[Review]

5-Aminolevulinic Acid: Pitfalls of Fluorescence-guided Resection for Malignant Gliomas and Application for Malignant Glioma Therapy

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Abstract **:** 5-Aminolevulinic acid (ALA) has been widely used as an intravital fluorescence marker in the fluorescence-guided resection of malignant gliomas. Although not a photosensitizer itself, 5-ALA is a prodrug that accumulates protoporphyrin IX (PpIX) in the mitochondria of glioma cells; PpIX acts as a photosensitizer. Fluorescence-guided resection for malignant gliomas has some pitfalls. Moreover, 5-ALA is not merely a fluorescence marker but has potential as a mitochondria-targeting drug for malignant glioma therapy. In this article, we review the literature related to 5-ALA, discuss the pitfalls of fluorescence-guided resection using 5-ALA for malignant gliomas, and describe the application of 5-ALA for malignant glioma therapy with personal opinions.

Keywords **:** 5-aminolevulinic acid, glioma, mitochondria-targeting drug, reactive oxygen species, radiosensitizer.

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Introduction

5-Aminolevulinic acid (ALA) is a natural biochemical precursor of heme, which is converted by the heme synthesis pathway to protoporphyrin IX (PpIX) in the mitochondria [1]. Following the systemic administration of 5-ALA, normal cells convert 5-ALA to PpIX and then quickly into heme. However, tumor cells cannot perform this conversion and consequently accumulate PpIX in the mitochondria. PpIX acts as a photosensitizer. Generally, photosensitizers have two characteristics: they 1) fluoresce under light exposure, and 2) produce cytotoxic oxygen (mainly singlet oxygen) under light exposure at different wavelengths. Thus, once tumor cells take in a photosensitizer, they can be visualized and cell death can be induced under light exposure. The former characteristic is used for fluorescence-guided resection (photodynamic diagnosis). 5-ALA induces high accumulation of PpIX in glioma cells; thus, fluorescence-guided resection using 5-ALA in malignant gliomas has been useful to determine tumor borders and to detect residual tumors, compared to conventional microsurgery [2, 3]. Fluorescence-guided resection of malignant gliomas using 5-ALA was first approved in 2007 by the European Medical Agency, and in 2013 by the Pharmaceutical Affairs of Japan. Since then, this procedure has been widely used in clinical settings. In 2017, it was also approved by the Food and Drug Administration of the United States of America. In Japan, fluorescence-guided surgery for bladder cancer was also approved as an additional indication in 2017.

In this review, we discuss the pitfalls of fluorescence-guided resection using 5-ALA for malignant

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gliomas and the role of 5-ALA in malignant glioma therapy with our personal opinions.

1. Why does 5-ALA fluoresce in gliomas?

5-Aminolevulinic acid (5-ALA) is metabolized by heme biosynthesis in cells. In this step, many kinds of porphyrin compounds, such as uroporphyrinogen III, coproporphyrinogen III, and PpIX, are produced (Fig. 1). After administration of 5-ALA to patients with malignant glioma, PpIX was the most often confirmed of these porphyrin compounds in tumor specimens [4]. Red fluorescence in malignant gliomas is observed under violet-blue light during surgery; this fluorescence originates from PpIX accumulating in tumors [4, 5]. However, this process is modulated by complex factors in the human body. Considering the mechanism of 5-ALA-induced fluorescence in glioma patients, the following two steps should be considered: 1) penetration through the blood-brain barrier (BBB), 2) pharmacokinetics of 5-ALA within glioma cells.

Although 5-ALA at high concentrations in the blood can cross the normal BBB through limited passive diffusion, its permeability is extremely low [6, 7]. Radiolabeled 5-ALA in the rat brain is thought to reach only approximately 1% of the plasma levels within 1 hour after administration, when the plasma concentration of radiolabeled 5-ALA is constant [6, 7]. On the contrary, BBB is disrupted in malignant gliomas, and a greater amount of 5-ALA can cross though the abnormal BBB, similar to the BBB dynamics of the gadolinium-based contrast reagent (GBCA) in magnetic resonance imaging [5, 7]. In contrast to 5-ALA dynamics within the brain parenchyma, 5-ALA-induced fluorescence is observed in the choroid plexus as well as in the ventricular wall in the rat brain tumor model [8]. This phenomenon involves leakage of tumor-secreted porphyrin into blood through an abnormal BBB or porphyrin generated in other organs such as the liver, followed by spreading through the cerebrospinal fluid to the choroid plexus, and consequent direct uptake into the ventricular wall [8, 9]. In fact, we confirmed 5-ALA-induced fluorescence on the surface of the normal ventricular wall in a patient with glio-

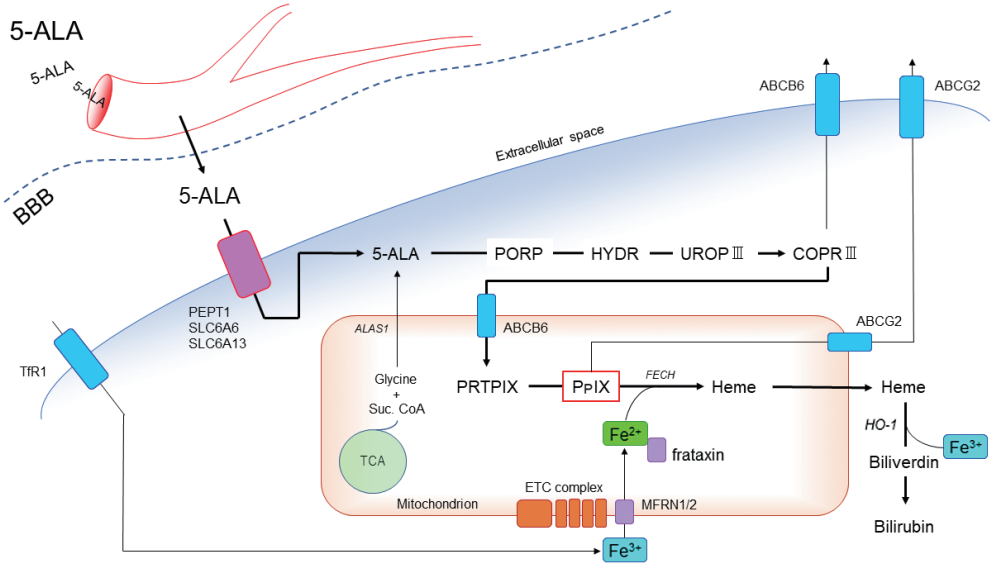

Fig. 1. Pathway of 5-Aminolevulinic acid metabolism and the associated membrane transporter in tumor cells. ABCB6: ATP binding cassette subfamily B member 6, ABCG2: ATP binding cassette subfamily G member 2, ALAS1: 5-aminolevulinic acid synthase 1, BBB: blood brain barrier, COPRIII: coproporphyrinogen III, ETC complex: electron transport chain complex, FECH: ferrochelatase, HYDR: hydroxymethylbilane, MFRN 1/2: mitoferrin 1/2, PORP: porphobilinogen, PRTPIX: protoporphyrinogen IX, PpIX: protoporphyrin IX, PEPT1: peptide transporter 1, SLC6A6: solute carrier family 6A6; taurine transporter, SLC6A13: solute carrier family 6A13; GABA transporter, Suc. CoA: Succinyl CoA, TfR1: transferrin receptor 1, UROPIII: uroporphyrinogen III.

blastoma during surgery [10]. Thus, 5-ALA-induced fluorescence in the ventricular wall does not necessarily demonstrate the presence of tumor cells. Thus, 5-ALA-induced fluorescence in the ventricular wall should be interpreted carefully during fluorescence-guided resection of malignant gliomas.

Next, unlike GBCA, 5-ALA is taken up and metabolized in glioma cells after the passing through the defective BBB in malignant gliomas. Although malignant gliomas can accumulate PpIX to a large extent within the tumors following 5-ALA administration, the precise mechanisms underlying this process have been unclear. Accumulation of 5-ALA-induced PpIX in tumor cells is dependent on four possible processes: 1) enzyme activity of heme synthesis, 2) membrane transporter functions (influx of 5-ALA, efflux of PpIX), 3) iron metabolism, and 4) turnover of heme synthesis. In the last step of heme synthesis, PpIX is converted to heme by ferrochelatase (FECH) with the insertion of Fe^{2+} within the inner mitochondrial membrane (Fig. 1). A previous study reported that FECH mRNA expression in glioblastomas is significantly downregulated compared to that in normal brain tissues [11]. This low activity of FECH restricts the conversion of PpIX to heme, thus leading to the accumulation of PpIX in glioblastomas [11, 12]. Oligo-peptide transporters (PEPT1 or SLC15A1) have been reported as influx transporters of 5-ALA in cancer cells [9, 13]. Overexpression of PEPT1 induced by the knockdown of cadherin 13 (CDH13) can enhance the accumulation of 5-ALA-induced PpIX in glioma cells [14]. In addition to PEPT1, neurotransmitter transporters of the SLC6A family, particularly a taurine transporter (SLC6A6) and GABA transporter (SLC6A13), are also associated with 5-ALA uptake in glioma cells [15]. The ATP-binding cassette (ABC) transporter ABCG2 has been reported as a transporter of anti-cancer reagents in cancer cells [16−18]. Although porphyrin is also secreted into the extracellular space by ABCG2, low activity of ABCG2 inhibits the secretion of 5-ALA-induced PpIX and leads to PpIX accumulation within glioma cells [19, 20]. Thus, overexpression of the influx transporter of 5-ALA (PEPT1, SLC6A6, SLC6A13) and suppression of the efflux transporter of PpIX (ABCG2) increases the accumulation of 5-ALA-induced PpIX in glioma. The ATP-binding cassette (ABCB6) is also overexpressed and is associated with intracellular accumulation of 5-ALA-induced PpIX in malignant gliomas [21]. However, ABCB6 is located on both the mitochondrial and plasma membranes and acts as a coproporphyrinogen III (CPIII)-specific transporter [22, 23]. Another study reported that hypoxic conditions cause overexpression of ABCB6 in the plasma membrane and the secretion of CPIII into the extracellular space, leading to decreased accumulation of intracellular CPIII and PpIX in adenocarcinoma cells [24]. Thus, the function of ABCB6 differs in the mitochondrial and plasma membranes in glioma cells, and further investigation is required.

In the final step of heme synthesis in the mitochondria, Fe^{2+} is inserted into PpIX to form heme by FECH (Fig. 1). The expression of mitoferrin 1/2, which transports iron ions into the mitochondria, and frataxin, which transports iron to FECH, is suppressed in cancer cells [25−27]. Thus, low activity of these iron transporters inhibits the transportation of Fe^{2+} to PpIX in glioma cells. Meanwhile, the expression of transferrin receptor protein 1, which binds iron, is increased in the plasma membrane of cancer cells, including glioma cells [28]. Taken together, even though glioma cells can take up significant amounts of iron, the cells cannot utilize iron for heme synthesis in the mitochondria. Thus, glioma cells may accumulate PpIX because of failure to use iron.

Finally, another recent study indicated a difference in the turnover rate of 5-ALA-induced PpIX between glioma and normal cells. Isocitrate dehydrogenase 1 (IDH1) mutation and overexpression of glutaminase 2 inhibit the activity of hemeoxygenase-1 (HO-1) via consumption of NADPH in glioma cells [29, 30]. Because of HO-1 dysfunction, heme cannot be converted to biliverdin, i.e., heme synthesis is stopped (Fig. 1). Consequently, glioma cells accumulate PpIX in the mitochondria. Thus, although numerous factors affect heme synthesis in glioma cells, the dominant mechanisms of 5-ALA-induced PpIX accumulation in malignant gliomas remain unclear.

2. Why does 5-ALA not fluoresce in gliomas?

During fluorescence-guided resection of malignant gliomas, no fluorescence is observed in the absence of

accumulation of 5-ALA-induced PpIX in gliomas or of tumor cells (except in the ventricular wall). Despite high accumulation of 5-ALA-induced PpIX in glioma cells, some cases exhibit no fluorescence during surgery. A photosensitizer cannot act by itself; it is activated and can fluoresce under light exposure at the appropriate wavelength. The intensity of light exposure depends on the distance between the tumor and light source and the direction of light exposure. In particular, the narrow corridor created after the debulking of tumors may disturb the exposure of laser light to the tumor cavity and result in decreased 5-ALA-fluoresence [31]. With continued light exposure to the photosensitizer, fluorescence is gradually decreased. This phenomenon is known as photobleaching, wherein the photosensitizer converts to another photoproduct [32]. During surgery for malignant gliomas, exposure to the conventional white light of the microscope, which contains specific wavelengths, results in photosensitizer exposure, and consequently induces photobleaching. In addition, hemorrhages covering the tumor surface intercept the violet-blue light required to excite the photosensitizer, owing to absorption of the violet-blue (Soret band) wavelength by hemoglobin [33]. Thus, in fluorescence-guided resection for malignant gliomas, it is important to regulate the appropriate environment for excitation of the photosensitizer contained within tumors.

3. Future prospects of 5-ALA for cancer therapy

5-Aminolevulinic acid (5-ALA) is a well-known fluorescence marker for fluorescence-guided resection and photodynamic therapy in neurosurgery [2, 34]. Interaction between 5-ALA-induced PpIX and external energy exposure, such as that owing to ultrasound, hyperthermia, and radiotherapy, has been reported in cancer therapy [35−40]. Porphyrin compounds, such as hematoporphyrin derivatives and photofrin, are well-known for their photosensitizing activities as well as their radiosensitizing effects in cancer cells [41−43]. However, the interaction between 5-ALA-induced PpIX and radiotherapy has been controversial [42, 44]. A recent study demonstrated that X-ray irradiation increased the production of reactive oxygen species such as superoxide, hydroxyl radicals, and singlet oxygen in PpIX solution via water radiolysis [45]. We demonstrated that the radiosensitizing effect of 5-ALA-induced PpIX after single dose ionizing irradiation was weak, but multi-dose ionizing irradiation with repeated 5-ALA administration enhanced the host antitumor response and strongly inhibited tumor growth in experimental gliomas [39, 40]. Although the precise mechanism of the radiosensitizing effect of 5-ALA-induced PpIX is not known, Takahashi *et al* demonstrated that a combination therapy with 5-ALA and radiotherapy induced cell-cycle arrest, enhancement of oxidative stress, and antitumor effects by microarray analysis using mouse melanoma models [38, 46]. We confirmed that 5-ALA-induced PpIX strongly enhanced the delayed intracellular production of reactive oxygen species (ROS) generated by ionizing irradiation in the mitochondria of glioma cells [47]. We also demonstrated that 5-ALA-induced PpIX shows anti-tumor effects via suppression of PGE2 production and expression of both cyclooxygenase-2 and microsomal prostaglandin E synthase-1 in glioma cells [48]. Thus, focal oxidative stress in mitochondria and host antitumor responses affect the radiosensitizing effect of 5-ALA-induced PpIX in gliomas. Following our data on the radiosensitizing effect of 5-ALA-induced PpIX in gliomas, this interaction was observed in studies on melanoma, colon cancer, breast cancer, prostate cancer, and lung cancer both *in vivo* and *in vitro* [49−54]. Thus, theoretically, a combination therapy using 5-ALA and radiotherapy may be effective for malignant tumors that accumulate 5-ALA-induced PpIX. Similarly, an interaction between 5-ALA and ultrasound, known as sonodynamic therapy, has been reported in cancer cells including gliomas [36, 55]. A combination of hyper thermotherapy and 5-ALA also enhanced the antitumor effect in Lewis lung carcinoma cells *in vivo* [35]. Thus, the method used to excite 5-ALA-induced PpIX accumulated within the mitochondria of glioma cells using external energy is very important.

Conclusion

5-Aminolevulinic acid (5-ALA) is not a photosensitizer itself but is a prodrug that selectively accumulates PpIX within the mitochondria of glioma cells. Then, 5-ALA acts as a selective fluorescence marker of mi-

tochondria, and an inducer of ROS production in the mitochondria of glioma cells under the exposure of external energy. The mitochondria is the most important producer of energy in glioma cells. Mitochondria are also the key organelles in cell metabolism producing ROS and inducing apoptosis [56]. Thus, 5-ALA should be considered not only as a fluorescence reagent, but also as a candidate drug for direct mitochondrial targeting in malignant glioma therapy.

Conflict of Interest

The authors declare that they have no conflict of interest.

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5-アミノレブリン酸：悪性神経膠腫に対する術中蛍光診断におけるピットフォールと悪性神経膠腫治療への応用

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産業医科大学　脳神経外科

要　　旨：5-アミノレブリン酸（5-ALA）は，悪性神経膠腫に対する術中蛍光診断における生体蛍光標識薬として広く利用されている．5-ALAそのものは光感受性物質ではなく，神経膠腫細胞のミトコンドリアにプロトポルフィリンIX（PpIX）を集積させるプロドラッグである．このPpIXが光感受性物質の性質を有する．5-ALAを用いた悪性神経膠腫に対する術中蛍光診断には，ピットフォールが存在する．また，5-ALAは，ただ単に蛍光診断薬ではなく，がん治療におけるミトコンドリア標的薬としての可能性を有する．本稿では，最新論文を基に5-ALAを用いた悪性神経膠腫に対する術中蛍光診断におけるピットフォールと5-ALAの悪性神経膠腫治療への応用について私見を加えて解説する．

キーワード：5-アミノレブリン酸，グリオーマ，ミトコンドリア標的薬，活性酸素種，放射線増感剤．

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