescence). The area under the curve (AUC) was 0.975. A cut-off of 0.575 for PPIX levels gave a sensitivity of 95.2% and specificity of 100% in detecting any fluorescence. The diagnostic accuracy of the test was 97.22% with a PPV and NPV of 100% and 93.75%. PLR of the test was also 1.49 which is greater than 1 hence confirming that it is effective for detecting tumors with any fluorescence. PPIX levels were also plotted for strong fluorescence (vs weak or no fluorescence). The AUC was 0.917. A cutoff of 1.995 PPIX level gave a sensitivity of 71% and specificity of 86%. The diagnostic accuracy of the test was 79.17% with a PPV of 78.57% and NPV of 79.55%. PLR and NLR were 4.85 and 0.34, respectively reinforcing the strong predictive value.

Patient based analysis (Table 3 and Fig. 5a, b)

Additional patient-wise analysis was also performed. The mean PPIX levels for each fluorescence type (in each patient) was used to discriminate between the strong or weak fluorescence. We had a total of 47 fluorescence (strong, weak or non-fluorescent) in 25 patients. Mean

Table 3 ROC analysis of pooled samples and patient wise fluorescence quality versus PPIX levels

	Cutoff	AUC	P-value	Sn	Sp	DA	PPV	NPV	PLR	NLR
Pooled sample wise analysis										
Any vs no florescence	0.575	0.975 (0.941–1.0)	< 0.001	95.2 (83.84–99.42)	100 (88.43–100)	97.22 (90.32–99.66)	100	93.75 (79.5–98.31)	a	0.05
Strong vs (weak + no florescence)	1.995	0.917 (0.86–0.98)	< 0.001	71 (0.52–0.86)	86 (0.71–0.94)	79.17 (68–87.84)	78.57 (62.87–88.82)	79.55 (68.8–87.25)	4.85	0.34
Patient wise analysis										
Any vs no fluorescence	0.615	0.98 (0.94–1)	< 0.001	96.55 (82.24–99.91)	100 (81.47–100)	97.87 (88.71–99.95)	100	94.74 (72.4–99.2)	a	0.03
Strong vs (weak or no fluorescence)	0.925	0.90 (0.81–0.99)	< 0.001	93.1 (77.23–99.15)	100 (81.47–100)	95.74 (85.46–99.48)	100	90 (70.26–97.17)	a	0.07

AUC area under curve, *Sn* sensitivity, *Sp* specificity, *PLR* positive likelihood ratio, *NLR* negative likelihood ratio, *PPV* positive predictive value, *NPV* negative predictive value, *DA* diagnostic accuracy

[a]PLR could not be ascertained (was infinite)

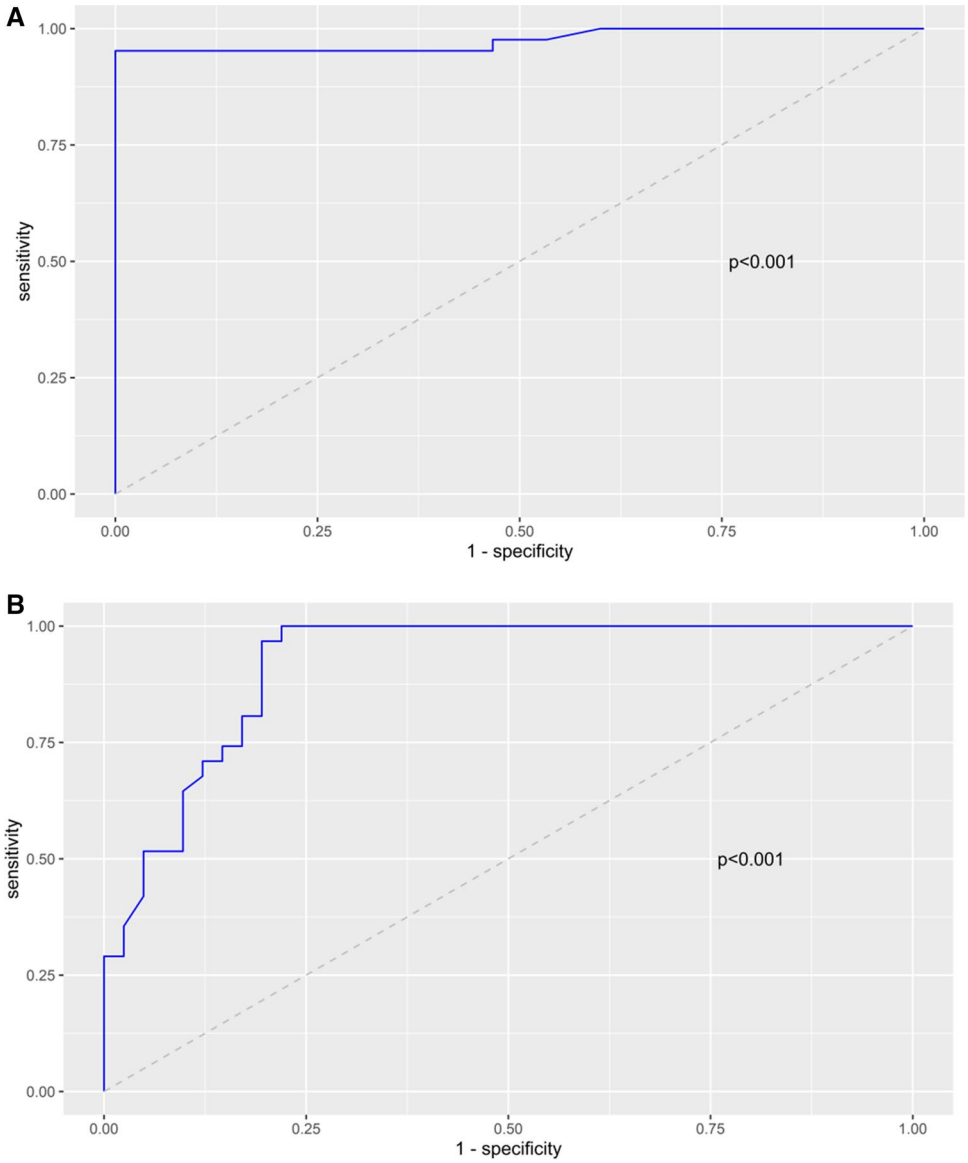

Fig. 4 a ROC curve for PPIX levels with any fluorescence (strong + weak fluorescence) for pooled samples. b ROC curve for PPIX in detecting only strong fluorescence for pooled samples

PPIX levels was plotted against any fluorescence. The ROC analysis gave an AUC of 0.98 and a cutoff of 0.615 with sensitivity of 96.6% and specificity of 100%. The overall accuracy of the test was 97.87%. PPV of 100% suggests that all patients with any fluorescence have Mean PPIX levels above 0.615. 94.74% of the patients with no fluorescence have mean PPIX levels less than 0.615. Mean PPIX levels was also plotted for strong fluorescence alone. The ROC analysis gave an AUC of 0.90 and a cutoff of 0.925 with sensitivity of 93.1% and specificity of 100%. The overall accuracy of the test was 95.74%. PPV of 100% suggests that all the all patients with strong fluorescence had Mean PPIX levels above 0.925. 90% of the patients with weak or no fluorescence had mean PPIX levels less than 0.925.

Intratumoral heterogeneity

For each pathological category, we correlated the visualized fluorescence and PPIX levels. Table 4 shows the distribution of the visualized and objective (PPIX) fluorescence properties. There was a wide variation not only of the subjective (visualized) but also objective (PPIX) fluorescence properties within the coalescent and infiltrative tumor biopsy sites. However coalescent tumor were more likely to have strong fluorescence (54%), and the infiltrative edge more likely (69%) to have weak or no fluorescence. ROC curves were plotted (AUC 0.6 for coalescent tumor and 0.56 for all tumor categories; data not shown) to look at the ability of PPIX in detecting tumor type. This was found to be non discriminatory.

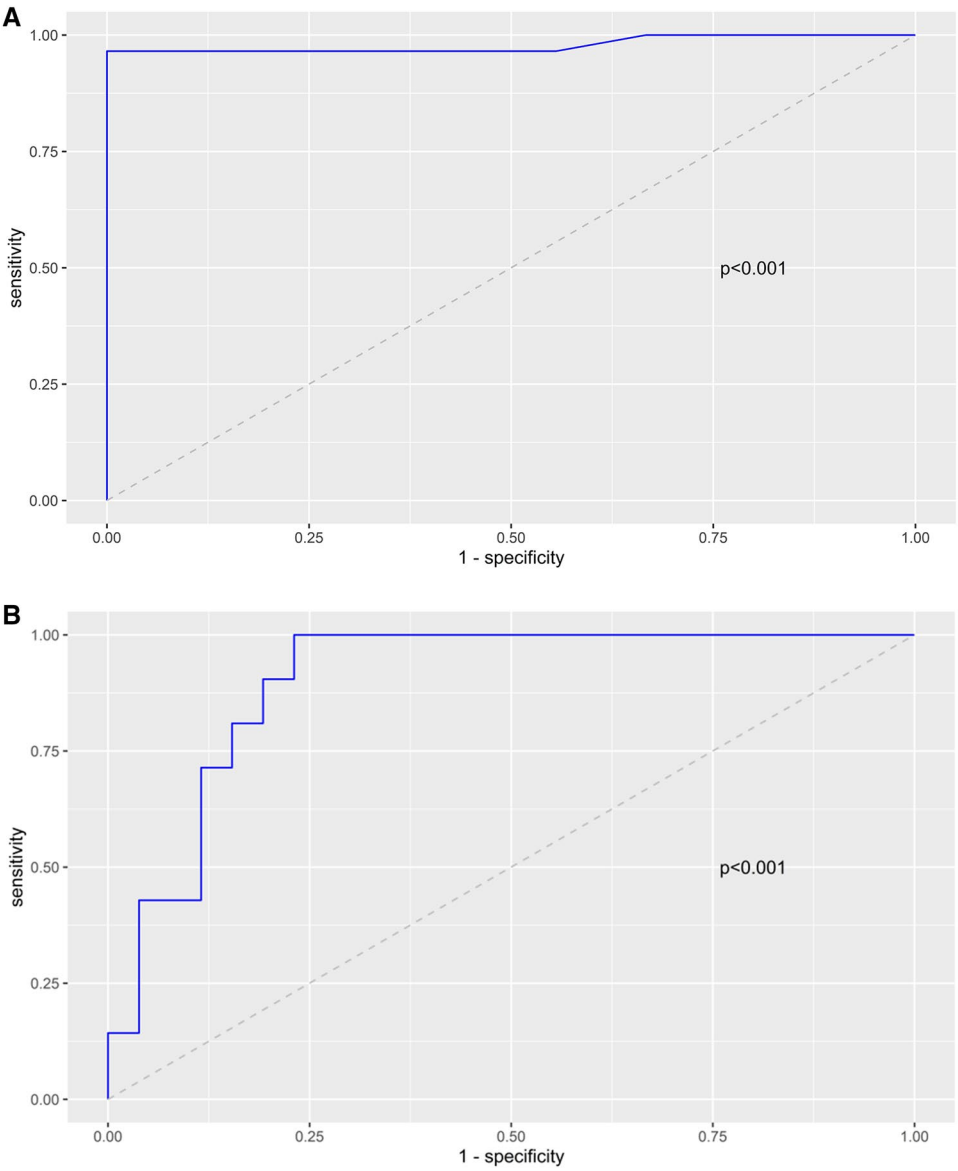

Fig. 5 a ROC curve for PPIX levels with any fluorescence (strong + weak fluorescence) for patient wise analysis. b ROC curve for PPIX levels with strong fluorescence for patient wise analysis

Table 4 Correlation between tumor pathology and fluorescence pattern as well as PPIX levels

Pathological type	Fluorescence type		
	Strongly fluorescent (n = 32)	Weakly fluorescent (n = 11)	Non fluorescent (n = 31)
Coalescent tumor (n = 48)	26	6	16
PPIX levels (range)	0.80–5.81	0.13–3.97	0.00–0.31
Infiltrative EDGE (n = 19)	5	3	11
PPIX levels (range)	3.02–4.20	0.92–2.77	0.08–0.35
Necrosis (n = 2)	1	0	1
PPIX levels (range)	3.2	–	0.11
Non-neoplastic (n = 5)	0	2	3
PPIX levels (range)	–	2.3–3.7	0.14–0.17

Of note, within the coalescent tumor areas, there was a variable distribution of fluorescence (both subjective as well as objective PPIX levels). 46% of coalescent tumor samples showed weak or no fluorescence at all and this was verified by objective PPIX assays. In 7 subjects this dichotomy was observed in histologically identical (i.e. coalescent tumor) yet spatially separated areas within the same tumor (paired samples showing histologically coalescent tumor but one with high PPIX and another with low/ negligible PPIX) (Table 5) 3 of these 7 were IDH positive, but otherwise there was no significant difference from the rest of the cohort.

Table 5 Details of the cases with discordance between biological and PPIX characteristics was seen

ID	Tumor histology and IHC	Biopsy	Flourescence properties	Tumor type	Mib LI (%)	PPIX levels
1	Glioblastoma (IDH wild type)	1	Strongly fluorescent	Predominant tumor	15–20	4.6
		2	Non fluorescent	Predominant tumor	10–12	0.28
5	Glioblastoma (IDH wild type)	1	Strongly fluorescent	Predominant tumor	50–60	1.11
		2	Non-fluorescent	Predominant tumor	50–60	0.00
6	Glioblastoma (IDH mutant)	1	Fluorescent tissue	Predominant tumor	8–10	1.26
		2	Non-fluorescent tissue	Predominant tumor	12–15	0.09
10	Glioblastoma (IDH mutant)	1	Non fluorescent	Predominant tumor	5–7	0.20
		2	Strongly fluorescent	Predominant tumor	4–6	2.31
18	Glioblastoma (IDH mutant)	3	Strongly fluorescent	Predominant tumor	3–5	2.3
		1	Non-fluorescent	Predominant tumor	3–5	0.22
20	Glioblastoma (IDH mutant)	4	Fluorescent tissue	Predominant tumor	15–20	2.1
		2	Non-fluorescent tissue	Predominant tumor	8–10	0.13
22	Glioblastoma (IDH wild type)	5	Fluorescent tissue	Predominant tumor	2–3	3.2
		3	Non-fluorescent tissue	Predominant tumor	6–8	0.16

Discussion

Using biometric analysis, we have demonstrated that fluorescence distribution in malignant gliomas is highly variable both subjectively (visualized fluorescence quality) as well as objectively (quantified with assay of PPIX levels). Practically, in the intraoperative setting, subjective evaluation of the operative field with a combination of white light appearance as well as visualized fluorescence intensity is useful. Whereas WL is much more sensitive in picking up any abnormal looking areas, presence of fluorescence (and particularly strong fluorescence) is very specific for detecting tumor bearing regions acting as a reliable guide during resection. However, interpretation of the visualized fluorescence quality remains subjective and this is more so in the weakly fluorescing regions where the reliability is very poor. This was confirmed by using objective assays for PPIX where again we showed that PPIX estimation is a very sensitive and specific method for detecting any fluorescence (and certainly for strong fluorescence) with reliable cutoff values for both pooled samples as well as patient-wise assays. Strongly fluorescing tissues show significantly higher PPIX levels and non-fluorescing areas show negligible PPIX, the weakly fluorescing zones are much more variable and therefore less reliable. Though PPIX assays can reliably correlate with the visualized fluorescent properties, this does not necessarily correlate with histological tumor type. Not all coalescent tumor areas show high PPIX. In fact, in seven of the analysed 25 cases we have documented areas within the same tumor which harbour similar tumor cell density (i.e. high grade coalescent tumor) and yet show diametrically different objective PPIX levels. That means that there are spatially separated and histologically similar (concordant) tumor areas bearing variable fluorescence properties. This highlights and confirms the biological heterogeneity (in terms of PPIX metabolism) which we have reported earlier [4].

5-ALA induced tumor fluorescence has become the standard of care for intraoperative visualization of malignant glioma tissue [1]. It has shown to significantly impact gross total resection rates as well as survival [8–12]. The effectiveness of FGR is based on the predictive value of strong fluorescence in reliably detecting tumor tissue [6]. Strong fluorescence has a very high positive predictive value in detecting coalescent tumor (or high grade tumor areas). Diffuse gliomas are notorious for intratumoral heterogeneity. In this context, tumor fluorescence has also been used for selectively targeting and identifying high grade areas within otherwise lower grade gliomas [2, 3]. Thus, it is very useful in unmasking the histological heterogeneity within gliomas and has proven to be a very useful adjunct during glioma surgery.

However there remain certain issues during FGR which could potentially pose practical limitations. Firstly, visualized fluorescence (VF) remains a very subjective interpretation. Kamp et al. [5] showed that most studies of FGR varied in their recording and interpretation of the quality of the VF. Strong (or bright red) fluorescence remained the most consistently reported quality and is probably the most useful interpretive result. Our results also corroborate these findings. White light appearances were a good screening tool after which finding strong fluorescence was a very specific finding ruling out normal tissue, thereby strengthening the surgical decision making. However as one moves along the spectrum towards the less bright /pink or vaguely/faintly fluorescing areas, the variability in reporting the quality of fluorescence also increases significantly (Fig. 6). This is reflected in the poor predictive value of vague/faint

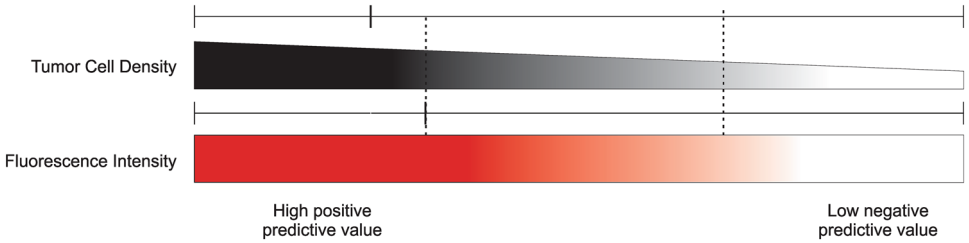

Fig. 6 Diagrammatic representation of relationship between tumor cell density and fluorescence intensity. The positive predictive value is best at the left extreme (strong fluorescence predicting presence of coalescent tumor cell mass). At the opposite end the negative predictive value is low (absence of visible fluorescence cannot rule out presence of tumor)

fluorescence as has been demonstrated in our study. In fact Stummer et al. [6] showed that such vague fluorescence could be present even at some distance beyond conventional MR defined tumor boundaries. Further, it must be noted that most human studies evaluating diagnostic accuracy of fluorescence are limited by the fact that true negatives are extremely difficult to obtain (as it may be difficult to sample areas because of their eloquent location). Therefore specificity (which is essentially the true negative rate) often cannot be reliably ascertained; but positive predictive value (and likelihood ratios) are much more reliable. Here again in our study, presence of strongly fluorescing tissue was most predictive of tumor (specificity 100% and PLR infinitesimally high). Overall, white light appearances were a good preliminary screening tool to look for abnormal areas requiring resection, after which presence of strong fluorescence was a reliable confirmatory finding. Weak fluorescence however, is a very unreliable finding because of the wide variability and subjectivity in its reporting. It could range from anything just less than intense fluorescence (which could show high grade tumor areas) to barely perceptible fluorescence in the peritumoral/reactive gliosis zone with no active tumor. It remains very difficult to uniformly quantify and grade the visualized fluorescence in such cases. For this reason, the surgeon needs to think twice before resecting such tissue, and only after excluding functionally eloquent sites. Similarly absence of fluorescence is also not a very reliable finding (corroborated by the fact that NPV is very low) seen in our study as well as others [6, 13].This low NPV persisted even when the fluorescence quality was reported as an all or none finding (i.e. present or absent). It has been shown that using more sensitive methods to detect it, fluorescence is demonstrable even in low grade tumors [14]. So, the poor NPV can be attributed to the relatively poorer sensitivity of visually diagnosed fluorescence. Objective measurement of tissue PPIX could potentially circumvent this drawback. We used the technique described earlier to accurately measure PPIX in each sample [7]. The AUCs for all fluorescence as well as strong fluorescence were very high (more than 0.9) in both the pooled sample as well as patient-based analysis indicating a very good relation between visualized fluorescence and PPIX levels. The cutoff for SF for the pooled samples (1.995) was lower than the mean PPIX level observed in the same sample set (mean PPIX = 3) indicating that there is a wide range of high PPIX values which are generally interpreted as strongly fluorescent when visually evaluated. The cutoff value for SF in the patient based dataset was lower (0.925) indicating that even the visually interpreted SF is not uniform across patients (hence some may be interpreted as SF even with a PPIX of 0.925 whereas others may have a very high PPIX as is also reflected in the wide range (0.8–5.8, Table 2) of PPIX values seen in SF samples). Nonetheless, all fluorescing samples had PPIX values above 0.9 except 2 cases where the values were 0.13 and 0.16 in the WF samples (again highlighting the subjectivity and lack of reliability in defining "weak fluorescence". So our findings showed that visualized fluorescence is good at distinguishing non-fluorescing from fluorescing areas, but was unreliable in discriminating between various grades of fluorescence, corroborating what was noted by Kamp et al. [5].

Whereas performing PPIX assays is not feasible intraoperatively for decision making in real-time, various attempts at quantifying (or semi-quantifying) the fluorescence have been described. Hefti et al. [15] used digital video data to measure the relative fluorescence intensity using a technique of single photon count. They created colour maps to semi-quantify the fluorescence intensity. They showed that vague fluorescence corresponds to 15–30% of the highest fluorescent intensity. Though semi-quantified, it remains offline and difficult to use in real-time. More objective assessment in real-time is possible using spectroscopy [6, 16]. However it is challenging to cover and scan all the visible region of interest using spectroscopy and too cumbersome to perform intraoperatively routinely. Confocal microscopy has been explored in low grade gliomas and holds promise in the future [14].

Though the more objective and sensitive techniques may improve the overall diagnostic accuracy of FGR, they may not be able to circumvent all the limitations. Our objective assay reinforced the utility of visually detected SF, but

still could not impact the poor NPV. When correlated with the pathological tumor type, PPIX assays did not have significant discriminatory ability. This was highlighted by the fact that one-thirds (16 of 48) of coalescent tumor samples showed no fluorescence (and low PPIX) and a quarter (5 of 19) infiltrating tumor samples showed strong fluorescence and high PPIX levels (Table 4). One of the reasons could have been the binary division of tumor type (into coalescent and infiltrating edge). Using a more semi-quantitative assessment of tumor pathology (for example based on categorizing tumor cell density depending on percentage) may have provided a better discriminatory ability as was reported Stummer et al. [6]. In their study, they were able to demonstrate a good correlation between visualized fluorescence, tumor cell density and spectroscopic in situ fluorescence assessment.

Even accounting for heterogeneity in various studies (including ours) regarding the interpretation of visualized fluorescence as well as the interpretation of tumor histology category, it cannot be overlooked that there are still high grade tumor components which do not show significant fluorescence. We have earlier documented this observation as have other papers [4, 17]. It is for this reason that for gliomas a multimodal approach using a combination of fluorescence and intraoperative imaging like MR or ultrasound is preferable in certain situations [18, 19].

More interestingly, our biometric study revealed that even with objective assessment, there exists a small but definite subpopulation of tumor cells with discordant biological properties (Fig. 7). Generally it is accepted that higher grade tumor areas have higher PPIX and hence fluoresce more strongly. Given the subjective nature of visual inspection of the fluorescence (which is what is routinely used intraoperatively), there could be erroneous findings if correlated with histology (as we have shown in this study above). However, even when controlling for that by doing objective analysis of PPIX levels in all tissue samples and correlating with histology, we still found areas of discordance. There were at least 7 cases where we had samples from within the same tumor (but geographically separate) which showed diametrically different fluorescent properties and PPIX levels despite having the same histological grade

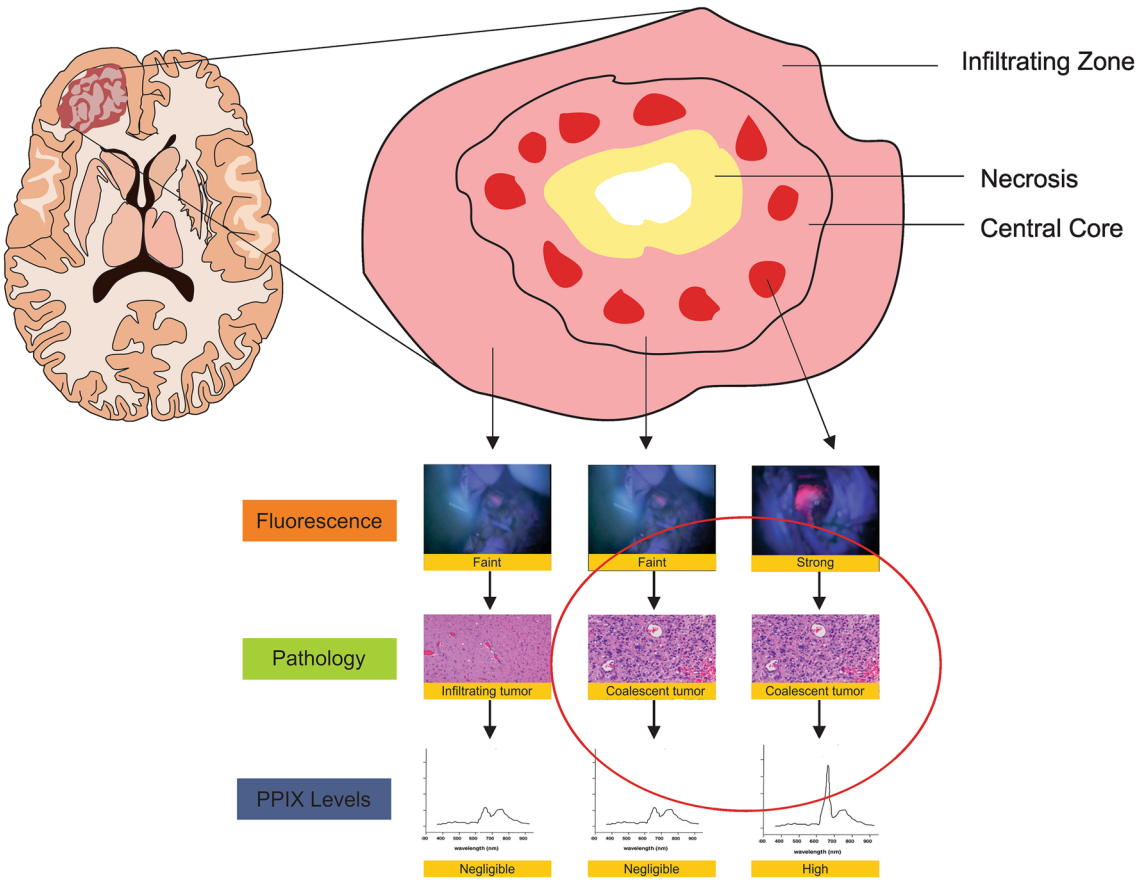

Fig. 7 Schematic representation of the heterogeniety within glioblastomas. Both histological (tumor cell density and proliferation) as well as biological (protoporpyrin IX distribution) heterogeniety exist and are depicted (the red circle highlights the biological heterogeniety in hitologically seemingly similar tumor areas)

(cell density and proliferation indices). Piccirillo et al. demonstrated that the cells in the fluorescent tumor mass is able to produce neurospheres in vitro as well as develop tumors in animals whereas those from the non-fluorescent margin only developed tumors in animals but could not form neurospheres in vitro suggesting that the former are more likely to have stem cell properties [20]. Rampazzo et al. [21] then used single cell sorting to confirm this finding. They also showed that both ALA positive as well as negative cells are variably distributed within the central tumor as well as peripheral zone and that not all tumor cells in the central tumor core stain with ALA. This corroborates our findings. This means that even with more objective methods of detecting fluorescence (as PPIX assays), it may still be possible to miss some tumor tissues. Therefore, by improving sensitivity of fluorescence detection methods as is being attempted by many groups, it may be unlikely that all the coalescent tumor would be visualized (as we show that some coalescent tumor biopsies show negligible PPIX levels). Some other biological factors are probably at play and need to be explored using various OMICS based approaches. Whether this is a function of variable uptake dynamics (related to BBB and cell transport characteristics) or truly a manifestation of variable metabolic machinery in different regions of the tumor is unclear. Identifying and modulating these could enhance fluorescence visualization and possibly aid resection and therapeutic targeting of these tumors. More concerted basic research into understanding this phenomenon is essential.

Limitations

This was a small study of selected patients. We included only cases with heterogeneous enhancement and thereby more diffuse and less amenable to complete resection. Whether this influenced the findings is questionable. But similar selection criteria has been used for the study published by Stummer et al. [6]. Further, considering the immense heterogeneity within gliobalstomas, theoretically it is possible that there could have been variations in the tumor biology amongst the samples sent for histology and corresponding PPIX assay. Practically since each sample was removed by a micro-forceps and immediately split into smaller bits, we assumed that this was not the case.

Conclusions

ALA-induced tumor fluorescence in gliomas is variable. Within the main tumor bulk, there exist a dichotomy clearly revealed by the fluorescence. Whereas unequivocally strong fluorescence is difficult to miss and will almost always delineate tumor tissue, weakly fluorescing areas are highly treacherous. Not only is the interpretation very subjective, but the pathological correlation is also ambiguous. Even if the subjectivity is eliminated using more reliable and accurate objective measures (such as PPIX assays) there remains the unresolved problem of biological heterogeneity (in terms of PPIX metabolism) within histologically seemingly similar tumor regions. Our results reveal this heterogeneity. The biological correlate of this phenomenon needs further elucidation. This could have important ramifications considering that photodynamic diagnosis as well as photodynamic therapy could be directly influenced by this. Unravelling the mechanisms of this biological heterogeneity could pave the way for augmenting PDD and PDT and thereby improve overall outcomes in gliomas.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. It was approved by the institute review board (IEC Approval: ACTREC IEC No. 139, dated 12th May 2014).

Informed consent It was obtained from all individual participants included in the study.

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