

Reactive oxygen species involved cancer cellular specific 5-aminolevulinic acid uptake in gastric epithelial cells

Hiromu Ito,¹ Masato Tamura,¹ Hirofumi Matsui,^{1,*} Hideyuki J. Majima,² Hiroko P. Indo² and Ichinosuke Hyodo¹

¹Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Ten-nohdai, Tsukuba, Ibaraki 305-8575, Japan

²Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan

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Photodynamic therapy and photodynamic diagnosis using 5-aminolevulinic acid (ALA) are clinically useful for cancer treatments. Cancer cells have been reported that 5-aminolevulinic acid is incorporated via peptide transporter 1, which is one of the membrane transport proteins, and has been reported to be significantly expressed in various gastrointestinal cancer cells such as Caco-2. However, the mechanism of this protein expression has not been elucidated. Concentration of reactive oxygen species (ROS) is higher in cancer cells in comparison with that of normal cells. We have previously reported that ROS derived from mitochondria is likely related to invasions and proliferations of cancer cells. Since 5-aminolevulinic acid is the most important precursor of heme which is necessary protein for cellular proliferations, mitochondrial ROS (mitROS) may be also related to peptide transporter 1 expressions. In this study, we used a rat gastric mucosal cell line RGM1 and its cancer-like mutated cell line RGK1, and we clarified the ALA uptake mechanism and its relations between mitROS and peptide transporter 1 expression in RGK1. We also used our self-established stable clone of cell which over-expresses manganese superoxide dismutase, a mitROS scavenger. We studied differences of the photodynamic therapy effects in these cells after ALA administrations to clear the influence of mitROS.

Key Words: aminolevulinic acid, reactive oxygen species, gastric epithelial cell, porphyrin, photodynamic therapy, mitROS

Photodynamic therapy (PDT) using 5-aminolevulinic acid (ALA) is one of the promising treatments for malignant tumor because of its selectivity, low toxicity, rapid effect, and rapid clearance from the body.⁽¹⁾ In traditional PDT, hematoporphyrin derivatives (HpDs) have been used.⁽²⁾ These photosensitizers show preferential tumor localization and light-activated tumor destruction via photosensitization.⁽³⁾ However, these have harmful effects such as cutaneous photosensitivity.⁽⁴⁾ ALA is a precursor of protoporphyrinIX (PpIX), which enzymatically converted ALA to PpIX in heme synthetic pathway.⁽⁵⁾ In many tumor cells such as gastric cancer cell and glioblastoma, an excess of the exogenous pro-drug ALA results in the accumulation of PpIX.^(6,7) PpIX shows a phototoxic property through producing singlet oxygen by exposing light at appropriate wavelength.⁽⁸⁻¹⁰⁾ On the other hand, ALA can avoid unexpected phototoxic property of PDT using a photosensitizer because ALA accumulates in tumor tissue selectively and shows a phototoxic property only when it converted to PpIX.⁽¹¹⁾ ALA-induced PDT has been successfully applied in various medical fields including gastroenterology, urology and dermatology.^(12,13) ALA has also been used as fluorescence marker for photodiagnosis of neoplasms.⁽¹²⁻¹⁴⁾

Several studies support to elucidate uptake-mechanism of

ALA.^(15,16) Two peptide transporters are reported to be coincided with the uptake; peptide transporter 1 (PEPT1) and peptide transporter 2 (PEPT2). PEPT1 and PEPT2 are mainly expressed in the small intestinal brush border membrane and luminal membrane of renal proximal tubes, respectively. PEPT1 and PEPT2 mediate the cellular uptake of dipeptides and tripeptides in a variety of tissues.⁽¹⁷⁾ However, the relations between ALA and these transporter expressions are not clarified.

Reactive oxygen species (ROS) are generated through cellular metabolism and played important roles in signaling pathways for inducing growth factors such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF).⁽¹⁸⁾ In cancer cells, ROS such as superoxide radical and hydroxyl radical are known to enhance the growth, invasion and metastasis directly and/or indirectly.^(19,20) Generally, cancer cells produce more ROS than normal cells⁽²¹⁾ because of mitochondrial dysfunction, especially complex I and III of electron transport chain.⁽²²⁾ We also reported that the relation between mitochondrial ROS (mitROS) and a tumor invasion using rat gastric mucosal cells, RGM1,⁽²³⁾ its chemically oncogenic cancer-like cells, RGK1⁽²⁴⁾ and manganese superoxide dismutase (MnSOD)-expressed RGK cells, RGK-MnSOD.⁽²⁵⁾ MnSOD is located in mitochondria and scavenges mitROS specifically.⁽²⁶⁾ ROS affects the expression of PEPT1,⁽²⁷⁾ however, the relation between uptake of ALA and mitROS has not clearly demonstrated.

In this study, we have clarified the relation between the uptake of ALA through PEPT1 and mitROS using RGM1, RGK1 and RGK-MnSOD. We mainly targeted not PEPT2 but PEPT1 because the expression amount of PEPT1 is larger in gastrointestinal tract such as intestine, and PEPT2 is mainly expressed in brain and kidney.⁽²⁸⁾ We investigated the expression of PEPT1, the uptake of ALA using RI-labeling technique, and the amount of synthesized porphyrin after exposing ALA and its PDT effect, using RGM1, RGK1 and RGK-MnSOD cells to elucidate the relation between the expression of PEPT1 and ALA-induced PDT effect.

Materials and Methods

Materials. Radioisotope of [4-¹⁴C]-ALA hydrochloride (American Radiolabeled Chemicals Inc., St. Louis, MO), 5-ALA hydrochloride (Cosmo Bio Co., Ltd., Tokyo, Japan), Cell Counting Kit-8 (DOJINDO, Tokyo, Japan), Pico-Fluor 40 (PerkinElmer Japan Co., Ltd., Kanagawa, Japan), Trizma® base (Sigma-Aldrich Japan K.K., Tokyo, Japan), NaCl (Wako Pure Chem. Ind. Ltd., Osaka, Japan), Triton X-100 (Sigma-Aldrich

*To whom correspondence should be addressed.
E-mail: hmatsui@md.tsukuba.ac.jp

Japan K.K.), sodium dodecyl sulfate (SDS) (Wako), deoxycholic acid (Wako), hydrochloric acid (Wako), NuPAGE® Novex® 12% Bis-Tris gels (Life Technologies Japan Ltd., Tokyo, Japan), PVDF Blocking Reagent for Can Get Signal® (TOYOBO CO., LTD., Osaka, Japan), Tris Buffered Saline with Tween® 20 (TBST-10X, Cell Signaling Technology Japan, K.K., Tokyo, Japan), PEPT1 antibody (Abcam plc., Cambridge, U.K.), Can Get Signal® Immunoreaction Enhancer Solution 1 (TOYOBO) horseradish peroxidase (HRP) linked anti-rabbit IgG antibody (Cell Signaling Technology Japan, K.K.), Lumina forte western HRP substrate (Millipore Co., Billerica, MA), anti-β-actin (Cell Signaling), and Can Get Signal® Immunoreaction Enhancer Solution 2 (TOYOBO) were purchased and used without further purification or modification.

Cell culture. RGM1 and RGK1 were cultured in DMEM/F12 with L-glutamine (Life Technologies Japan Ltd.) and DMEM/F12 without L-glutamine (Sigma-Aldrich Japan K.K.), respectively. These culture media contained 10% of inactivated fetal bovine serum (FBS) (Biowest LLC, Kansas City, MO) and 1% of penicillin/streptomycin (Life Technologies). All cells were cultured in 5% CO₂ cell culture incubator at 37°C.

Cellular uptake of ALA. Cellular uptake of ALA was examined by use of radio isotope-labeled ALA ([4-¹⁴C]-ALA hydrochloride: RI-ALA). Cells were cultured on 6-well plate at 1 × 10⁵ cells/well and incubated for overnight. The medium was exchanged to flesh one which contained 0.03 mM of RI-ALA and incubated at 37°C for 0.5, 1, 3 and 6 h. After incubation, cells were washed by PBS, detached by trypsin/EDTA and collected to vials containing liquid scintillation fluid (Pico-Fluor 40). Radiation dose of the samples was determined in a liquid scintillation counter (LSC-7200, Hitachi Aloka Medical, Ltd., Japan).

Porphyrin accumulation in cells. Synthesized porphyrin in cells was measured as follows; Cells were incubated for overnight on 6-well plate at 5 × 10⁵ cells/well. The medium was exchanged to flesh one which contained 1 mM of 5-ALA hydrochloride and incubated for 0.5, 1, 3 and 6 h. After incubation, cells were dissolved with 100 μl of RIPA buffer which composed with 25 mM of Tris-HCl solution (pH 7.6), 150 mM of NaCl, 1%(v/v) of Triton X-100, 0.1%(w/v) of sodium dodecyl sulfate (SDS) and 0.2%(w/v) of deoxycholic acid. The cell homogenates were transferred to 96-well plate and then the fluorescence intensity of porphyrin was measured by a Varioskan micro plate reader (Thermo Fisher Scientific K.K., Kanagawa, Japan). The measurement wavelength of excitation and emission were 409 nm and 634 nm, respectively.

Western blotting analysis of PEPT1. Western blotting analysis was performed according to previous reports.^(25,29,30) Briefly, 15 μl of cell lysed solution (10 μg) from each cells were prepared with NuPAGE LDS Sample buffer containing Sample Reducing Agent (Invitrogen Japan K.K., Tokyo, Japan) and boiled at 70°C for 10 min. For SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the cell lysed solutions were added into well of NuPAGE® Novex® 12% Bis-Tris gels. Since the gel was electrophoresed at 200 V for 30 min, protein were transferred onto a polyvinylidene difluoride (PVDF) membrane (EMD Millipore Co., Billerica, MA) by electrophoresis at 4.0 A·cm⁻² for 10 min. The sandwich immune assay was performed by SNAP i.d. (Millipore), which is a suction type immune reaction system and antibody reaction steps could be performed for about 30 min. After 15 ml of PVDF Blocking Reagent for Can Get Signal® was exposed to membrane and aspirated, an anti-rabbit PEPT1 antibody (1:500), which was primary antibody in this experiment, was added in Can Get Signal® Immunoreaction Enhancer Solution 1 and the solution was exposed to the membrane for 10 min. After the primary antibody solution was aspirated, the membrane was washed three times by 15 mL of 1 X TBST. HRP-linked anti-rabbit IgG antibody was cultured in Can Get Signal® Immunoreaction Enhancer Solution 2. The solution was exposed to the membrane for 10 min. The membrane was immersed in Lumina forte

western HRP substrate (Millipore), luminescence was then observed by LAS4000 (GE Health Care Japan, Tokyo, Japan). β-actin was detected with anti-β-actin as a control for protein loading.

Cell viability test after PDT. Cell viability test after PDT was performed according to manufacturer's protocol and previous report.⁽³¹⁾ Briefly, cells were incubated for overnight on 96-well plate at 2 × 10⁴ cells/well. The medium was exchanged to flesh one which contained 1 mM of ALA and incubated for 0.5, 1, 3 and 6 h. After incubation, cells were washed by PBS twice and the flesh medium without phenol red (Life Technologies) was added. Subsequently, cells were irradiated to the excimer dye laser light (630 nm, 1 J/cm²) for PDT by use of PDT EDL-1 (Hamamatsu Photonics K.K., Hamamatsu, Japan). After photo-irradiation, cells were incubated for 24 h. The medium was replaced to flesh one containing 10% of Cell Counting Kit-8 and then further incubated for 2 h. The absorbance of 450 nm was measured by the Varioskan micro plate reader (Thermo Fisher Scientific K.K.).

Static analysis. Static significant value (*p* value) was calculated using SPSS software (IBM Corp., Armonk, NY) followed by Turkey HSD.

Results

Cancer cellular specific uptake of ALA. In order to investigate cellular uptake manner of ALA in association with mitROS, cellular uptake of ALA was examined using RI-labeled ALA (¹⁴C-ALA). Fig. 1 showed cancer cell specific uptake of ALA: ¹⁴C-ALA was particularly incorporated into the cancerous cell in a time-dependent manner to show significant difference between the normal cells after 3 h. In RGK-MnSOD cells, the amount of this cancer specific ALA accumulation was significantly less than RGK1 cells after 3 h. After 1 mM ALA exposure, the cells expressed a slight cytotoxicity (data not shown), and the differences of cell viability among the cells were negligible. Therefore, 1 mM ALA was used for the following experiments.

Measurement of porphyrin fluorescence synthesized in cells. To study the influence of mitROS to the porphyrin synthetic efficiency from ALA, fluorescence intensity of porphyrin synthesized in each cell after ALA exposure was measured. Fig. 2 showed the relation between an exposed time of ALA and the 634 ± 6 nm intensity of fluorescence which was indicative of

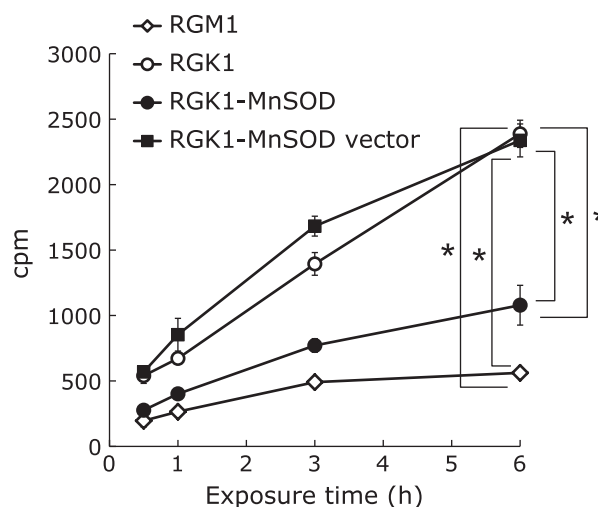


Fig. 1. Cellular uptake of ¹⁴C-labeled ALA. Cells were exposed to culture medium containing 0.03 mM of RI-labeled ALA. This figure showed cancer cells specific uptake of ALA and its significant decrease in MnSOD expressed cancer cells. *n* = 4, Error bar; SD. **p* < 0.001.

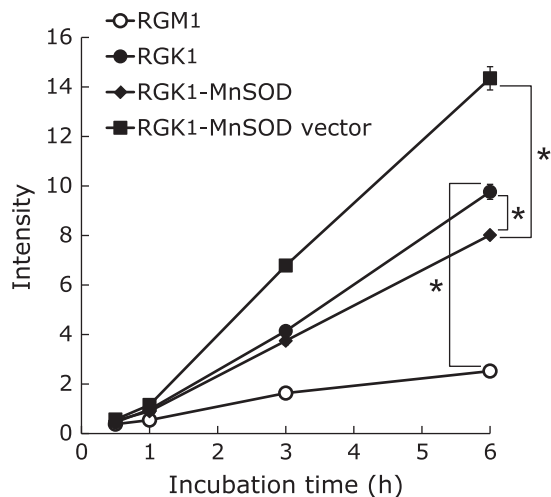


Fig. 2. Porphyrin accumulation in cells after 1 mM of ALA exposure. Cells were incubated in 1 mM of ALA-containing medium for each hour and dissolved in RIPA buffer. Then fluorescence of porphyrin was measured by micro platereader. Cancer cells produced much greater amounts of porphyrin than normal cells, and a little greater than MnSOD expressed cells. $n = 4$, Error bar; SD. * $p < 0.001$.

biosynthesized porphyrins. Porphyrin's fluorescence intensity in cancer cells was greater than that of normal cells. Furthermore, this fluorescent intensity of synthesized porphyrin in RGK-MnSOD cells was lower than in the cancer cells. This result showed the same tendency as the result of cellular uptake of ALA (Fig. 1). This phenomenon is thus likely to be caused by incorporated ALA.

PEPT1 protein expression in cells. Due to elucidation of the uptake mechanism of ALA, PEPT1 expressions in RGM1, RGK1 and RGK-MnSOD cells were determined by western blotting analysis. Fig. 3a showed the protein expression of PEPT1 and β -actin. Fig. 3b showed the expression ratio of PEPT1 and β -actin. PEPT1 expression level in RGK1 was greater than that in RGM1. Additionally, the protein expression was decreased in RGK-MnSOD suggesting that mitROS was likely to upregulate the expression of PEPT1 using these cells.

Cancer cell specific death by PDT. Photodynamic therapies were performed after the ALA treatments and their efficacies were studied by WST assay. Fig. 4 showed that the longer time of ALA incubation caused the lower cell viability in RGK cells besides RGM1 and RGK-MnSOD cells did not. These results suggested that the uptake of ALA and porphyrin accumulation is controlled by the mitROS levels, thus the mitROS levels regulated ALA-PDT effect.

Discussion

In this study, we demonstrated for the first time that mitROS plays an important role for both cellular ALA uptake and porphyrin accumulation in gastric epithelial cells. We examined cellular ALA uptake for following three cell lines; normal gastric epithelial cells RGM1, its mutated cancer-like cells with chemical carcinogen RGK1 and MnSOD overexpressing RGK1 (RGK-MnSOD). The amounts of incorporated ALA in cancer cells were about five times greater than that in normal cells after 6 h incubation. Furthermore, the amounts were decreased by MnSOD overexpression (Fig. 1). These results indicated that mitROS was most likely to accelerate ALA uptake.

In this study, the amount of porphyrin accumulation in cancer cells was significantly higher than that of normal cells 6 h after

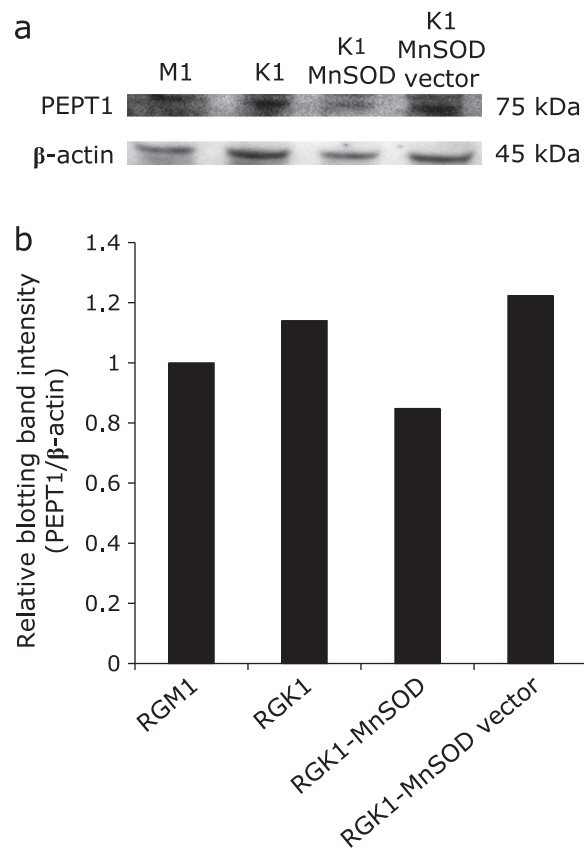


Fig. 3. Western blot analysis of PEPT1 protein expression in each cell. PEPT1 protein bands of each cell (a), and PEPT1 expression levels in each cell were represented by means of graph (b). PEPT1 was expressed greater in cancer cells and suppressed in normal and MnSOD expressed cells. These results were relative to uptake of ALA.

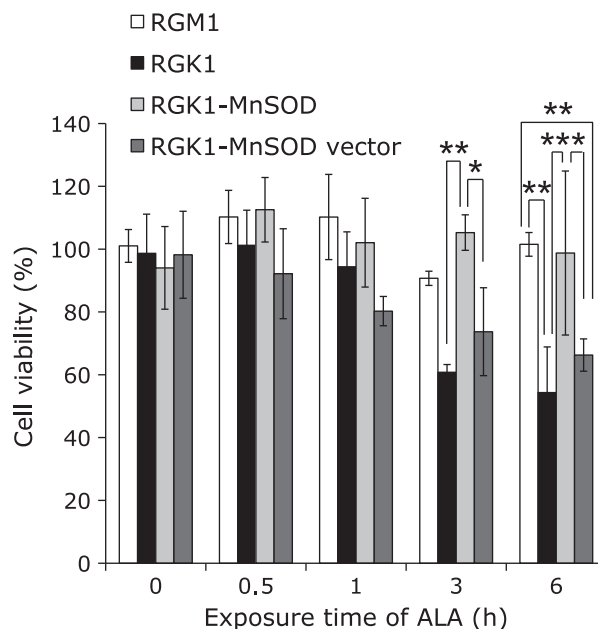


Fig. 4. PDT effect was examined after exposing ALA. Cell viability was evaluated after ALA exposure and photo-irradiation. Cancer specific death was observed and the death was suppressed by expressing MnSOD. $n = 4$, Error bar; SD. * $p < 0.05$, ** $p < 0.01$.

ALA treatment. MnSOD overexpression significantly restrained this cancer specific porphyrin accumulation (Fig. 2). We have previously reported that cancer specific high concentration of nitric oxide (NO) involved the cancer specific porphyrin accumulation. A key enzyme in heme biosynthesis is ferrochelatase. An active site of this enzyme is 2Fe-2S cluster which can be inactivated by NO to create dinitrosyl iron complexes (DNICs) irreversibly. Thus the conversion from protoporphyrin into heme can be inhibited to decrease porphyrin accumulation in cancer cells because inducible NO synthase used to be upregulated in cancer cells. Intracellular NO can react with superoxide anion to create peroxynitrite as following chemical formula: $\text{NO} + \text{O}_2^- \rightarrow \text{ONOO}^-$.⁽³⁰⁾ Therefore NO concentration can increase when superoxide anion is scavenged. Since peroxynitrite cannot react with 2Fe-2S cluster, NO concentration increase after superoxide anion suppression probably accelerates porphyrins accumulation. However, superoxide anion suppression by MnSOD overexpression decreases the amount of intracellular porphyrins in this study. Therefore, not only the inactivation of ferrochelatase, but also ALA incorporation is likely to be important for the cancer specific porphyrin accumulation.

ALA itself has been reported to involve mitochondrial ROS production. Onuki *et al.*⁽³³⁾ reported that exogenous ALA treatment causes cellular ROS production to injured rat liver mitochondria which derived generation of mitROS secondly. Since ROS after mitochondrial damage with functional inhibition of electron transport system used to be superoxide anion, the kind of ROS secondly involved by ALA treatment is also likely to be it. Since Fernando *et al.*⁽³⁴⁾ reported that ALA induced ROS production in rat cerebellum tissue and it was suppressed by exogenous SOD which scavenges superoxide anion, the primary generated ROS after ALA treatment were suggested to be superoxide anion. Taken together, ALA treatment is likely to accelerate ALA uptake via an induction of intracellular high ROS concentration after mitochondrial injury. In our study, overexpression of MnSOD decreased cancer cellular specific ALA uptake. This phenomenon seems to be involved not only by cancer specific high ROS concentration, but also by ALA-derived ROS. We propose that the amount of ALA uptake is able to maintain by the regulation of MnSOD expression.

ALA is transported into cells via PEPT1.⁽³⁵⁾ Ziegler *et al.*⁽³⁶⁾ reported that colonic mucosal PEPT1 expression was upregulated in patients with inflammatory bowel disease (IBD). Additionally,

ROS concentration in the intestinal mucosa of IBD patients is higher in comparison with normal mucosa.⁽³⁷⁾ This IBD mucosal specific ROS has been reported to be derived from mitochondria.⁽³⁸⁾ Since cancer cellular ROS concentration used to be higher as described above, PEPT1 expression is probably higher in comparison with normal cells. In fact, our result clearly demonstrated that cancer cells particularly overexpressed PEPT1 protein, and MnSOD overexpression significantly decreased it (Fig. 3). These results indicated the amounts of ALA uptake in gastric cells were dependent on the amounts of PEPT1-expression which was regulated by mitROS.

We also examined influence of mitROS on ALA-PDT effect with MnSOD overexpressing cells. Kim *et al.*⁽³⁹⁾ reported ALA-PDT induced cell death in various cancer cells such as squamous cell carcinoma, esophageal carcinoma and glioma, effectively. Since generation of singlet oxygen, which was a key ROS to derive cancer cellular apoptosis, was dependent on the amount of porphyrins, the uptake of ALA, a precursor of porphyrins, was very important factor to decide the PDT effect. In this study, suppression of mitROS with MnSOD overexpression significantly decreased the effect of PDT. At that time, the amounts of following three important factors also decreased significantly; PEPT1 expression, ALA uptake and intracellular porphyrins accumulation (Fig. 4). Therefore, we concluded that mitROS regulated the effect of PDT via the amount control of PEPT1 to derive ALA incorporation. We proposed that the effect of ALA-PDT could be increased by the regulation of mitROS.

In conclusion, we have demonstrated that PEPT1 is most likely to be a membrane transporter for cancer specific ALA incorporation. Cancer specific high mitROS concentration plays an important role for this phenomenon. Now we are undergoing to establish a therapeutic method to involve cancer specific acceleration of mitROS for more effective PDT.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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