Histopathological implications of ventricle wall 5-aminolevulinic acid-induced fluorescence in the absence of tumor involvement on magnetic resonance images

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Received February 9, 2016; Accepted March 28, 2016

DOI: 10.3892/or.2016.4881

Abstract. During 5-aminolevulinic acid (ALA)-guided glioblastoma multiforme (GBM) surgery, we encountered fluorescence in ventricular walls that lacked enhancement on magnetic resonance (MR) images and were free of macroscopic invasion of tumor cells. However, the meaning of ventricular wall fluorescence during 5-ALA-guided surgery is still unknown. The aim of this study was to investigate the relationship between intraoperative 5-ALA fluorescence and histopathological findings of ventricular walls free of enhancement on MR images. Nineteen patients with newly diagnosed GBM located near the lateral ventricle underwent 5-ALA fluorescence-guided surgery. During the surgery, the ventricle wall was opened and investigated with the aid of a surgical microscope equipped with optical filters to examine 5-ALA fluorescence of the ventricular wall. Twenty-five ventricular wall tissues that were apparently free of tumor involvement by MR imaging and macroscopic observation were obtained during surgery. Among the 19 cases with brightly fluorescing tumor masses, 11 patients (57.9%) exhibited 5-ALA-induced fluorescence in the ventricular wall. Of the 25 ventricular wall samples, 11 exhibited 5-ALA-induced fluorescence; upon pathologic examination, tumors were present in 5 samples (45.5%), but the remaining 6 (54.5%) were free of tumor cells. A pathologic examination revealed no tumor cells in the 14 samples that lacked 5-ALA-induced fluorescence. Our data suggest the possibility that glioma cells exhibiting 5-ALA fluorescence are present in the ventricle wall, despite no signs of tumor involvement in MR images. Further investigation of non-tumor cells from tissues with 5-ALA fluorescence is needed to understand the nature of this unexpected ventricular wall fluorescence.

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Key words: 5-aminolevulinic acid, fluorescence, glioblastoma, pathology, ventricular wall

Introduction

5-Aminolevulinic acid (5-ALA) is a natural metabolic precursor in the heme biosynthesis pathway. Oral administration of a large volume of 5-ALA overloads the heme pathway and induces the synthesis and selective accumulation of the fluorescent protoporphyrin IX (PpIX) in tumor cells and epithelial tissue (1,2). Increased vascular permeability attributable to disruption of the blood-brain barrier (BBB) in the tumor and decreased levels of ferrochelatase activity in tumor cells contribute to this phenomenon in glioblastoma multiforme (GBM) (3-6). PpIX in GBM tumors, present at higher levels compared to normal brain tissue, emits red-violet fluorescence under blue light, visually distinguishing tumor margins and allowing intraoperative, objective assessment of tumor infiltration (7,8). Evidence demonstrating the high sensitivity and specificity of 5-ALA-induced PpIX fluorescence in GBM provides support for the diagnostic accuracy of 5-ALA. As a result, 5-ALA fluorescence guidance has come to be widely used to improve the extent of tumor resection (9).

Since a number of studies have demonstrated that the extent of resection (EOR) is correlated with improved prognosis of patients with GBM, neurosurgeons have sought to maximize the EOR, an objective constrained by the difficulties of resection arising from the invasive nature of GBM (10-15). A large, randomized, controlled, multicenter phase III trial has shown that 5-ALA fluorescence-guided surgery for malignant glioma leads to a significantly higher resection rate, resulting in prolonged progression-free survival compared with conventional microsurgery guided by white light (16). In addition, several techniques, including intraoperative neuronavigation (17), intraoperative magnetic resonance (MR)

imaging (18), intraoperative ultrasound (19) and intraoperative electrical stimulation (20), have been introduced to facilitate optimal resection, thereby maximizing safe EOR and improving survival in patients with GBM. Increasing the EOR of GBM to achieve these survival benefits brings with it a greater chance of entering the ventricular system during the course of cytoreduction. In some cases of 5-ALA-guided GBM surgery, upon ventricular entry we encountered fluorescence in ventricular walls that lacked enhancement on MR images and were free of macroscopic invasion of tumor cells. However, the implications of this 5-ALA-induced fluorescence of the ventricle wall are not yet fully understood.

In this study, we obtained 25 ventricular wall tissues from 19 patients with newly diagnosed GBM that showed no enhancement on MR images, and investigated the relationship between intraoperative 5-ALA fluorescence and histopathology findings of these non-enhancing ventricular wall tissues.

Materials and methods

Patient information. Nineteen patients who underwent fluorescence-guided surgery with 5-ALA for newly diagnosed GBM at our hospital from December 2012 to May 2015 were included in this study. Approval was given by the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine. Informed consent was provided according to the Declaration of Helsinki. Of these 19 patients, 12 were males and 7 were females, and their age ranged from 45 to 74 years (mean, 58.5 years). All patients were newly diagnosed with GBM, and had no prior history of treatment with surgery, chemotherapy, or radiotherapy. All tumors showed typical enhancement patterns of GBM in MR images after the administration of contrast medium. In all cases, the ventricle was opened during resection of the tumor, which was located near the lateral ventricle. The characteristics of the 19 patients are summarized in Table I.

Surgical procedure. Three hours prior to induction of anesthesia, 5-ALA (Gliolan; Photonamic GmbH & Co. KG, Wedel, Germany) was administered orally at a dose of 20 mg/kg body weight. Patients were protected from direct exposure to light sources for 24 h after intake of 5-ALA to avoid skin phototoxicity. Preoperative, high-resolution, contrast-enhanced, T1-weighted axial MR images were obtained for each patient on the day of the procedure. All tumor resections were performed under the guidance of a neuronavigation system [StealthStation Treon (Medtronic, Minneapolis, MN, USA) or Stryker (Stryker Instruments, Kalamazoo, MI, USA)] using the MR images. Additional functional MR images and diffusion tensor images were used as appropriate, depending on tumor location. Zeiss OPMI Pentero microscopes (Carl Zeiss Surgical GmbH, Oberkochen, Germany) equipped with BLUE 400 fluorescence technology, which enabled switching from conventional standard white xenon light to filtered violet-blue excitation light for visualization of fluorescence, were used in all patients. In all cases, the tumor was resected to the extent possible consistent with safety, and supratotal resection was performed in some patients with a tumor in a non-eloquent area. After the opening of lateral ventricles, the fluorescence of the ventricular wall was examined with a microscope by switching between white light and violet-blue excitation light. Regions were annotated as non-visible, weak, or strong fluorescence for 5-ALA-induced fluorescence by the operating neurosurgeon, and samples of these regions were collected for histopathological analysis. Before sampling, regions of ventricular walls were checked macroscopically for tumor involvement and confirmed with the aid of the neuronavigation system. Tumor involvement was defined as the presence of macroscopic invasion of tumor or enhancement on the preoperative contrast-enhanced T1-weighted MR images used for neuronavigation.

Histopathological analysis. Specimens from patients with GBM were freshly obtained from the operating room. Samples of ventricular walls for histopathological analysis were categorized according to the presence or absence of 5-ALA-induced fluorescence by the operating neurosurgeon and were forwarded to the neuropathology department. Histopathological analyses were performed on hematoxylin and eosin (H&E)-stained, formalin-fixed, paraffin-embedded tissues from the main tumor mass and ventricular wall. Immunohistochemical staining for glial fibrillary acidic protein (GFAP), the proliferation marker Ki-67, epidermal growth factor receptor (EGFR), and p53 was also carried out to establish a definitive diagnosis. O^6-methylguanine DNA methyltransferase (MGMT) promoter methylation status and isocitrate dehydrogenase 1 (IDH1) mutations were analyzed by polymerase chain reaction (PCR), and loss of heterozygosity (LOH) at chromosomes 1p and 19q was determined by fluorescent *in situ* hybridization. One experienced neuropathologist diagnosed the type and grade of each sample based on the World Health Organization (WHO) 2007 grading criteria (21). To reduce bias, the neuropathologist was blinded to the 5-ALA fluorescence status. Samples in which tumor cells could not be identified and where increased levels of proliferation were not detectable were considered tumor-free.

Results

Ventricle wall fluorescence and sample collection. In each case, the tumor was clearly revealed in the surgical field and exhibited 5-ALA-induced fluorescence under blue light. Seventeen cases showed strong fluorescence of the main tumors and 2 cases showed weak fluorescence of the main tumors. 5-ALA-induced fluorescence in the ventricular wall was identified in 11 patients (57.9%) after opening the lateral ventricle. The fluorescent areas of the ventricular wall varied from one case to another. In these 11 patients, 5 showed strong fluorescence of the ventricular walls and 6 showed weak fluorescence of the ventricular walls. However, there was no correlation between the fluorescence intensity of the ventricular wall and that of the main tumors. A total of 25 samples of the ventricular wall, which is divided into 5 regions (anterior horn, body, atrium, occipital horn, and temporal horn) along the rostrocaudal axis (22), were collected intraoperatively from the 19 GBM patients: five from the anterior horn, one from the atrium, 2 from the occipital horn, and 12 from the temporal horn. Of these 25 samples, 11 showed observable intraoperative 5-ALA-induced fluorescence, whereas 14 samples did

Table I. Clinical and molecular characteristics of the 19 patients with glioblastoma multiforme.

Case no.	Age (years), gender	Tumor location	Extent of resection	Tumor contact to lateral ventricle	IDH1 mutation	EGFR	p53 mutation (%)	Ki-67 LI (%)	MGMT promoter methylation	1p LOH/ 19q LOH
1	61, M	Rt. FT	Subtotal	Yes	No	2+	40	60	Unmethylated	No/Yes
2	66, F	Rt. TP	Total	Yes	No	3+	3	50	Unmethylated	Yes/Yes
3	61, M	Rt. temporal	Total	Yes	No	3+	5	20	Unmethylated	Yes/No
4	70, F	Rt. TPO	Subtotal	Yes	No	3+	Negative	30	Methylated	No/No
5	62, F	Rt. frontal	Supratotal	No	No	3+	Negative	5	Methylated	No/No
6	53, M	Lt. temporal	Total	Yes	No	3+	2	60	Unmethylated	No/No
7	45, M	Rt. frontal	Total	Yes	No	3+	Negative	50	Methylated	Yes/No
8	45, M	Rt. TPO	Subtotal	No	No	3+	30	40	Unmethylated	No/No
9	58, F	Lt. TP	Total	Yes	No	2+	60	30	Methylated	Yes/Yes
10	56, M	Rt. temporal	Supratotal	Yes	No	1+	2	5	Methylated	No/No
11	51, M	Lt. FT	Subtotal	Yes	No	2+	40	15	Unmethylated	No/No
12	51, M	Lt. frontal	Total	Yes	Yes	0	25	7	Methylated	Yes/Yes
13	62, F	Rt. temporal	Subtotal	Yes	No	1+	5	20	Unmethylated	No/No
14	58, F	Lt. TP	Total	Yes	No	3+	Negative	3	Unmethylated	No/No
15	67, M	Rt. TPO	Subtotal	Yes	No	3+	90	40	Methylated	Yes/Yes
16	74, M	Lt. temporal	Subtotal	Yes	No	1+	70	30	Unmethylated	No/No
17	65, F	Rt. temporal	Total	No	No	0	1	3	Unmethylated	No/No
18	46, M	Rt. temporal	Total	No	No	3+	5	20	Unmethylated	No/No
19	60, M	Rt. PO	Supratotal	Yes	No	3+	20	25	Unmethylated	No/No

FT, frontotemporal; TP, temporoparietal; TPO, temporo-parieto-occipital; PO, parieto-occipital; LOH, loss of heterozygosity; LI, labeling index.

not (Table II). In all cases, the ventricular wall areas sampled showed no macroscopic evidence for tumor involvement and were free of enhancement on MR images.

Histopathological analysis of ventricular wall samples. Histopathological assessments of the main tumor mass confirmed WHO Grade IV GBM in every case (Fig. 1). Eleven ventricular wall samples with observable intraoperative 5-ALA-induced fluorescence were analyzed; 5 (45.5%) were positive for the presence of tumor cells, whereas the remaining 6 (54.5%) were free of tumor cells. Of the 5 ventricular wall samples that showed the presence of tumor cells, 2 (40%) corresponded to low-grade glioma, and 3 (60%) corresponded to high-grade glioma. Fourteen ventricular wall samples without observable intraoperative 5-ALA-induced fluorescence were analyzed; no tumor cells were identified in any of the 14 samples (Table II). 5-ALA exhibited a sensitivity of 100% and specificity of 70% [95% confidential interval (CI): 45.72-88.11%] in detecting tumor invasion of ventricular wall samples. Positive predictive values and negative predictive values were 45.5% (95% CI: 16.75-76.62%) and 100%. The overall accuracy of this method was 76%. However, we did not find any correlation between the fluorescence intensity and the pathological finding of the ventricular wall in this study.

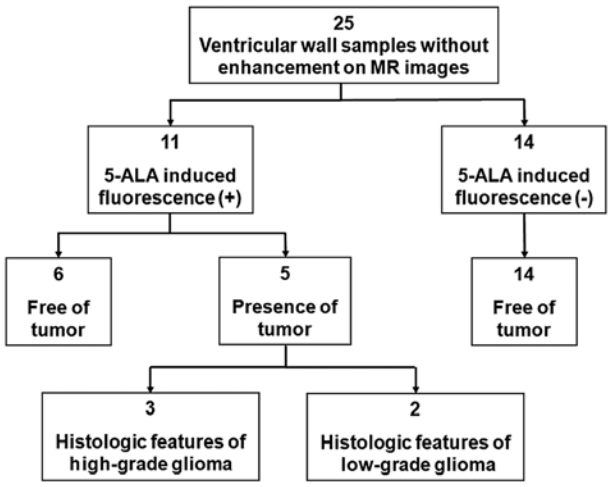

Figure 1. Summary of histopathological assessments of the ventricular wall samples.

Illustrative cases

Patient 1. A 62-year-old woman (case 13) presented with a 1-month history of headache and progressive left hemiparesis. Preoperative MR images revealed a right temporal enhancing mass extending to the frontal lobe and insula (Fig. 2A). A right fronto-temporal craniotomy was performed with 5-ALA fluorescence guidance and the tumor was subtotally removed with a temporal lobectomy. A histopathological analysis confirmed diagnosis of the main tumor mass as GBM. During resection of the tumor, we entered the temporal horn of the left lateral ventricle (Fig. 2B), and detected apparent fluorescence on some parts of the ventricular wall (Fig. 2C). Ventricular wall

Table II. 5-ALA fluorescence characteristics and pathological findings of the 19 patients with glioblastoma multiforme.

Case no.	Ventricular wall sampling site	5-ALA fluorescence of tumor	Ventricular wall tissue		Presence of tumor cells
			No. of samples	5-ALA fluorescence	
1	Temporal horn	Strong	1	Strong	Yes (low-grade)
2	Temporal horn	Strong	1	Strong	No
3	Temporal horn	Strong	2	Weak Non-visible	Yes (low-grade) No
4	Temporal horn	Strong	2	Strong Non-visible	Yes (high-grade) No
5	Anterior horn	Strong	1	Non-visible	No
6	Temporal horn	Strong	2	Weak Non-visible	No No
7	Anterior horn	Weak	1	Non-visible	No
8	Occipital horn	Strong	1	Weak	No
9	Temporal horn	Weak	1	Non-visible	No
10	Temporal horn	Strong	2	Weak Non-visible	No No
11	Anterior horn	Strong	1	Non-visible	No
12	Anterior horn	Strong	1	Non-visible	No
13	Temporal horn	Strong	2	Weak Non-visible	Yes (high-grade) No
14	Atrium	Strong	1	Strong	Yes (high-grade)
15	Temporal horn	Strong	2	Strong Non-visible	No No
16	Temporal horn	Strong	1	Non-visible	No
17	Temporal horn	Strong	1	Non-visible	No
18	Temporal horn	Strong	1	Non-visible	No
19	Occipital horn	Strong	1	Weak	No

samples obtained as part of the planned temporal lobectomy were analyzed histopathologically. The presence of tumor consistent with high-grade glioma was confirmed in a sample of weakly fluorescent ventricular wall tissue without contrast enhancement on MR images (Fig. 2D). The pathological diagnosis of strongly fluorescent ventricular wall tissue with contrast enhancement on MR images was GBM (Fig. 2E). No tumor cells were identified in the sample from the non-fluorescent ventricular wall tissue lacking contrast enhancement on MR images (Fig. 2F). A postoperative MR image showed that the tumor was resected to the greatest possible extent, and an additional right temporal lobectomy was performed (Fig. 2G).

Patient 2. A 67-year-old man (case 15) presented with a 2-week history of confusion, left homonymous hemianopsia, and left hemiparesis. Preoperative MR images revealed a right temporo-parietal enhancing mass that involved the posterior part of the temporal horn of the right lateral ventricle (Fig. 3A and B). The tumor was resected under the guidance of 5-ALA fluorescence, and showed strong red fluorescence under blue light. The histopathological diagnosis of the main tumor mass was GBM. After the ventricle was opened (Fig. 3C and D), we encountered fluorescence in the ventricle walls that were free of enhancement on MR images (Fig. 3E and F). Ventricular wall samples obtained as part of the planned lesionectomy and temporal lobectomy were analyzed histopathologically. The pathological diagnosis of strongly fluorescent ventricular wall tissue with contrast enhancement on MR images was GBM (Fig. 3G). No tumor cells were identified in the samples from either strongly fluorescent ventricular wall tissue or non-fluorescent ventricular wall tissue lacking contrast enhancement on MR images (Fig. 3H and I). A postoperative MR image showed that the tumor was resected to the extent possible, and an additional right temporal lobectomy was performed (Fig. 3J and K).

Discussion

Because 5-ALA has been widely used for improving surgery of malignant gliomas, it is not rare to detect 5-ALA-induced fluorescence of the ventricle wall in cases where the ventricle is

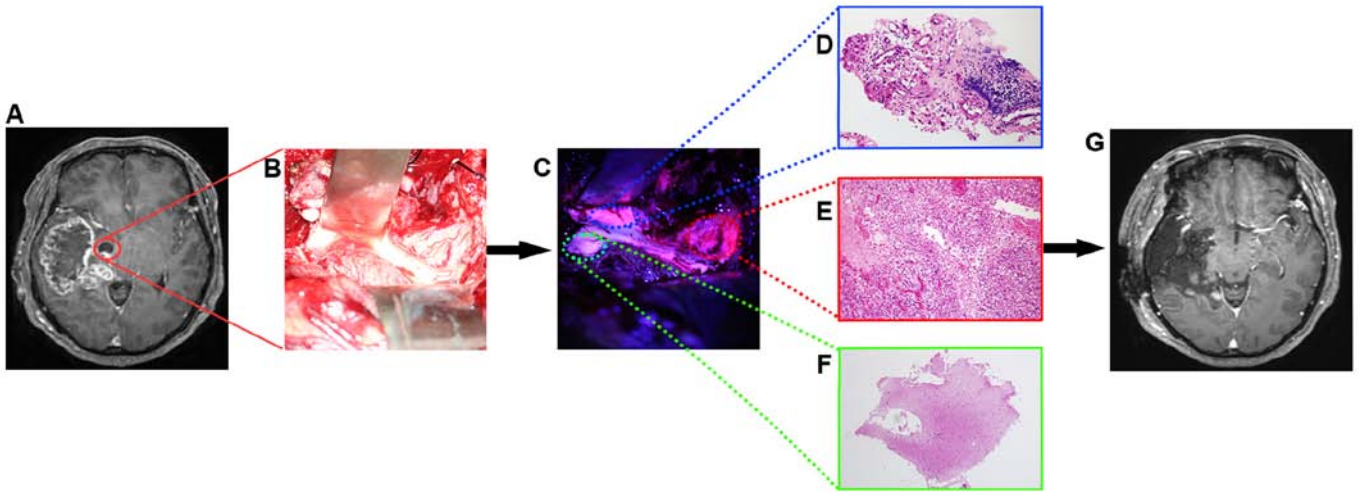

Figure 2. Case 13. (A) Preoperative, axial, T1-weighted, post-contrast MR image showing a tumor located near the lateral ventricle. (B) Intraoperative microscopic image under white light illumination showing the exposed temporal horn of the lateral ventricle. (C) Intraoperative microscopic image under blue light showing ventricular walls with varying intensity of fluorescence and no fluorescence. (D) Histopathological analysis of weakly fluorescent ventricular wall tissue lacking contrast enhancement on MR images, confirming the presence of a tumor consistent with high-grade glioma. (E) Histopathological analysis of strongly fluorescent ventricular wall tissue exhibiting contrast enhancement on MR images, confirming the presence of a tumor consistent with GBM. (F) Histopathological analysis of non-fluorescent ventricular wall tissue lacking contrast enhancement on MR images, confirming the absence of tumor. (G) Axial T1-weighted post-contrast MR image showing a tumor resection that includes ventricular wall tissues as part of the planned margin of resection.

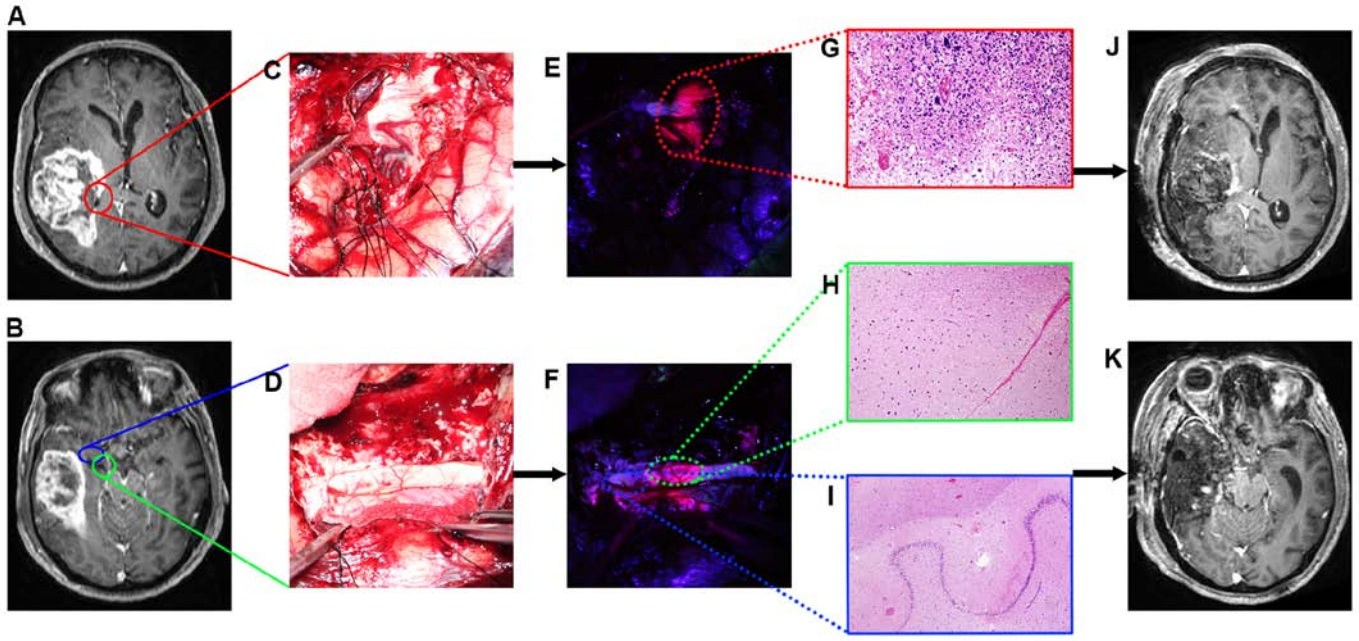

Figure 3. Case 15. (A) Preoperative, axial, T1-weighted, post-contrast MR image showing tumor involvement in the lateral ventricle. (B) Preoperative axial T1-weighted post-contrast MR image showing a temporal horn of the lateral ventricle without tumor involvement. (C) Intraoperative microscopic image under white light illumination showing the exposed lateral ventricle. (D) Intraoperative microscopic image under white light illumination showing the exposed temporal horn of the lateral ventricle. (E) Intraoperative microscopic image under blue light showing ventricular walls with strong fluorescence. (F) Intraoperative microscopic image under blue light showing ventricular walls with and without fluorescence. (G) Histopathological analysis of strongly fluorescent ventricular wall tissue exhibiting contrast enhancement on MR images, confirming the presence of a tumor consistent with GBM. (H) Histopathological analysis of strongly fluorescent ventricular wall tissue lacking contrast enhancement on MR images, confirming the absence of tumor. (I) Histopathological analysis of non-fluorescent ventricular wall tissue lacking contrast enhancement on MR images, confirming the absence of tumor. (J) Axial, T1-weighted, post-contrast MR image demonstrating resection of a tumor. (K) Axial T1-weighted post-contrast MR image showing a tumor resection that includes ventricular wall tissues as part of the planned margin of resection.

opened during the operation (23,24). Although the relationships between 5-ALA-induced fluorescence and pathologic parameters in gliomas and peritumoral tissues have been extensively investigated, few studies have addressed the histopathological implications of 5-ALA-induced fluorescence in ventricular wall tissues that show no tumor involvement. In a series of 7 patients with periventricular GBMs, Hayashi *et al* (23) found that most ventricle wall tissues with unenhanced MR images that showed 5-ALA-induced fluorescence were positive for the presence of tumor cells, whereas all tissues showed disrup-

tion of ependymal cell layers of the ventricle wall. On the basis of this study, these authors suggested that tumor cells in the ventricular wall or environmental changes around the ventricle could lead to 5-ALA fluorescence of the ventricular wall. In a separate study, Tejada-Solís *et al* (24) concluded that ventricular wall fluorescence does not always indicate glioma cell invasion of the ventricular wall, based on the observation that 3 out of 8 (37.5%) cases that underwent selective biopsy of fluorescent ventricle wall exhibited an intact ependymal layer and no tumor cells. However, these studies were limited by the small number of cases and the lack of negative control specimens.

In the present study, we compared pathological findings of fluorescent ventricular walls with those of non-fluorescent ventricular walls using a relatively large number of samples in a homogeneous group of patients. To maintain the homogeneity of the patient population, we only included patients with newly diagnosed GBM. To eliminate the possibility of pseudo-positive 5-ALA-induced fluorescence in ventricular wall samples, we excluded patients diagnosed with recurring GBM that had undergone prior treatment, including surgery and radiotherapy, because areas showing infiltration of inflammatory cells associated with surgical and radiation interventions can also accumulate PpIX and show 5-ALA-induced fluorescence (25,26).

Compared with the previous studies described above, we obtained a relatively large number of samples from various domains of the lateral ventricle, including non-fluorescent ventricular wall samples as a negative control group. Most of the ventricular wall samples were excised during surgery as part of the planned margin of resection surrounding the GBM. Maximizing the extent of resection likely extends time to progression and increases survival, and supratotal resection of gliomas in non-eloquent regions can be beneficial for clinical outcomes (10,14,15,27-29). Accordingly, we tried to increase the extent of resection during neurosurgical procedures. Because we sought to achieve supratotal resection and additional lobectomy if possible during the removal of GBMs in non-eloquent areas, most ventricular wall samples were consequently included in the resected area. Therefore, we could safely harvest samples from ventricular walls. Additionally, for safety we collected intraoperative samples from ventricular walls in accordance with certain *a prior* criteria. First, we did not intentionally open the ventricle during the surgical procedure to check for fluorescence in the ventricular wall or to obtain ventricular wall samples. Because ventricular opening during surgical procedures can be a risk factor for postoperative hydrocephalus and leptomeningeal dissemination, which may potentially worsen the clinical condition and decrease survival (30-35), opening of the ventricular system was performed only in cases where it was needed for radical supratotal resection of the tumor. Second, ventricular wall samples in the safe area were collected carefully to prevent postoperative neurological sequelae. Functionally important areas, including the fornix, were excluded from ventricular wall sampling, because neurological sequelae can occur due to direct damage to the area. We also excluded certain areas with vascular structures, such as internal cerebral veins and thalamostriate veins, to prevent ischemic insult. Third, we tried to obtain a sufficient ventricular wall sample to clearly assess histopathology, while limiting the depth of tissue resection to <5 mm to prevent damage to structures under the surface.

In our series, ventricular wall fluorescence was detected in a majority (57.9%) of patients, but not in all patients. Considering the variety of locations in the lateral ventricle that showed fluorescence, the frequency of existence of ventricular wall fluorescence may be underestimated since we did not explore the entire lateral ventricle. Five of the 11 (45.5%) fluorescent ventricular wall samples contained glioma cells, whereas none of the 14 non-fluorescent samples showed infiltration of tumor cells (Fig. 1). In contrast to previous studies of the relationships between 5-ALA fluorescence and the pathology of tissue in GBM (9,36,37), the low positive predictive values (PPVs) and high negative predictive values (NPVs) in our series indicate that non-fluorescent ventricular walls under blue light should lack invading tumor cells. On the other hand, fluorescent ventricular walls could possibly reflect the presence of tumor cells, even though the fluorescence is not always related to the infiltration of tumor cells into the ventricular wall. Because the NPV depends greatly on the non-fluorescent tissue biopsy algorithm, the high NPV in our series may imply that ventricular wall samples remote from the tumor and not invaded by tumor cells were properly collected. The likelihood of not finding tumor cells should be higher if sampling sites are remote from the tumor than would be the case if they are close to the tumor. Our results suggest that, in cases where the ventricular wall is fluorescent, there is a chance of tumor cell infiltration into the ependymal spaces; accordingly, we recommend close follow-up with imaging studies and monitoring of the patient's clinical status after the surgical resection of GBM if 5-ALA-induced fluorescence of the ventricular wall is detected during surgery. Since fluorescence can be an indication of ependymal glioma cell invasion or leptomeningeal seeding in some cases, a biopsy should be performed to decide treatment options if feasibility and safety allow. However, postoperative radiotherapy covering the whole ventricle system based on the presence of ventricular wall fluorescence should be avoided because it may not always indicate pathological glioma cell invasion.

Six of the ventricular wall samples in our series were falsely fluorescing, showing no evidence of the presence of tumor cells. This raises questions regarding the meaning of false-positive fluorescence in the ventricular wall. False-positive fluorescence previously described by others was related to infiltration of inflammatory cells and reactive astrocytes, necrosis, prominent vasculature, or peritumoral edema (26,36,37). However, we did not observe such findings in our false-positive ventricular wall samples and found no difference between falsely fluorescing ventricular wall samples and non-fluorescent control samples from the standpoint of histopathology. The intraoperative 5-ALA-induced fluorescence in the 2 samples corresponding to low-grade glioma might be a false-positive finding because the vast majority of low-grade gliomas do not exhibit visible intraoperative fluorescence under a surgical microscope (38-40). A better understanding of 5-ALA-induced fluorescence in the ventricular wall will require further investigation, including a molecular characterization, of these falsely fluorescing samples. In a study with GBM patients by Piccirillo *et al* (41), the histologic analysis and genomic characterization revealed that the fluorescent subependymal zone contained tumor-initiating

cells, however most samples from the fluorescent subependymal zone were truly fluorescing as they were included in the contrast enhancing lesion on MR images and pathologically confirmed to be involved by tumor. Studies with samples from 5-ALA-induced fluorescent ventricular wall tissues which show no tumor involvement are still lacking.

One limitation of this study is its subjective assessment of intraoperative 5-ALA-induced ventricular wall fluorescence. We adopted a trinary approach - non-visible, weak, or strong fluorescence - to assess fluorescence. However, this approach is subjective as fluorescence was estimated by a surgeon using only a surgical microscope with a specific filter. Overcoming this limitation would require quantitative or semiquantitative fluorescence measurements capable of objectively discriminating fluorescence intensity. These modalities, such as intraoperative spectrometry or confocal microscopy, have proven to be more sensitive for the determination of 5-ALA-induced fluorescence than the surgical microscope used here, although these modalities for quantitative determination of fluorescence still need to be validated for clinical use (37-39,42,43). Another limitation of this study is that infiltration of the tumor was judged based on enhancement on T1-weighted, contrast-enhanced MR images. GBMs have an infiltrative nature, and their presence is not always associated with a disrupted BBB; thus, contrast enhancement may not show invasive areas of GBM because it only depicts areas with a disruption in the BBB. Accordingly, active tumor tissue can exist beyond the area of contrast enhancement. T2-weighted and fluid-attenuated inversion recovery MR images may depict invasive areas of GBM; however, it is difficult to determine the extent of the non-enhancing component of the tumor using these approaches owing to peritumoral edema (44,45). On this account, there is the possibility of tumor invasion into ventricular wall samples in some cases, despite the fact that we obtained ventricular wall samples that were definitely remote from the enhanced lesion in MR images. Errors in the neuronavigation system related to image-to-patient registration error and intraoperative brain shift are also a limitation of this study. In all cases, the ventricle was opened and a considerable amount of cerebrospinal fluid was drained out during the surgery, which might subsequently degrade navigational accuracy over the course of a surgical procedure. We tried to compensate for such errors using intracranial anatomical landmarks; however, this method is subjective and could not eliminate the error completely.

In summary, our data suggest the possibility that glioma cells are present in ventricle walls exhibiting 5-ALA fluorescence despite the absence of tumor involvement in MR images. Ventricular walls lacking 5-ALA fluorescence and enhancement on MR images may be free of tumor, so a decision to resect non-fluorescent ventricular walls should be made carefully. Further investigations of non-tumor cells from tissues exhibiting 5-ALA fluorescence are needed to understand the nature of ventricular wall fluorescence.

Acknowledgements

This study was supported by grants from the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2013R1A1A2055597), and the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (HI13C1509).

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