

Intraoperative photodynamic diagnosis of lymph node metastasis in esophageal cancer patients using 5-aminolevulinic acid

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Abstract. Lymph node metastasis is the strongest prognostic factor in esophageal cancer patients who have undergone esophagectomy. The accurate diagnosis of lymph node metastasis is important, but the pre-operative diagnostic accuracy is poor. The intraoperative diagnosis based on histopathological examination of frozen tissue specimens is complicated and time-consuming. Therefore, the establishment of a simple and rapid intraoperative diagnostic method is essential. Exogenous application of 5-aminolevulinic acid (ALA) causes a selective accumulation of protoporphyrin IX, which is a fluorescent substrate, in cancer cells. The present study evaluated the feasibility of photodynamic diagnosis using ALA (ALA-PDD) for lymph node metastasis in esophageal cancer. A total of 292 lymph nodes were analyzed from 8 esophageal squamous cell cancer patients treated with esophagectomy. The patients were administered ALA orally prior to surgery. Excised lymph nodes were cut in half and examined by spectrometer. The diagnostic results of ALA-PDD were compared to those of the histopathological examination. Among the 292 lymph nodes, 19 nodes (6.5%) were histologically metastatic and 21 nodes (7.2%) were PDD-positive. The sensitivity and specificity of ALA-PDD were 84.2% (16/19) and 98.2% (268/273), respectively. The area of cancer nests of the PDD-negative lymph nodes was <2 mm². Metastatic lymph nodes, including cancer nests >4 mm², were correctly diagnosed by ALA-PDD. In conclusion, this study demonstrated that ALA-PDD of lymph node metastasis in patients with esophageal cancer is feasible. Further investigation would make this method a simple and rapid intraoperative diagnostic tool.

Introduction

Surgical resection is currently the optimal curative treatment in esophageal cancer patients without distant metastases. However, even after curative resection by esophagectomy with extended three-field lymphadenectomy, the recurrence of cancer is found in ~50% of patients (1). Nodal status, including the metastatic lymph node number and extent, is the strongest prognostic factor in esophageal cancer patients who have undergone esophagectomy (1). Accurate assessment of lymph node status is essential. However, pre-operative evaluation of lymph node metastasis is difficult, as smaller lymph nodes cannot be detected by radiographic examination. Indeed, the pre-operative diagnostic accuracy of lymph node metastasis by computed tomography or endoscopic ultrasonography is 60-80% and remains unsatisfactory (2,3).

The intraoperative diagnosis of lymph node metastasis is currently based on histopathological examination of frozen tissue specimens stained with hematoxylin and eosin. Recently, genetic analysis, such as polymerase chain reaction of molecular markers specific to cancer cells, has been used to detect lymph node metastasis (4,5). However, these methods are complicated and time-consuming, and not suitable for examining a large number of specimens (4,5). Therefore, the establishment of a simple and rapid intraoperative diagnostic method of lymph node metastasis is important.

Photodynamic diagnosis using 5-aminolevulinic acid (ALA-PDD) has recently emerged as a promising technique for detecting cancer nests. ALA is the natural precursor of the heme pathway. Exogenous application of ALA causes a selective accumulation of a heme precursor, protoporphyrin IX (PPIX), in cancer cells due to an increase in porphobilinogen deaminase activity and a decrease in ferrochelatase activity. PPIX is a fluorescent substrate that emits a red fluorescence at ~635 nm on blue light excitation (6). ALA-PDD has proven useful in various types of malignancies. In early bladder cancer, the introduction of ALA cystoscopy has increased the recurrence-free survival rate compared with white-light cystoscopy after the transurethral resection of superficial bladder cancer (7,8). In gastric cancer, our previous study reported that staging laparoscopy using ALA improved the detection sensitivity of peritoneal metastasis (9). In malignant

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glioma, intraoperative ALA-induced fluorescence guidance was useful for the complete removal of tumors and improved progression-free survival (10). However, to the best of our knowledge, the usefulness of ALA-PDD in human esophageal cancer has not been investigated. The present study evaluated the feasibility of ALA-PDD of lymph node metastasis in patients with esophageal cancer.

Materials and methods

Uptake of ALA in a cell line. First, ALA-induced PPIX red fluorescence was confirmed in an esophageal cancer cell line. Human esophageal squamous cell cancer TE-1 cells were cultured in RPMI-1640 medium (Gibco Life Technologies, Tokyo, Japan) in a 6-well plate at a concentration of 140,000/ml and incubated at 37°C in a humidified atmosphere of 5% CO₂ for one night. Next, the medium was changed to RPMI-1640 containing 0, 0.25, 0.5 or 1.0 mg/ml ALA hydrochloride (Cosmo Bio Co., Ltd., Tokyo, Japan), and the cells were incubated at 37°C for 60 min. Once the medium had been changed to RPMI-1640 containing no ALA, 5 µl Hoechst 33342 (Sigma-Aldrich, Tokyo, Japan) was added to each dish. Finally, the TE-1 cells were subjected to observation under a fluorescence microscope (BZ-8100; Keyence, Tokyo, Japan). An OP-66834 BZ fluorescence filter (excitation, 360 nm; emission, 460 nm; dichroic mirror, 400 nm; Keyence) and an OP-66838 BZ fluorescence filter (excitation, 560 nm; emission, 630 nm; dichroic mirror, 595 nm; Keyence) were used for Hoechst and PPIX observations, respectively.

Fluorescence microscopy of metastatic lymph nodes. Second, the study confirmed whether the distribution of ALA-induced PPIX red fluorescence agreed with the presence of cancer nests in apparently metastatic lymph nodes in patients with esophageal squamous cell cancer at Osaka Medical Center for Cancer and Cardiovascular Diseases (Osaka, Japan). Between June 2010 and August 2010, ALA hydrochloride (1 g/body; Cosmo Bio Co., Ltd.) was dissolved in 20 ml of a 5% glucose solution and orally administered to 2 patients with esophageal squamous cell cancer 3 h prior to esophagectomy. Patients were protected from direct sunlight for 24 h to avoid phototoxic reactions. Surgical therapy consisted of en bloc esophagectomy via a right thoracotomy with two- or three-field lymphadenectomy, and reconstruction using the stomach, jejunum or colon. Excised metastatic lymph nodes were immediately embedded in TissueTek OCT medium (Sakura, Tokyo, Japan) and frozen at -80°C for 10 min. In a dark room, 2 cryosections of an 8-µm thickness were prepared. One section was stained with hematoxylin and eosin to confirm the presence of cancer. Another section was stained with hematoxylin and Hoechst 33342 (Sigma-Aldrich, Tokyo, Japan). Each section was subjected to observation by fluorescence microscopy (BZ-8100; Keyence). An OP-66834 BZ fluorescence filter (excitation, 360 nm; emission, 460 nm; dichroic mirror, 400 nm; Keyence) and an OP-66838 BZ fluorescence filter (excitation, 560 nm; emission, 630 nm; dichroic mirror, 595 nm; Keyence) were used for Hoechst and PPIX observation, respectively.

Intraoperative PDD of lymph node metastasis. Next, intraoperative PDD of lymph node metastasis was performed in

8 patients with esophageal squamous cell cancer between June 2010 and February 2012. The administration of ALA and surgical treatment were performed as aforementioned. Excised lymph nodes were sliced through the center and subjected to PDD. ALA-induced PPIX fluorescence was analyzed using a spectrometer (VLD-M1; M&M Co., Ltd., Tokyo, Japan) and its accessory software (BWSpec V3.24; B&W Tek, Inc., Newark, DE, USA). Lymph nodes were exposed to a laser light that exhibited a peak wavelength of 405 nm, and those which exhibited a peak wavelength of 635 nm were defined as metastasis-positive on PDD, as shown in Fig. 1. Following PDD, each lymph node was fixed with 10% formalin and subjected to a routine histopathological examination, which was regarded as the gold standard diagnostic procedure. The study protocol was approved by the Human Ethics Review Committee of Osaka Medical Center for Cancer and Cardiovascular Diseases. Written informed consent was obtained from all patients.

Results

PPIX fluorescence in a cell line. ALA-induced PPIX red fluorescence was observed in the TE-1 cells incubated with 0.25, 0.5 and 1.0 mg/ml ALA, but not in the TE-1 cells incubated without ALA (Fig. 2).

Distribution of PPIX in metastatic lymph nodes. The distribution of ALA-induced PPIX red fluorescence in apparently metastatic lymph nodes was analyzed in 5 patients with esophageal squamous cell cancer. The distribution of red fluorescence was identical to that of the metastatic focus (Fig. 3).

Comparison of PDD of lymph node metastasis with pathological diagnosis. Patient characteristics are shown in Table I. Tumor location, depth of tumor and lymph node metastasis were based on the 7th edition of the Union for International Cancer Control tumor-node-metastasis classification guidelines (11). A total of 3 patients were diagnosed with clinical T2 disease and 5 patients with clinical T3 disease. While 2 patients were clinically node-negative, 6 patients were node-positive. All patients were pathologically node-positive. Of all the 292 lymph nodes, 19 nodes were pathologically metastatic and the remaining 273 nodes were pathologically non-metastatic. Table II shows the comparison of PDD with the pathological diagnosis. The sensitivity and specificity of PDD were 84.2% (16/19) and 98.2% (268/273), respectively. Furthermore, PDD was compared with the clinical diagnosis in pathologically metastatic nodes. All the clinically-positive nodes were correctly diagnosed by PDD. Among 10 clinically-negative but pathologically-positive nodes, 7 nodes were diagnosed as positive by PDD and the remaining 3 nodes were diagnosed as negative by PDD (Table III). Fig. 4 shows the area of cancer nests in the pathologically metastatic lymph nodes. The metastasis size of the PDD-negative lymph nodes was <2 mm². Metastatic lymph nodes, including cancer nests >4 mm², were correctly diagnosed by ALA-PDD.

Discussion

This is the first study demonstrating that ALA-PDD of lymph node metastasis in patients with esophageal squamous cell

Table I. Patient characteristics.

Characteristics	Value
Gender, n	
Male/female	7/1
Median age (range), years	65 (52-78)
Tumor location, n	
Upper/middle/lower	2/4/2
Depth of tumor, n	
cT2/cT3	3/5
Lymph node metastasis, n	
cN0/cN1/cN2/cN3	2/3/2/1

Tumor location, depth of tumor and lymph node metastasis were based on the 7th edition of the Union for International Cancer Control tumor-node-metastasis classification guidelines (11).

Table II. Comparison of the PDD of lymph nodes with the pathological diagnosis.

ALA-PDD	Histology		Total
	Positive	Negative	
Positive	16	5	21
Negative	3	268	271
Total	19	273	292

ALA-PDD, photodynamic diagnosis using 5-aminolevulinic acid.

Table III. Comparison of the PDD of lymph nodes with the clinical diagnosis.

ALA-PDD	Clinical diagnosis		Total
	Positive	Negative	
Positive	3	0	3
Negative	7	9	16
Total	10	9	19

ALA-PDD, photodynamic diagnosis using 5-aminolevulinic acid.

cancer is feasible. The present study determined that the time required for diagnosing the metastatic status of one lymph node by ALA-PDD is only a few minutes. When compared with conventional histopathological examination of frozen tissue specimens, ALA-PDD is simple and useful for examining a large number of lymph nodes. ALA is metabolized and excreted within 24-48 h, and the time of shielding from strong light is short compared with other photosensitizers, such as hematoporphyrin and Photofrin. In this study, no adverse events, including cutaneous photosensitization, were

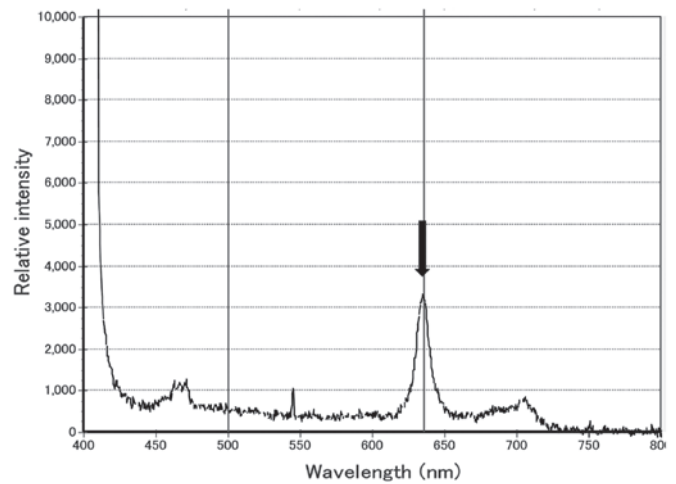


Figure 1. Photodynamic diagnosis (PDD) of lymph nodes using a spectrometer. 5-aminolevulinic acid-induced protoporphyrin IX emits red fluorescence at an ~635-nm wavelength. Lymph nodes that exhibited a peak wavelength of 635 nm (arrow) were defined as metastasis-positive on PDD.

observed. Therefore, ALA-PDD is a safe and promising method of intraoperative diagnosis of lymph node metastasis.

In the present study, the excised lymph nodes were sliced prior to performing PDD. Usually, lymph nodes are surrounded by a number of connective tissues. The penetration depth of red light ranges from 0.2 to 2 cm, with a mean depth of ~0.6 cm, and that of blue light is even shallower (12,13). The penetration depth of excitation blue light is too short to reach metastatic lesions buried in tissues. Previously, we performed PDD without cutting the lymph nodes, but the detection rate of PPIX fluorescence was poor (unpublished data). In gastric cancer, Koizumi *et al* performed fluorescence imaging of the cut surface of lymph nodes (14), as did the present study. Further examination is necessary for intraoperative PDD of lymph node metastasis without excision of the lymph nodes.

In the present study, all the clinically-positive nodes were correctly diagnosed by PDD. False-negative results were observed in 3 clinically-negative nodes. The size of the metastatic focus of these 3 lymph nodes was <2 mm² and relatively small. It is considered that the smaller the cancer nest, the smaller the amount of accumulated PPIX. Furthermore, a small cancer nest may be easily affected by photobleaching, a phenomenon whereby the photosensitizer is photochemically destroyed by light. To induce higher intracellular PPIX levels, chemical modifications of ALA, such as esterification with aliphatic alcohols, may be useful for developing a more sensitive method of PDD (15).

Additionally, a number of false-positive cases were observed in the present study. A non-metastatic lymph node was observed, in which PPIX red fluorescence was observed in the marginal sinus by fluorescence microscopy observation (data not shown). This may cause a false-positive result. Koizumi *et al* reported that they observed some non-metastatic lymph nodes presenting accumulation of PPIX red fluorescence in normal lymphoid follicles (14). This phenomenon may have also occurred in the present study. However, fluorescence microscopy examination was only performed on a small number of lymph nodes.

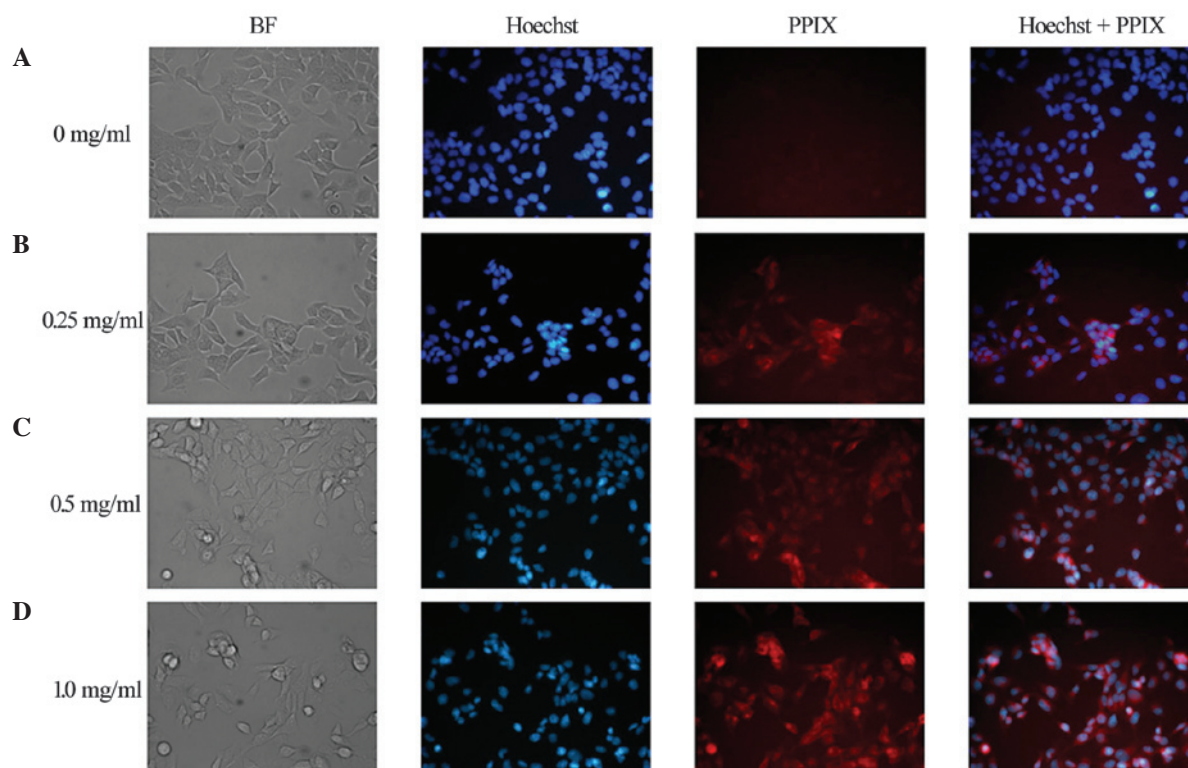


Figure 2. Uptake of ALA in the esophageal squamous cell cancer cell line. ALA-induced PPIX red fluorescence was not observed in TE-1 cells incubated (A) without ALA, but was observed in TE-1 cells incubated with (B) 0.25, (C) 0.5 and (D) 1.0 mg/ml ALA. Original magnification, x100. ALA, 5-aminolevulinic acid; BF, buffered formalin; PPIX, protoporphyrin IX.

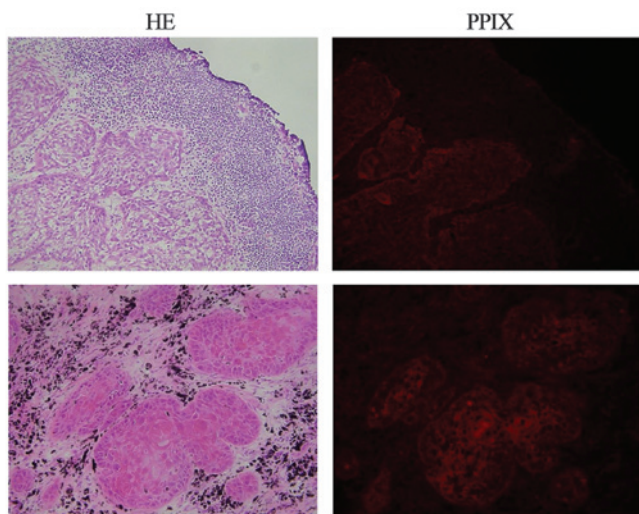


Figure 3. Representative images of microscopic observations of the metastatic lymph nodes. The distribution of 5-aminolevulinic acid-induced protoporphyrin IX red fluorescence was identical to that of the metastatic focus. Original magnification, x100. HE, hematoxylin and eosin; PPIX, protoporphyrin IX.

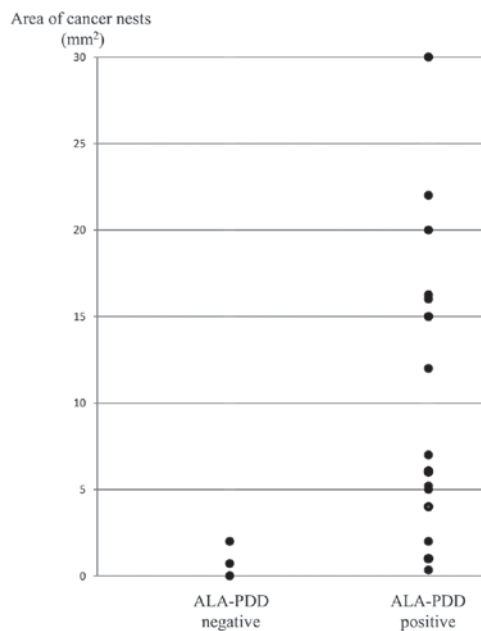


Figure 4. Distribution of the area of cancer nests in the metastatic lymph nodes. ALA-PDD, photodynamic diagnosis using 5-aminolevulinic acid.

Previous studies demonstrated that PPIX tends to accumulate at an inflamed site (16). The detailed mechanism of PPIX accumulation in non-cancerous lesions is unclear.

For the further application of ALA-induced fluorescence in esophageal cancer surgery, intraoperative PDD of resection margins, such as in the aorta, trachea and recurrent laryngeal nerve, are considered to be important. Preliminary ALA-PDD

was performed in the present study for evaluating remnant cancer in the surgical margins around the trachea, but the evaluation was extremely difficult. One of the problems of evaluating the surgical margin is hemorrhage, as hemoglobin absorbs blue excitation light. Several problems remain to be resolved prior to applying ALA-PDD to the surgical margin of

esophageal cancer. ALA-induced fluorescence has been used not only for diagnosis but also for treatment. In nodular basal cell carcinoma, PDT therapy (PDT) using methyl aminolevulinic acid cream is effective (17). The complete response rate of clinically assessed lesion clearance did not differ significantly between the PDT group and the surgically-treated group (17). Pech *et al* reported that the excellent long-term results of ALA-PDT in patients with superficial Barrett's esophageal cancer or high-grade intraepithelial neoplasia and ALA-PDT may be an alternative treatment to esophagectomy and endoscopic resection, particularly in cases with multifocal Barrett's neoplasia (18). In patients with advanced esophageal cancer, intraoperative ALA-PDT for surgical margins where microscopic cancer nests may be left has potential as a novel treatment option.

In conclusion, the present study demonstrated that ALA-PDD of lymph node metastasis in patients with esophageal cancer is feasible. Further investigation would make this method a simple and rapid intraoperative diagnostic tool.

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