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Quantitative Spectroscopic Analysis of 5-Aminolevulinic Acid-Induced Protoporphyrin IX Fluorescence Intensity in Diffusely Infiltrating Astrocytomas

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Abstract

The fluorescence of protoporphyrin IX (PpIX) induced endogenously by 5-aminolevulinic acid (5-ALA) administration has recently been used for the intraoperative visualization of glioma tissues. To increase the sensitivity of photodetection, the emission spectra of 5-ALA-induced PpIX fluorescence was quantitatively measured in tissues taken from six cases of en bloc resected diffusely infiltrating astrocytomas (2 diffuse astrocytomas, 2 anaplastic astrocytomas, and 2 glioblastomas), and the correlation assessed between the fluorescence intensity and histological features. A total of 65 slices were analyzed by ex vivo spectroscopy. The ratio of the peak emission intensity to reflected excitation intensity or fluorescence intensity ratio was less than 0.001 for all 36 non-tumor tissues. The tissues with glioblastoma morphology had a fluorescence intensity ratio in excess of 0.090. The spectroscopic fluorescence intensity was positively correlated with the MIB-1 labeling index as an indicator of proliferation activity, the CD31-microvessel density as a pan-endothelial marker, and the vascular endothelial growth factor expression as an angiogenetic factor. The MIB-1 proliferation index was the most powerful determinant, suggesting that higher cell proliferation may govern preferential PpIX accumulation in glioma cells. This preliminary study suggests that spectroscopic analysis may be useful for optimizing the removal of diffuse gliomas.

Key words: 5-aminolevulinic acid, diffusely infiltrating astrocytoma, fluorescence, protoporphyrin, spectroscopy

Introduction

Diffusely infiltrating astrocytomas, which include diffuse astrocytoma (World Health Organization [WHO] grade II), anaplastic astrocytoma (WHO grade III), and glioblastoma (WHO grade IV), are the most common neoplasms of the human nervous system and arise preferentially in the cerebral hemispheres of adults.[1] Several clinical studies incorporating early postoperative magnetic resonance (MR) imaging have shown the survival advantage of extensive cytoreductive surgery.[2,3] However, radical removal is often difficult to achieve due to the intrinsic tendency for diffuse infiltration of the neighboring brain structures, largely irrespective of the histological malignancy grade.[3,4]

Recently, a novel method using 5-aminolevulinic acid (5-ALA), a metabolic precursor of protoporphyrin IX (PpIX), has been exploited for the intraoperative visualization of tumor tissues.[3,4] Malignant glioma cells are likely to selectively synthesize and accumulate highly fluorescent PpIX following exogenous administration of 5-ALA.[4] Significantly better survival was found among 52 patients with glioblastomas in whom visible fluorescent tissue was completely removed, independent of the other clinical variables examined.[3] Therefore, 5-ALA-induced PpIX fluorescence could be used to detect the extent of infiltrating tumors and to enhance the completeness of tumor removal. Unfortunately, the clinical practicality of this application is impaired

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possibility for overcoming such limitations in the sensitivity is the quantitative measurement of fluorescence signals emitted specifically from the tumor tissue.

This preliminary study examined various biopsied tissues taken from six en bloc resected diffusely infiltrating astrocytomas for quantitative analysis of the 5-ALA-induced PpIX fluorescence signal by ex vivo spectroscopy, and assessed the correlation of the fluorescence intensity with the histological characteristics.

Patients and Methods

The study protocol was approved by the Clinical Research Ethics Committee, and all patients gave informed consent. The tumor specimens were obtained from six patients with tumors totally removed by en bloc gyrectomy, with a histological diagnosis of supratentorial diffusely infiltrating astrocytoma.

Patients received 20 mg 5-ALA/kg body weight (Cosmo Bio Co., Ltd., Tokyo) dissolved in 20 ml glucose solution administered orally at 1 hour before the induction of anesthesia. Tumor removal was completed within 6 hours after the drug administration. Immediately after completion of the en bloc resection, tissue slices were brought to a darkroom and were illuminated with violet-blue light (405 ± 1 nm) delivered from a VLD-M1 device (M&M Co., Ltd., Tokyo). At least 10 sections per case were randomly chosen and were subjected to analysis by PpIX-fluorescence employing visual light spectroscopy (C9183; Hamamatsu Photonics K.K., Hamamatsu, Shizuoka). As shown in Fig. 1, the spectrum of the PpIX-fluorescent tissue peaks at 635 nm. To quantify the fluorescence intensity of each sample, the fluorescence intensity ratio was calculated as the ratio of the peak emission intensity to reflected excitation light intensity after subtracting the extrapolated autofluorescence curve. The fluorescence intensity ratio was measured at least three times in each region, but no decrease in intensity was found. The macroscopic fluorescence intensity was simultaneously observed using a long-pass filter and was recorded as: , absence of fluorescence; , pink fluorescence; or , bright red fluorescence.

Individual sections were fixed with 10% buffered formalin and embedded in paraffin to correlate the fluorescence findings and histological features of the same section stained with hematoxylin and eosin. Any specimen that contained obvious tumor cells in the hematoxylin and eosin-stained section was subjected to immunohistochemical analysis of

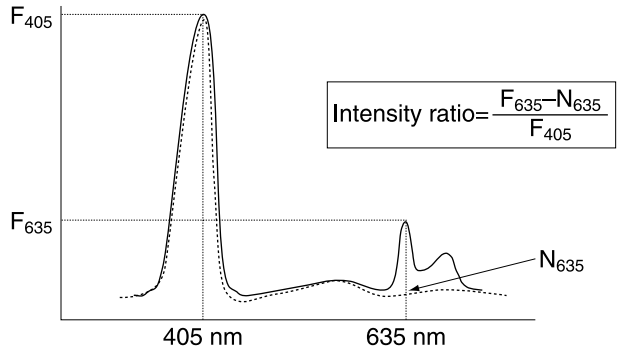

Fig. 1 Spectrum of protoporphyrin IX-fluorescent tissue demonstrating the peak at 635 nm. The fluorescence intensity ratio was calculated as the ratio of the peak emission intensity to reflected excitation light intensity after subtracting the extrapolated autofluorescence curve. F_{405} and F_{635} indicate the absolute values of the fluorescence intensity at 405 nm and 635 nm, respectively; N_{635}, the autofluorescence at 635 nm.

the MIB-1 labeling index, CD31-microvessel density, and vascular endothelial growth factor (VEGF) expression. Sections were deparaffinized in xylene and rehydrated in a graded ethanol series. Endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxidase in methanol for 30 minutes at room temperature. For MIB-1 and VEGF immunohistochemistry, sections were immersed in 0.01 M citrate buffer (pH 6.0) and heated in a microwave oven, then incubated at room temperature with antibodies to MIB-1 (1:50, M7240; Dako Corp., Tokyo), CD31 (1:100, JC-70A; Dako Corp.), and VEGF (1:40, Ab-2; Calbiochem, San Diego, Calif., U.S.A.). The reaction was visualized using a Vectastain ABC kit and diaminobenzidine (Vector Laboratories, Burlingame, Calif., U.S.A.), and sections were counterstained with hematoxylin. To determine the labeling index of MIB-1 and VEGF, the percentage of immunoreactive tumor cells per 100 tumor cells was estimated in at least 10 fields per preparation, and the mean was calculated. For VEGF, tumors with a labeling index of more than 10% were considered as positive.[6] The microvessel density assessed by CD31 staining was calculated as reported previously.[5]

Results

A total of 65 slices were obtained from the six en bloc resected tumors, including two diffuse astrocytomas, two anaplastic astrocytomas, and two glioblastomas. Histological examination of the hematox-

Table 1 Summary of findings for the 29 tumor specimens

Case No.	Histology	Age (yrs)/ Sex	Tumor size* (cm)	Specimen No.	Macroscopic fluorescence	Fluorescence intensity ratio	MIB-1 labeling (%)	CD31-microvessel density	VEGF expression
1	DA	57/M	5 × 3	1	−	0.017	0.3	83.6	−
				2	−	0.018	0.5	82.6	−
				3	−	0.008	0.3	76.5	−
				4	−	0.023	0.2	75.8	−
				5	−	0.031	0.2	88.7	−
2	DA	48/M	7 × 5	1	−	0.040	6.0	65.7	−
				2	−	0.014	0.1	87.9	−
				3	−	0.017	0.9	79.7	−
				4	−	0.040	2.9	62.8	−
				5	−	0.015	0.1	72.0	−
3	AA	62/M	6 × 4	1	−	0.011	0.9	83.7	−
				2	+	0.055	4.6	82.6	+
				3	+	0.051	4.3	79.0	+
				4	−	0.026	3.4	70.7	−
				5	−	0.005	2.9	69.8	−
4	AA	55/M	7 × 6	1	−	0.049	5.2	75.6	−
				2	−	0.014	0.8	86.0	−
				3	−	0.010	0.4	66.8	−
				4	+	0.081	9.6	85.5	−
				5	+	0.088	9.6	81.3	+
5	GB	71/F	6 × 4	1	++	0.130	27.8	92.1	+
				2	++	0.090	20.1	95.2	+
				3	−	0.033	6.0	88.6	+
				4	+	0.054	15.7	81.3	−
				5	++	0.110	22.5	84.3	+
6	GB	74/F	6 × 5	1	+	0.054	9.2	66.8	−
				2	++	0.120	32.4	98.2	−
				3	++	0.094	14.3	86.6	−
				4	++	0.110	22.6	82.1	−

*Tumor size was assessed by the largest cross-sectional diameter of en bloc resected tissue. AA: anaplastic astrocytoma, DA: diffuse astrocytoma, GB: glioblastoma, VEGF: vascular endothelial growth factor.

ylin and eosin-stained sections failed to demonstrate obvious tumor cells in 36 specimens. Of these, 28 specimens corresponded to the adjacent normal brain tissue and eight revealed areas of gross tumor necrosis. No PpIX-fluorescence signal was found macroscopically in any of these 36 slices, and the fluorescence intensity ratio measured by ex vivo spectroscopy was less than 0.001.

Table 1 summarizes the 29 slices including neoplastic tissues. Visible PpIX-fluorescence was noted in 12 specimens, all of which had a fluorescence intensity ratio of more than 0.05. None of the diffuse astrocytoma slices contained macroscopic fluorescent tissue. In contrast, the glioblastoma tissues were characterized by bright red fluorescence, and the fluorescence intensity ratio exceeded 0.090.

Although the number of samples was too small to perform rigorous statistical analysis, the fluorescence intensity ratio was positively correlated with MIB-1 labeling index ($p < 0.001$ and R 0.929 by Pearson's correlation coefficient test) (Fig. 2) and CD31-microvessel density (p 0.0067 and R 0.487 by Pearson's correlation coefficient test). Tumors with VEGF expression had a median fluorescence intensity ratio of 0.0880 as opposed to a median fluorescence intensity ratio of 0.0245 in tumors without VEGF expression (p 0.0100 by the Mann-Whitney test). The median CD31-microvessel density was 84.3 in tumors with VEGF expression and 80.5 in tumors without VEGF expression (p 0.0665 by the Mann-Whitney test). The independent influences of each variable on the fluorescence intensity ratio were examined by multiple regression analysis. The percentage of immunoreactive tumor cells was employed as a continuous variable for VEGF expression. Only the MIB-1 labeling index was significantly related to the fluorescence inten-

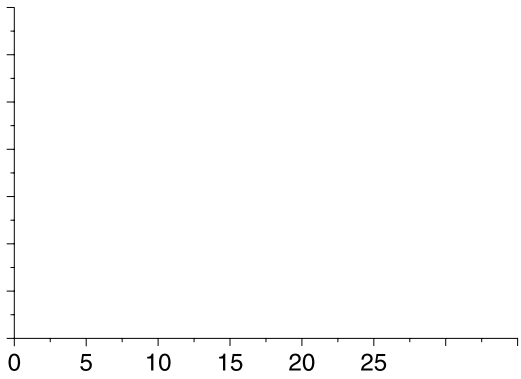

Fig. 2 Relationship between the fluorescence intensity ratio and MIB-1 labeling index ($p < 0.0001$, R 0.929).

sity ratio ($p < 0.0001$).

Discussion

The present quantitative ex vivo spectroscopy analysis of 5-ALA-induced PpIX fluorescence signal in various en bloc resected diffusely infiltrating astrocytomas indicated a positive correlation between the fluorescence intensity ratio and histological malignancy grade. Furthermore, the fluorescence intensity ratio was positively correlated with the MIB-1 labeling index as an indicator of proliferation activity, the CD31-microvessel density as a pan-endothelial marker, and the VEGF expression as an angiogenetic factor. Multiple regression including these three variables showed MIB-1 labeling index was the most important factor associated with fluorescence accumulation. These findings suggest that spectroscopic analysis has potential for the intraoperative identification of residual tumor.

Although the mechanism regulating specific PpIX accumulation in glioma cells following exogenous administration of 5-ALA remains to be definitively elucidated, abnormal blood-brain barrier associated with endothelial proliferation may be a prerequisite for the accumulation of PpIX fluorescence.[1] The present study demonstrated a significant correlation of fluorescence intensity ratio to the presence of VEGF expression and higher CD31-microvessel density. However, the MIB-1 proliferation index was more closely correlated to the fluorescence intensity ratio, suggesting that proliferation may offer a more powerful determinant of fluorescence accumulation than blood-brain barrier abnormality or neovascularization.

In the present study, ex vivo spectroscopy showed a high degree of specificity, with a fluorescence spectrum ratio of less than 0.001 in the non-neoplastic tissues, more than 0.05 in the visible fluorescent tissues, and more than 0.09 in the tissues with glioblastoma morphology. Nevertheless, this method cannot be directly adapted to intraoperative applications for the photodetection of glioma tissues, since the PpIX fluorescence signals in the operative field could be easily affected by various factors including migration of external light, bleaching of the fluorescence by excessive microscope illumination, and contamination from blood that contains considerable amounts of PpIX.[4] Further investigations to evaluate in vivo spectroscopy, as well as the development of an optical fiber probe that could be easily integrated with the tumor resection apparatus, are required to facilitate assessment of the tissue fluorescence in the clinical setting.

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Commentary

5-Aminolevulinic acid (ALA)-induced protoporphyrin IX (PpIX) has recently been used as a photosensitizer for the intraoperative visualization of glioma tissues. This study investigates the sensitivity of photodetection and the tumor tissue proliferation activity in 6 patients with gliomas by analyzing PpIX fluorescence induced by orally administered ALA. The authors found that higher cell proliferation may induce more PpIX accumulation in diffuse gliomas.

I think such spectroscopic analysis is a promising and valuable tool for optimizing the removal of diffusely infiltrating astrocytomas by detecting the boundaries of the infiltrate when standard fluorescence techniques fail. This technique offers the potential to be advantageous over other non-optical techniques in terms of providing real-time diagnosis by in situ monitoring.

Although 5-ALA and its derivatives have been widely studied and applied in clinical photodynamic therapy (PDT), a common problem encountered when using fluorescence detection in real sample analysis is that the matrix may contain compounds that autofluoresce or that can be excited at the wavelengths of commonly employed fluorescent reporter molecules. This causes an increase in background fluorescence, which in turn tends to compromise the detection limits of the system. Because the PpIX fluorescence signals could be easily affected by various factors, further investigation is needed to evaluate in vivo spectroscopy.

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The authors have thoroughly conducted a study of the comparison of the fluorescence intensity derived from 5-aminolevulinic acid (ALA) administration to glioma patients compared with MIB-1 index, CD31-microvessel density, and vascular endothelial proliferation factor (VEGF) expression. Although 5-ALA has become an increasingly popular method for detection of the tumor margin, the exact uptake mechanism is still not fully clarified. The strong point of this study is that the authors have used an ex vivo semi-quantitative method for evaluation of the fluorescence intensity. Many studies have used macroscopic evaluation of the fluorescence and/or semi-quantitative evaluation during operation. The fluorescence intensity may be unstable if used for in situ evaluation. In contrast, the ex vivo data is a stable method for evaluating the fluorescence intensity if the specimen is correctly handled. Therefore, the high correlation of the fluorescence intensity and MIB-1 index is one of the important findings to understand the mechanism of fluorescence in tumor tissue. The negative or weak correlation of the fluorescence with CD-31 microvessel density and VEGF are also important negative findings in understanding the underlying mechanism for 5-ALA photodynamic diagnosis (PDD).

There are still open questions about positive and negative fluorescence in 5-ALA PDD, for example, the influence of steroid therapy, and positive fluorescence in benign tumors such as meningiomas and other benign tumors. I am looking forward to further studies from the authors to clarify the mechanism for 5-ALA PDD and to improve the sensitivity and specificity of the PDD for better evaluation of tumor margin.

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This report is idealistic about radical removal of diffusely infiltrating astrocytomas by use of protoporphyrin IX fluorescence. As we know, astrocytoma cells usually infiltrate into the white matter without obviously destroying the fibers, therefore, the surgery following the guide of fluorescence may induce serious damage. In fact, this study is still using ex vivo spectroscopy, and the number of cases is still small, and the methods cannot be directly used in the intraoperative field. However, the MIB-1 proliferation index of the tumors with fluorescence intensity ratio may have more significance in research.

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