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Original article

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Masahiro Kitada*, Yoshinobu Ohsaki, Syunnsuke Yasuda, Masahiro Abe, Nana Takahashi, Satoshi Okazaki, Kei Ishibashi, Satoshi Hayashi

Respiratory Center, Asahikawa medical University, Japan

A B S T R A C T

Background: Visceral pleural invasion (PL) is a prognostic factor in lung cancer. In the lung, lymph flows along the pleura, in addition to the flow toward the pulmonary hilum just as the pulmonary arteries and veins run toward it. Even with the same tumor diameter, a PL1 or higher level of pleural invasion is indicative of a more advanced disease stage. Final diagnosis based on the PL level is made by pathological examination of excised specimens. However, if an intraoperative diagnosis can be established, proper selection of the surgical procedure can be made, and unnecessary surgeries for disseminated lesions can be avoided. We investigated optical diagnostic techniques for identifying the presence or absence of visceral pleural invasion in lung cancer by capitalizing on the phenomenon of 5-amino-levulinic acid (5-ALA) being metabolized to a photosensitizing substance or protoporphyrin IX within malignant tumors, generating red luminescence in response to excitation light.

Method: This study included 38 patients with primary lung cancer who underwent surgery. They received 5-ALA (20 mg/kg) orally 4 h before surgery and then we assessed the presence or absence of pleural invasion using an autofluorescence observation system. At visceral pleural invasion sites, we were able to confirm tumor sites visualized in red with a clear border in contrast to the green autofluorescence generated in normal tissues.

Result: Red luminescence could be confirmed in 100% of PL1-PL3 patients (14/14) and 41.6% of PL0 patients (10/24) with primary lung cancer. PL0 patients in whom visualization was possible were preoperatively diagnosed as having PL1 and many of them showed vascular channel invasion. The sensitivity, specificity, positive predictive value, and negative predictive value of this diagnostic technique were 100%, 58.0%, 63.1%, and 100%, respectively. Red fluorescence emission was observed significantly more often in pleural invasion cases.

Conclusion: Accurate intraoperative diagnosis for visceral pleural invasion in lung cancer may contribute to determining the indications for limited operations such as segmental resection. In addition, accurate local diagnosis has the possibility of being applicable to photodynamic therapy.

1. Introduction

In the lung, lymph flows along the pleura, in addition to the flow toward the pulmonary hilum just as the pulmonary arteries and veins run toward it. Visceral pleural invasion means that the disease stage is more advanced even if the tumor diameter is the same, and pleural invasion (PL) is one of the prognostic factors of disease progression in lung cancer. Since intraoperative macroscopic diagnosis and diagnostic imaging techniques such as computerized tomography (CT), magnetic resonance imaging (MRI), and positron emission computerized-tomography (PET) have limitations, more accurate diagnostic methods have been eagerly awaited. Currently, the final diagnosis is made by pathological examination of excised specimens, but if intraoperative diagnosis is possible, selection of the optimal surgical procedure becomes possible. We have been investigating a novel photodynamic diagnosis (PDD) technique that uses an autofluorescence observation system, focusing particularly on autofluorescence [1], and have been making efforts to achieve further accuracy because the visualization of lesions

* Corresponding author at: Department of Respiratory Center, Asahikawa Medical University, Midorigaoka-Higashi 2-1-1-1 Asahikawa Hokkaido 078-8510, Japan.
E-mail addresses: k1111@asahikawa-med.ac.jp (M. Kitada), yohsaki@asahikawa-med.ac.jp (Y. Ohsaki), s-yasuda@asahikawa-med.ac.jp (S. Yasuda), a080046@al.asahikawa-med.ac.jp (M. Abe), n-takahashi@asahikawa-med.ac.jp (N. Takahashi), syugoshin.19851221@gmail.com (S. Okazaki), kei_dod@hotmail.com (K. Ishibashi), shayashi@asahikawa-med.ac.jp (S. Hayashi).

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and the border between normal tissues and malignant lesions remain unclear. We thus turned our attention to a photosensitizer, i.e. 5-aminolevulinic acid (5-ALA). Once 5-ALA is ingested exogenously, it is metabolized to a heme precursor or protoporphyrin IX, which stays within malignant cells and emits red fluorescence at 630 nm [2]. This form of research is currently being carried out in the field of thoracic surgery [3] focusing on topics such as malignant pleural disease, in the field of neurosurgery with targets such as brain tumors [4], and in the field of urology for diseases such as bladder and prostate cancers [5,6]. After patients with lung cancer had ingested 5-ALA orally, we compared the diagnosis of lesions with visceral pleural invasion and the pathological diagnosis of the resected lesions to assess the usefulness of this novel method. Reports are also found in which visceral pleural invasion factors influence prognosis [7,8]. The purpose of this study is to investigate whether intraoperative judgment of pleural invasion is one factor to determine adaptation of reduction surgery for lung cancer such as segmentectomy.

2. Material and methods

From among patients with primary lung cancer who underwent surgery from July 2015 to April 2017, we enrolled 38 in whom the tumor invaded the pleura or was relatively close to the pleura on preoperative diagnostic imaging. According to the preoperative diagnosis, 13 patients were PL0, 17 were PL1, and 4 each were PL3 and PL4. Patients were administered 20 mg/kg of 5-ALA orally 4 h prior to surgery and we used a thoracoscope equipped with an autofluorescence imaging system immediately after the start of surgery to observe the pleural space through a 12 mm port hole. Observations were made in pulmonary contraction state under isolated lung ventilation. One case was stage IV with disseminated metastasis and was not resected. In all other cases, standard surgery for lobectomy and mediastinal lymph node dissection was performed. In addition of pl3 cases, the wall side pleura of the local invasion was excised in addition to standard surgery.

2.1. Autofluorescence observation system

Autofluorescence is a spontaneous emission of light generated by biological structures such as mitochondria and lysosomes when they absorb excitation light. As to the sources of autofluorescence in systemic tissues, collagen, and fibronectin, nicotinamide-adenine dinucleotide phosphate (NADPH) and flavin-adenine dinucleotide (FAD), have also been reported [9,10]. In normal tissues, green autofluorescence at about 520 nm is observed in response to a blue excitation wavelength of 400–500 nm. At the sites of cancer lesions, diminished green autofluorescence and color tone changes involving the fluorescence generated are observed due to thickening of the mucosal epithelium, a decrease in green autofluorescent substances, and increases in fluorescence absorbing substances. Imaging of this diminished fluorescence and changes in the wavelength for observation is the principle underlying the autofluorescence observation system (Fig. 1).

This study aimed to establish a diagnostic method that can contrast red sites of 5-ALA taken into malignant lesion sites in the pleural space and green autofluorescence in normal tissues, using a thoracoscope (rigid scope) equipped with an autofluorescence observation system. As to the autofluorescence observation system used in this study, we modified a conventional endoscopic color fluorescence system, PDS-2000 (Hamamatsu Photonics, Shizuoka, Japan) [11,12] and equipped it with a camera using a CCD sensor, which allowed us to observe the target with white light as well as autofluorescence through the filter (Fig. 2). A color fluorescence camera was attached to a thoracoscope using portions of the Olympus endoscopic system to achieve attachment The LED light source capable of delivering an excitation light with a peak wavelength around 405 nm was used.

2.2. 5-Ala:

5-ALA, the starting substance in the synthetic pathway of 5-porphyrin, is a natural amino acid within the body. 5-ALA is an endogenous amino acid synthesized by glycine and succinyl CoA in mitochondria and is a precursor of hemoglobin. When 5-ALA is ingested exogenously, it is rapidly metabolized to heme in normal tissues. In contrast, a fluorescent substance or protoporphyrin IX (PpIX) accumulates selectively in malignant cells because they have high porphobilinogen deaminase (PBGD) activity and low ferrochelatase (FECH) activity. As a result, red fluorescence at about 630 nm is emitted in response to an excitation light wavelength of about 405 nm (Fig. 3). In this study, we diagnosed lesions by contrasting green autofluorescence and red fluorescence emitted by 5-ALA to determine the presence or absence of visceral pleural invasion in lung cancer.

2.3. PL category

The PL category was determined by diagnostic imaging (CT diagnosis), and the pl category was then confirmed by pathological examination after surgery. Preoperative diagnosis was determined using CT photograph in consideration of the contact condition of the tumor to the pleura. PL categories is shown in Table 1, and refers to TNM Classification for Non-Small Cell Lung Cancer [13]. This study was approved by the Ethics Committee of the Asahikawa Medical College. Informed consent was obtained from each patient prior to surgery. Statistical processing was done by Chi-squared test

3. Results

There were no adverse events attributable to oral administration of 5-ALA.

1) Degree of visualization: Even when lesions were indistinguishable under white light in patients with pleural invasion, the autofluorescence camera visualized tumor sites in red with a clear border in contrast to the green autofluorescence generated in adjacent

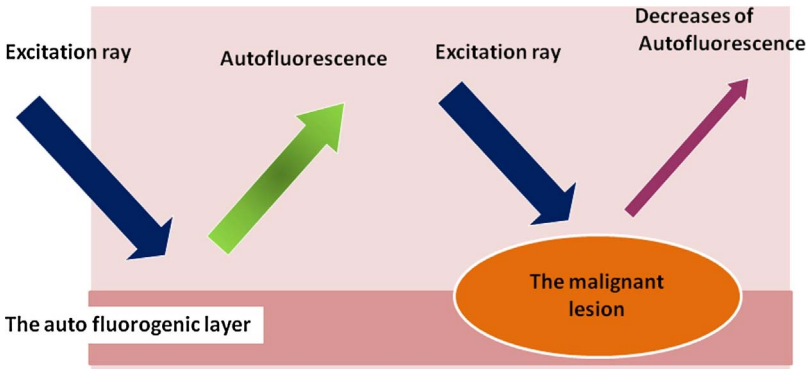

Fig. 1. The principle of autofluoresence observation.
Normal tissues: Green autofluorescence at about 520 nm was observed in response to a blue excitation light at about 400–450 nm. Lesion sites: Green autofluorescence diminished and the color tone of the generated fluorescence changed due to thickening of the mucosal epithelium, a decrease in autofluorescent substances, and an increase in fluorescence absorbing substances

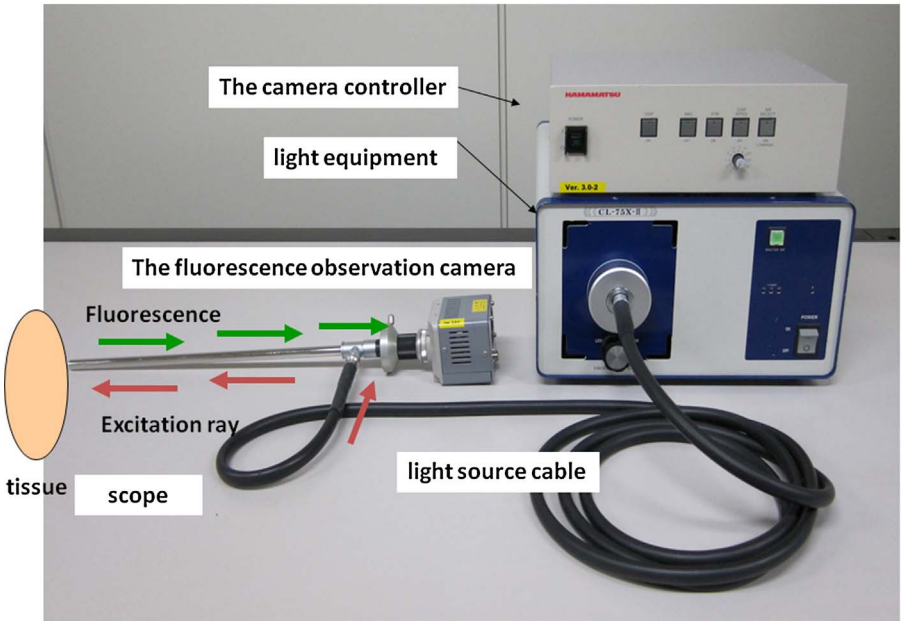

Fig. 2. The autofluorecence imaging system.
The endoscopic color fluorescence system (PDS- 2000) equipped with a small CCD camera allows clinicians and researchers to observe targets under white light as well as autofluorescence through the filter. A color fluorescence camera was attached to a thoracoscope employing attachment portions of the Olympus endoscopic system.

normal tissues. The pl0 cases showed mild marginal and pale pink tones(Fig. 4). The pl1 case was clearly marginally and showed a slightly red color tone(Fig. 5). The pl2 cases showed margin + surrounding invasion shadow and red tone(Fig. 6). Fig. 4 through 6 show preoperative CT images, taken under white light, with the fluorescence camera for Pl0, Pl1, and Pl2 lung cancer cases, respectively.

2) Degree of visualization of red fluorescence

Red luminescence could be confirmed in 100% of PL1-PL3 patients (14/14) and 41.6% of PL0 patients (10/24) with primary lung cancer, though many had been preoperatively diagnosed with PL1. Sensitivity, specificity, positive predictive value, and negative predictive value for this procedure were 100%, 58.0%, 63.1%, and 100%, respectively. A significant difference in red fluorescence was seen in patients preoperatively diagnosed as having pleural invasion ($p = 0.004789$).

Table 1
PL category: pleural invasion of lung cancer.

PL0: Tumor within the subpleural parenchyma or, invading superficially into the pleural connective tissue below the elastic layer.
PL1: Tumor invades beyond the elastic layer.
PL2: Tumor invades to visceral pleural surface.
PL3: Tumor invades the parietal pleura.

4. Discussion

The standard surgeries for lung cancer are pulmonary lobectomy and dissection of mediastinal lymph nodes, but proactive limited surgery, so-called segmental resection, has been increasingly performed for lung tumors as small as 2.0 cm. Some researchers have asserted that the prognosis after limited resection is not inferior to that after lobectomy, but their experiences suggest that the results should be interpreted carefully because the targets differed among reports [14,15]. If patients currently have any possibility of having visceral pleural invasion, indications for limited surgery should be considered carefully.

The stage of lesion progression is one of the major factors determining the survival prognosis of patients with lung cancer. Visceral pleural invasion is one of the factors that determine the stage of progression, and accurate diagnosis is critical for planning an optimal treatment strategy However, preoperative diagnostic imaging and intraoperative diagnosis under direct vision or under thoracoscopic vision have limitations, and a more accurate intraoperative diagnostic method has been sought. Thus, we have been investigating diagnostic methods for malignant tumors of the lung or the pleura, with a particular focus on autofluorescence [1]. We found that green autofluorescence was emitted in normal tissues, while green autofluorescence diminished and the color tone changed to red-purple in malignant lesions due to thickening of the mucosal epithelium, a decrease in green autofluorescent substances, and an increase in fluorescence absorbing substances. However, the boundaries of lesion sites as well as their

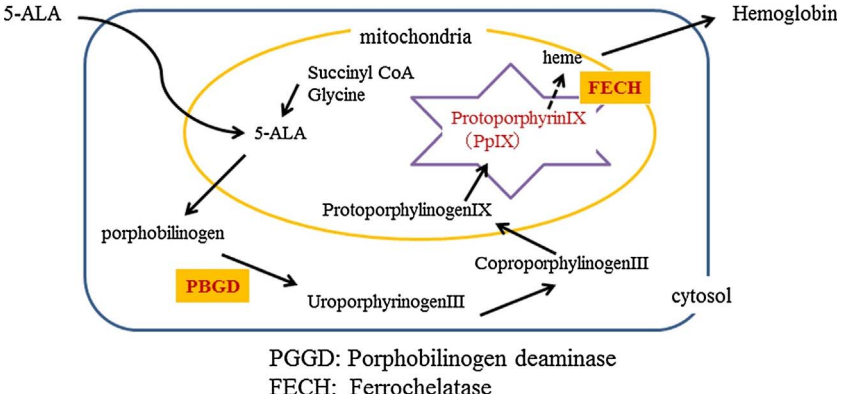

Fig. 3. The 5-ALA metabolic pathway.
When 5-ALA is ingested exogenously, it is rapidly metabolized to heme in normal cells. In contrast, in cancer cells that have high porphobilinogen deaminase (PBGD) activity and low ferrochelatase (FECH) activity, a fluorescent substance or protoporphyrin IX (PpIX) selectively accumulates to emit red to pink fluorescence at about 630 nm.

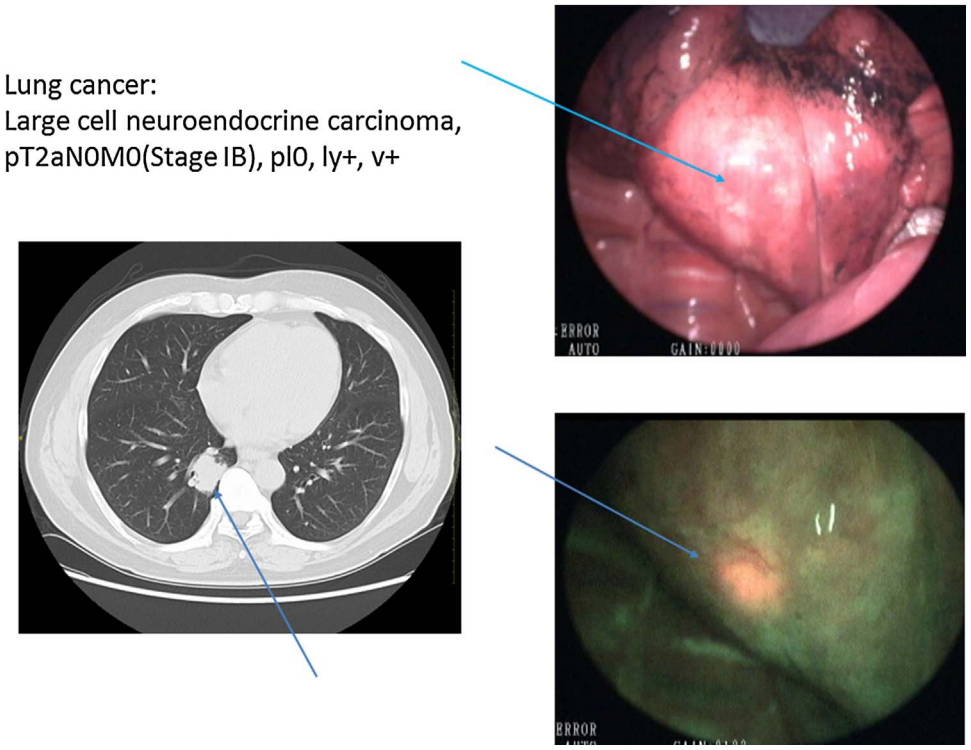

Fig. 4. Lung cancer (a case with pl0).
Pathology: Large cell neuroendocrine carcinoma, pT2aN0M0 (Stage IB), pl0, ly+, v+ Pink tone change was observed in a portion of the lesion. This portion of the lesion was close to the pleura on the preoperative CT image.

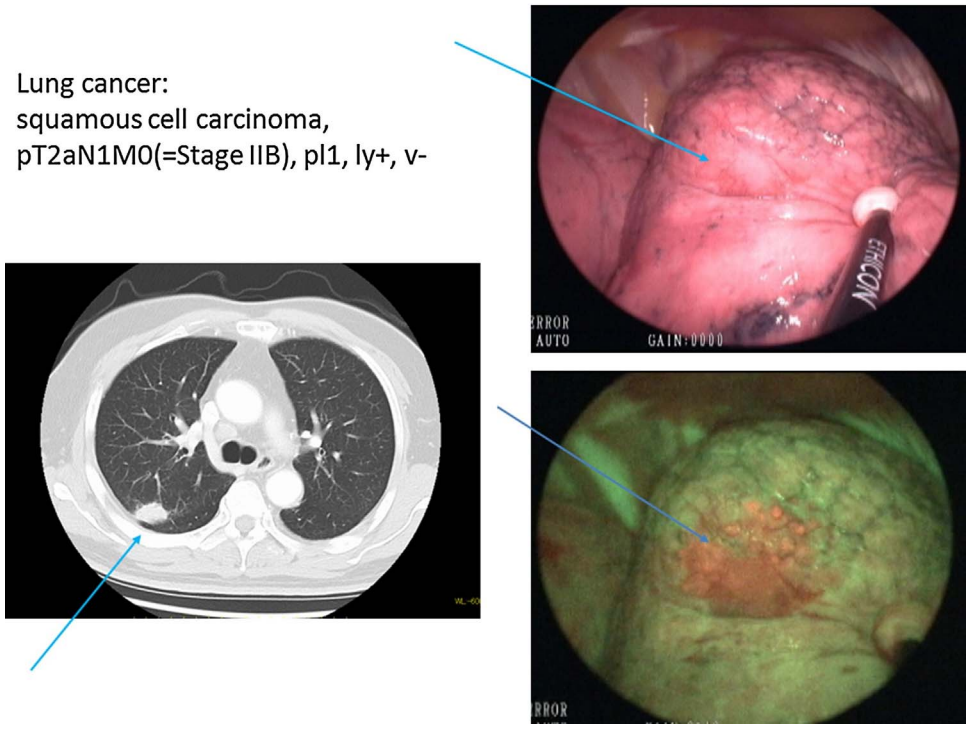

Fig. 5. Lung cancer (a case pl1).
Pathology: Squamous cell carcinoma, pT2aN1M0 (= Stage IIB), pl1, ly+, v- Red tone change corresponding to pleural invasion was observed.

properties could not be assessed in detail relying only on autofluorescence observation. We therefore proposed using a photosensitizing substance and 5-ALA simultaneously.

Photodynamic diagnosis (PDD) using 5-ALA was reportedly applied to localization diagnosis of malignant tumors in the field of thoracic surgery, in the field of neurosurgery surgery for brain tumors, and in the field of urology for malignancies such as bladder and prostate cancers [2,6], and this method is currently being investigated. This study targeted peripheral lung cancer lesions located relatively close to the pleura. Localization diagnosis was definitely possible in patients with PL1-PL3, but visualization of a tumor that was not adjacent to the visceral pleura was difficult. As to patients in whom PL0 tumors could be visualized, most of their tumors were close to the pleura on preoperative CT images and they had been diagnosed as having PL1. Also, many such patients had lymphatic invasion. The distance between a tumor and the pleura was considered to be 2 mm or less in the PL0 cases in which color tone change was observed, but further advancements in the instruments may ultimately allow for the visualization of deeper

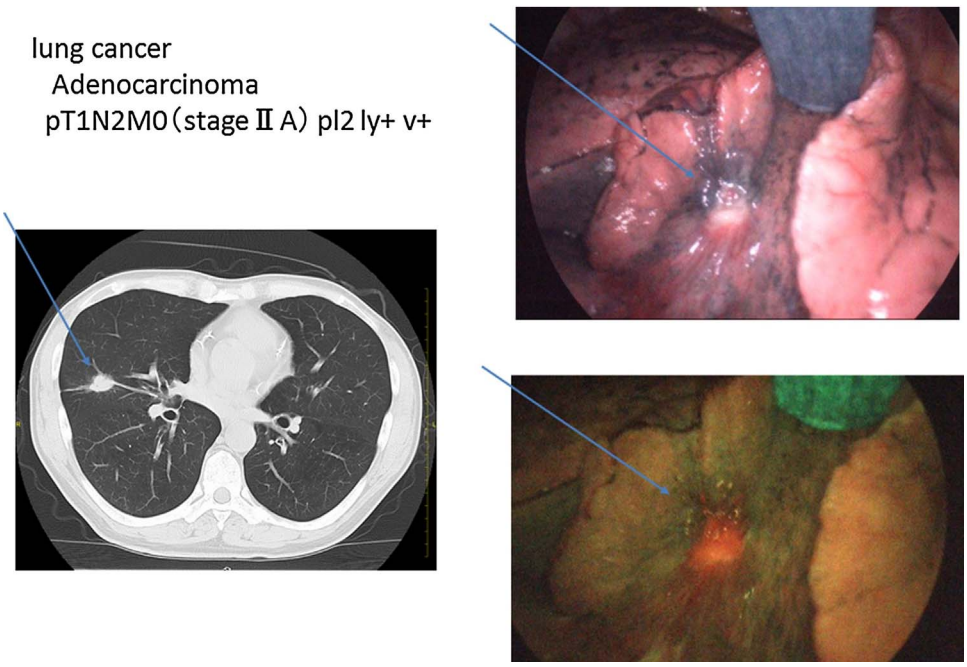

Fig. 6. Lung cancer (a case of pl2).
Pathology: Adenocarcinoma, pT1N2M0(stageIIA) pl2 ly+ v + Tumor invasion was observed in the surrounding area, along with red tone change in the tumor.

Table 2
Patients Baseline Characteristics.

Primary lung cancer : 38	
Mean age: 69.1(46–79)	
Men : Women: 22 : 16	
Pathology :	
Adenocarcinoma 25	Squamous cell carcinoma 10
Largecell carcinoma 2	Pleomorphic carcinoma 1
p-stage I/II/III/IV : 19/16/2/1	
PL factor : PL0/PL1/PL2/PL3: 13/17/4/4	
pl factor: pl0/ pl1/ pl2/ pl3 : 23/8/3/4	

Table 2 displays a summary of patient baseline characteristics. In the lung cancer patients, it showed a disease staging and pathological finding.

lesions. Although no significant relationships with histological type, the grade of malignancy (nuclear grade), ki-67, and vascular channel invasion were detected, this issue should be examined in future studies. Also, it was our impression that visualization was poor in lungs showing prominent emphysematous change. The capacity for visualization was considered to be affected by the degree of pulmonary damage. Furthermore, as to non-malignant tumors, benign lesions such as fibrous thickening and neurogenic tumors could clearly be distinguished from malignant tumors. However, inflammatory masses such as IgG4-related disease showed a high standardized uptake value (SUV) on PET [16], and differential diagnosis may require more extensive examination in such cases. In recent years, the means of optical diagnosis has expanded, and researches on ICG, extrinsic fluorophores and the like are actively carried out. In this thesis, we studied more accurate localization diagnosis and diagnosis of pleural invasion factor by contrasting pink to red color tone change of 5ALA taken in malignant tumor and green autofluorescence emitted by normal part.

In this study, we examined the utility of photodynamic diagnosis using 5-ALA for visceral pleural invasion of lung cancer. If an intraoperative diagnosis with higher accuracy can be established, examining its application to the treatment region (Photo Dynamic Therapy: PDT) would be also possible. Currently, PDT mainly uses 2 oncotropic photosensitizers, i.e., Photofrin (porfimer sodium) [17] and Laserphyrin (talaporfin sodium) [18], but there is also a report noting that ALA-PDT may reduce EGFR expression and the degree of invasion of cancer cells [19]. PDT is a treatment method that attacks malignant cells with reactive oxygen which is generated in the cell recovery phase by photosensitizers activated by laser light. Conventionally, this was a procedure for irradiation using laser light of a wide area such that treatment was delivered only to the site where malignant cells are present, based on the principle that oncotropic photosensitizers accumulate in malignant cells. Research is currently ongoing since selective localization diagnosis using 5-ALA has the potential to contribute to lung cancer treatment, particularly proactive limited surgery for patients with relatively poor lung functions (Table 2).

5. Conclusion

We examined the efficacy of photodynamic diagnosis using 5-ALA for pleural invasion in lung cancer. Patients with PL1 or more advanced disease could be diagnosed with significantly superior accuracy. By accurate lung cancer pleural invasion factor diagnosis, accurate adaptive selection of reduction surgery such as segmentectomy or partial resection is considered to be effective.

Conflict of interests

The authors declare that they have no competing interests.

Authors contributions

MK have operated this case and analyzed all data. MK and YO did the improvement of the equipment and the analysis of the data. KI and SH, SO, NT, MA, SY did the assistant of the operation.

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