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ORIGINAL ARTICLE—ALIMENTARY TRACT

Differences of image enhancement in image-enhanced endoscopy: narrow band imaging versus flexible spectral imaging color enhancement

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Abstract
Background Narrow band imaging (NBI) can emphasize images of the surface microvasculature of lesions, because the central wavelengths of the NBI filter are 415 and 540 nm and these wavelengths are well absorbed by hemoglobin. Flexible spectral imaging color enhancement (FICE) increases the contrast in depictions of mucosal lesions. However, quantitative evaluation of the image enhancement shown by NBI and FICE has not been reported. The aim of this study was to measure and compare the degrees of image enhancement in NBI and FICE.
Methods We compared the visibility of human blood diluted with distilled water between that shown by white-light imaging (WLI) and that shown by NBI or FICE. One milliliter of human blood was plated onto a 12-well transparent plastic plate to set up doubling dilutions, from 1/2 to $1/2^{23}$. High-definition endoscopes were used for each imaging method. A total of 11 endoscopists independently evaluated the visibility of the diluted blood. The median dilution was defined as the limit of visibility in each image.
Results NBI enabled clearer visualization of the presence of blood compared with conventional WLI. NBI recognized blood contamination up to a $1/2^{14}$ dilution, whereas conventional WLI recognized blood contamination up to a $1/2^{11}$ dilution. In contrast, FICE did not improve the visualization of diluted blood and recognized blood contamination up to a $1/2^{10}$ dilution.
Conclusions NBI more effectively enhanced images of diluted blood compared to conventional WLI, while FICE did not improve the visualization of the diluted blood. These data suggest the usefulness of NBI for the early detection of gastrointestinal neoplasia, which is accompanied by abundant neovascularization.

Keywords Image-enhanced endoscopy · Narrow band imaging · Flexible spectral imaging color enhancement

Introduction

Image-enhanced endoscopy (IEE) can be divided into dye-based and equipment-based approaches; the latter is a newly developed technology [1]. The diagnostic accuracy of equipment-based IEE is expected to improve in combination with magnifying endoscopy. In particular, narrow band imaging (NBI) is an equipment-based IEE approach that uses optical technology and depends on hemoglobin absorption wavelengths of 415 and 540 nm exclusively [2, 3]. The combination of NBI with magnifying endoscopy enables the clear visualization of very small mucosal structures and the microvasculature [4].

Superficial cancers in the head and neck region and in the esophagus show neovascularization and morphological changes in microvascular architecture, and novel endoscopic diagnosis by NBI has been established for such cancers [4–9]. We recently showed that NBI provided superior detection and higher diagnostic accuracy compared with conventional white-light imaging (WLI) for lesions in the head and neck region and the esophagus [10].

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Most pharyngeal and esophageal superficial cancers can be recognized as well-demarcated brownish areas which are associated with the development of irregular microvessels. Confirmation of this finding leads to a cancer diagnosis with very high accuracy. However, no reports have objectively quantified the impact of red blood cells contained in the lesions on the acquisition of the images.

Flexible spectral imaging color enhancement (FICE) is also an equipment-based IEE that employs image post-processing technology to increase the contrast of the depictions of mucosal lesions [11]. However, there are no reports documenting the superiority of FICE over conventional WLI for the detection and diagnosis of cancers in the head and neck region and in the esophagus. Additionally, images acquired using FICE require processing, but are not influenced by the amounts of red blood cells or hemoglobin. However, color enhancement in the absorption range for hemoglobin is possible in FICE images; thus, visualization of the vasculature should be facilitated with FICE. However, objective quantitative evaluation of the image enhancement shown by FICE compared with that shown by NBI has not been reported.

In the present study, we measured the degree of image enhancement in samples with different amounts of red blood cells in diluted water, comparing the results among WLI, NBI, and FICE.

Methods

To measure differences in the degree of image enhancement between IEE and conventional WLI, we compared endoscopic images of human blood diluted with distilled water. Endoscopic WLI and IEE images of diluted blood were taken under the same conditions in a dark room.

Dilution series

One milliliter of human blood (containing 5×10^7 red blood cells per μL) was plated onto a 12-well transparent plastic plate (Cellstar; Greiner Bio-one, Tokyo, Japan) to set up doubling dilutions, from 1/2 to $1/2^{23}$ (Fig. 1). The human blood was taken from one healthy volunteer (H.H.) and was divided into two experimental samples, to be used for NBI and FICE.

Endoscopic systems

High-definition endoscopes (H260Z; Olympus Medical Systems, Tokyo, Japan, and EC-590ZW; Fujifilm Medical, Tokyo, Japan) were used, with corresponding light sources (LUCERA; Olympus Medical Systems and ADVANCIA; Fujifilm), respectively. The magnifying function was not used in this study, because this study aimed to evaluate the overall visibility.

WLI and IEE conditions

WLI

Color enhancement was employed at level 0 for WLI in the Olympus system. In the Fujifilm Medical system, color enhancement was not set for WLI. While the function of the color enhancement by the Olympus system indicates hemoglobin enhancement, that of the Fujifilm system indicates enhancement of color tone. In this study, to avoid the influence of color enhancement on the results, the color enhancement function was not set in either system.

NBI

Color tone was employed at level 1 for NBI. This setting is recommended for the visualization of microvessels in the upper gastrointestinal tract in the Olympus instruction manual.

FICE

The red, green, and blue (RGB) settings for FICE were $R = 525$ nm (gain 3), $G = 495$ nm (gain 4), and $B = 495$ nm (gain 3). This setting was one of the best recommendations for the enhancement of microvascular architecture provided in the Fujifilm instruction manual.

Evaluation of the endoscopic images

The WLI endoscopic images provided by both Olympus and Fujifilm Medical; NBI; and FICE were independently reviewed by 11 endoscopists who were blinded to the information on each imaging method. The median dilution that was estimated as the limitation of the visibility of diluted blood by each endoscopist was defined as the limitation of visibility with each imaging method.

Results

In general, NBI yielded darker images than WLI in the Olympus system (Fig. 1). However, NBI enabled clear visualization of the presence of blood, which was not visible by WLI (Fig. 1). In contrast, the brightness of FICE was similar to that of WLI in the Fujifilm system (Fig. 2) and the presence of blood was also similarly observable with WLI and FICE (Fig. 2).

In the Olympus system, blood dilutions up to around $1/2^{11}$ were recognizable by WLI; however, it was difficult

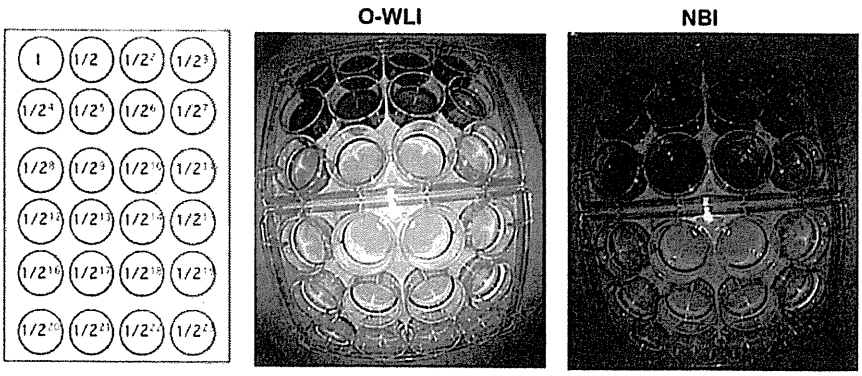

Fig. 1 Comparison of the visibility of blood diluted with distilled water using narrow band imaging (*NBI*) and conventional white light imaging (*WLI*) in the Olympus system. *O-WLI* WLI by Olympus system

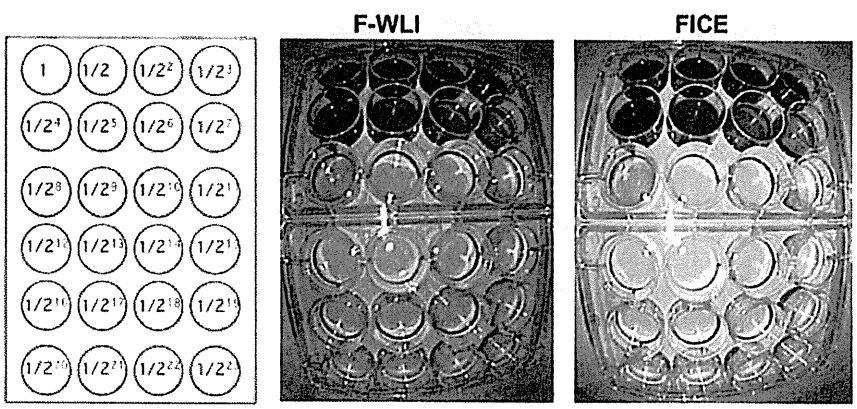

Fig. 2 Comparison of the visibility of blood diluted with distilled water using flexible spectral imaging color enhancement (*FICE*) and conventional WLI in the Fujifilm system, *F-WLI* WLI by Fujifilm system

to detect blood contamination at greater dilutions. In contrast, blood contamination up to a dilution of around $1/2^{14}$ was visualized using NBI (Fig. 1). The limit of visibility of the diluted blood in the water was $1/2^{11}$ and $1/2^{14}$ by WLI and NBI, respectively (Fig. 3). Thus, there was a 2^3-fold difference in sensitivity between these two methods.

To avoid any undesirable effect due to curved endoscopic images, we selected the demonstrable images around the limit of the visibility (Fig. 4). At a dilution of $1/2^9$, blood contamination was recognized in a relatively easy manner by WLI, but the detection was clearer using NBI. At a dilution of $1/2^{10}$, blood contamination was only marginally recognizable by WLI; in contrast, it was clearly observable using NBI. Furthermore, at a dilution of $1/2^{11}$, blood contamination was barely detectable by WLI, whereas it remained clearly visible using NBI. In addition, the presence of blood in distilled water was indicated by a brownish color in NBI images.

In the Fujifilm system, blood dilutions up to around $1/2^{10}$ were recognizable by both WLI and FICE; however, it was difficult to detect blood contamination at greater dilutions by FICE (Fig. 2). The limit of visibility of the diluted blood in the water was $1/2^{10}$ with both WLI and FICE (Fig. 3).

Similar to the Olympus system, to avoid any undesirable effect due to curved endoscopic images, we selected the

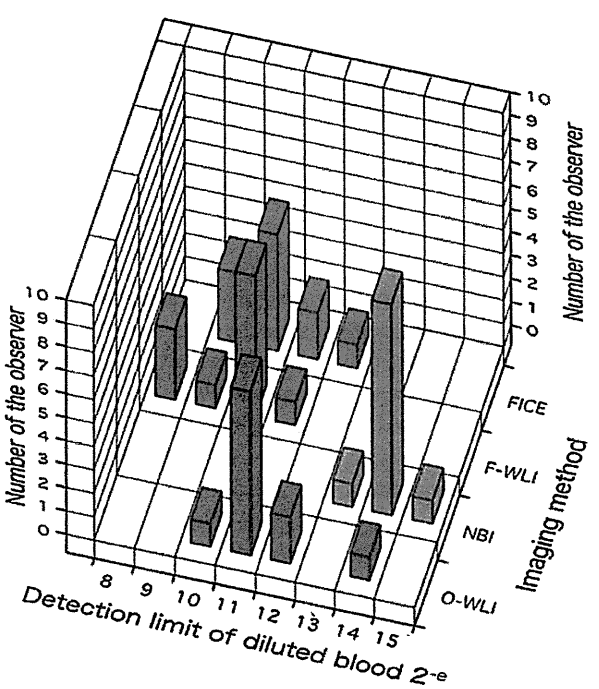

Fig. 3 Estimation of the limitation of the visibility of diluted blood by each endoscopist. *O-WLI* WLI by Olympus system, *F-WLI* WLI by Fujifilm system, $2^{-e} = 2^{-8 \sim 15}$

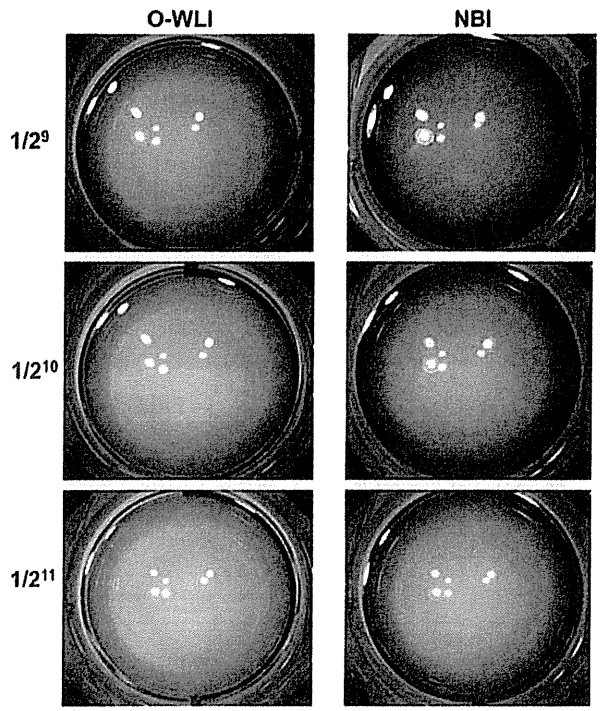

Fig. 4 Comparison of the visibility of blood dilutions from $1/2^9$ to $1/2^{11}$ using NBI and conventional WLI

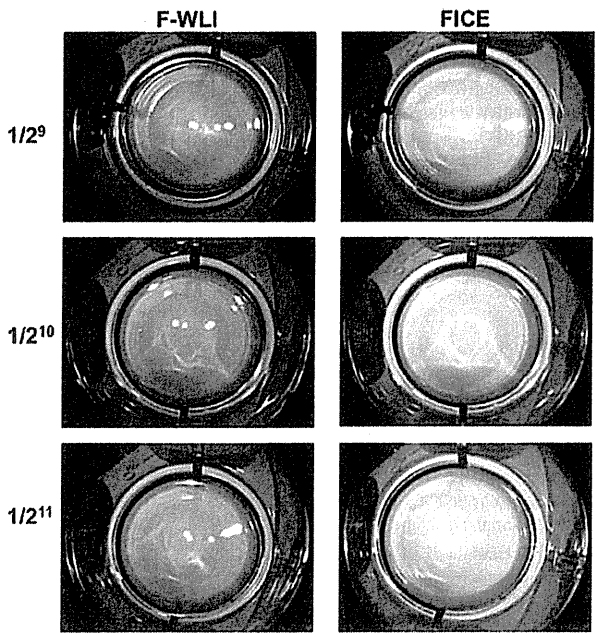

Fig. 5 Comparison of the visibility of blood dilutions from $1/2^9$ to $1/2^{11}$ using FICE and conventional WLI

demonstrable images around the limit of the visibility (Fig. 5). At a dilution of $1/2^9$, blood contamination was marginally recognized by both WLI and FICE. At a dilution of $1/2^{10}$, blood contamination was difficult to detect by both WLI and FICE. Indeed, at dilutions of $1/2^9$–$1/2^{11}$, FICE failed to provide better visualization than NBI (Figs. 4, 5).

Discussion

In this study, by using a quantitative approach, we demonstrated for the first time that the presence of blood was detectable by NBI with a high sensitivity, which was 2^3 times greater than that of conventional WLI. Since NBI light is well absorbed by hemoglobin, it follows that NBI will enhance blood detection. In addition, as blood is the content of the human microvasculature, NBI could theoretically enhance the detection of microvessels.

As cancer arising from squamous cell epithelia in the head and neck region and in the esophagus is accompanied by abundant neovascularization, the present results suggest the usefulness of NBI for the sensitive detection of these cancers [10]. Furthermore, abnormal microvessels are also observed in early cancers of the stomach and colorectum, and NBI with magnification endoscopy has been reported to be more useful for cancer diagnosis than WLI in these regions [12–14]. The present data may also confirm such clinical usefulness.

In contrast to the findings with NBI, when using FICE we did not detect blood with greater sensitivity compared with that for WLI. While the FICE preset mode in this study was one of the best recommendations for the imaging of microvessels, this result might suggest that FICE has limitations for diagnoses based on neovascularization and morphological changes in the microvascular architecture.

In general, the evaluation of visibility is subjective and varies among different individuals. Therefore, in the present study, the visibility of the diluted blood in the sterilized water was independently evaluated by a total of 11 endoscopists. While seven of them (63.6%) evaluated that the limit of visibility of the diluted blood was $1/2^{11}$ in the WLI Olympus system, nine of them (81.8%) evaluated the limit at $1/2^{14}$ with NBI. This means that visibility was objectively improved by NBI. In contrast, evaluation of the visibility of the diluted blood was similar for WLI and FICE. This means that visibility was not objectively improved by FICE.

Most superficial squamous cell carcinomas in the head and neck region and in the esophagus are recognized as brownish areas by NBI. The reason for this remains unclear. In the present study, diluted blood in distilled water was recognized as a brownish color by NBI, even at the dilution level at which the identification of blood contamination was difficult with conventional WLI. One possibility may be that most squamous cell carcinomas in

these regions exhibit marked neovascularization, and the lesions are supplied by abundant red blood cells; as a result, the lesions may be recognized as brownish areas.

The limitation of this study was that the evaluation was performed in vitro. In the human body, cancerous lesions contain not only red blood cells but also collagen tissue, inflammatory cells, and so on. Furthermore, a patient's movement and the existence of mucus will influence the image. Therefore, the possibility cannot be denied that the content of other materials in the tissue and the light condition affect the advantages of NBI. However, there are no structural components that will be absorbed by specialized light as well as NBI. And the patient's condition can be managed in the clinical setting. Thus, the presence of blood might be a strong enhancer for the visualization provided by NBI.

The disadvantage of NBI is its darkness. However, when we visualize the microvascular architecture, the magnifying function is necessary and therefore the darkness will be omitted in the conditions of close observation. Thus, the clinical implications of the present results might not be influenced for the detection but for the detailed observation by magnifying function. Also the present results could provide a plausible explanation for the better visibility of microvessels shown by NBI.

In conclusion, this study demonstrated that NBI is superior to WLI for the identification of diluted blood in an in vitro assay, whereas FICE did not have better visibility of diluted blood than WLI. These results deepen our understanding of the superiority of NBI compared to conventional WLI and also support the evidence that NBI provides better visibility of changes in the microvascular architecture of gastrointestinal neoplasia [9], which is accompanied by abundant neovascularization. Conversely, the data suggest that the visualization of a lesion lacking vascularization would not be improved by NBI.

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Macroscopic estimation of submucosal invasion in the esophagus

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KEYWORDS:
EUS;
Esophageal cancer;
Submucosal invasion

In esophageal squamous cell carcinoma, the depth of invasion into the wall is closely associated with metastasis to lymph nodes. Esophageal cancer invading the muscularis mucosae could be curably treated by endoscopic submucosal dissection but cancer with submucosal invasion necessitates surgical resection and/or chemoradiotherapy. Therefore, pretreatment diagnosis of invasion depth is crucially important for selecting appropriate treatment strategies for each individual patient. To estimate the depth of cancer invasion for early squamous cell carcinoma of the esophagus, standard endoscopy with image enhancement and endoscopic ultrasound are currently considered the best methods.

According to the staging manual published by the American Joint Committee on Cancer,[1] cancer confined to the mucosa or the muscularis mucosae is categorized as mucosal cancer (T1a). T1a esophageal cancer comprises carcinoma in situ (high-grade intraepithelial neoplasia), cancer invading the lamina propria mucosae, and cancer invading the muscularis mucosae. T1b cancer includes cancer with submucosal invasion.

In esophageal squamous cell carcinoma, the depth of invasion into the wall is closely associated with metastasis to lymph nodes (Figure 1).[2] It is important to note that the frequency of metastasis in the lymph nodes in cancer confined to the mucosa is not zero, but 3%. The risk increases to 12% in cancer invading the muscularis mucosae and markedly increases to 26%-46% in patients with submucosal invasion. Because cancers confined to the mucosal layer correlate with a low frequency of metastasis and because surgery confers a high risk of morbidity and mortality, they are considered excellent candidates for minimally invasive treatment by endoscopic mucosal resection or endoscopic submucosal dissection (ESD). Cancer invading the muscularis mucosae may still be treated by ESD, but cancer with submucosal invasion necessitates surgical resection and/or chemoradiotherapy.[3,4] Given that endoscopic resection has some risks of bleeding and perforation, pretreatment diagnosis of invasion depth is crucial for selecting appropriate treatment strategies for each individual patient.

To estimate the depth of cancer invasion for early squamous cell carcinoma of the esophagus, standard endoscopy with image enhancement and endoscopic ultrasound (EUS) are considered the best methods. Other methods such as the "barium meal," computed tomography, and positron emission tomography[5] are considered less accurate.

Indications

Conventional endoscopy with image-enhanced endoscopy is quite accurate to diagnose cancer in situ (high grade-intraepithelial neoplasia) or cancer with minimal subepithelial invasion[6,7]; in those cases, EUS is rarely indicated. In our practice, we used high-magnification endoscopy with narrow-band imaging to view the surface

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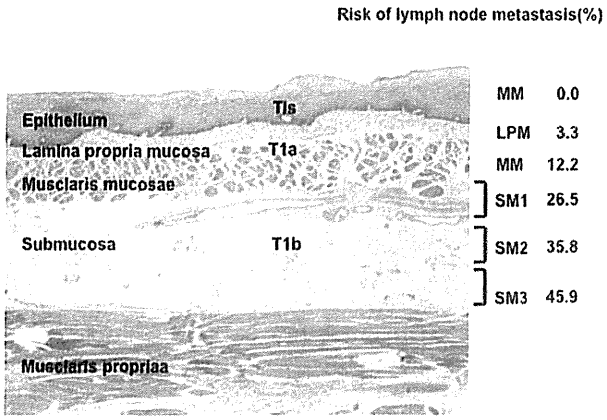

Figure 1 Risk of lymph node metastasis in the esophagus. In esophageal squamous cell carcinoma, the depth of invasion into the wall is closely associated with an increasing risk of lymph node metastasis. (Color figure is available online at www.techgiendoscopy.com.)

microvessels of early esophageal carcinoma. The patterns of these surface microvessels—called intraepithelial papillary capillary loops (IPCL)—have been shown to be predictive of the degree of mucosal and submucosal invasion.[8]

IPCL can be classified into eight different patterns; thus, the use of IPCL classification can be complex. A simpler way might be to classify the IPCL as regular and irregular. Early squamous cell carcinoma typically appears as a patch of mucosa with irregular IPCLs, which is clearly demarcated from the surrounding normal mucosa with regular IPCLs. When the carcinoma is limited to the mucosa, its surface is typically smooth and pliable. When the carcinoma has invaded the submucosa, its surface has nodular elevation, reddishness, and deeper depression. EUS can be helpful in these cases to rule out deeply submucosal invasive cancers.

Preparation

When peristalsis interferes with observation, an antispasmodic agent is required. Sedation is also necessary for stable observation.

Instruments

To visualize the distinct tissue layer of the esophageal wall, 20- or 30-MHz miniature probes should be used. This high-resolution imaging demonstrates 9-layered echo structures (Figure 2). Generally, the tumor can be seen as a low echoic mass by EUS. If the cancerous lesion invades to the submucosal layer, EUS deliver a low echoic mass in the high echo layer corresponding to the submucosal layer. A balloon can be attached to the tip of the endoscope to keep deaerated water in the esophageal lumen and prevent regurgitation to the pharynx. An endoscope with a water jet function is desirable to keep the esophageal lumen distended to obtain a clear image.

Techniques

To obtain clear images, several useful methods have been formulated. The most important issue is to keep air or air bubbles away from the scanning site.

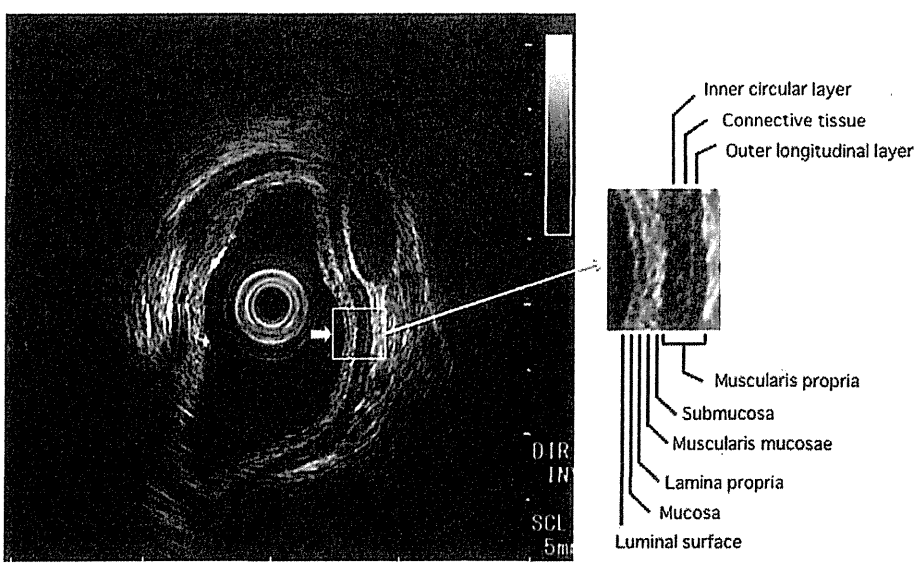

Figure 2 EUS image of the normal esophageal wall by 20-MHz miniprobe demonstrates 9-layered structures (arrow). The first 5 layers correspond to the echogenic luminal surface (high echo), mucosa (low echo), lamina propria (high echo), muscularis mucosae (low echo), and submucosa (high echo). Next are the inner circular (low echo) and outer longitudinal layers (low echo) of the muscularis propria. They are separated by a thin hyperechoic layer of connective tissue (high echo).

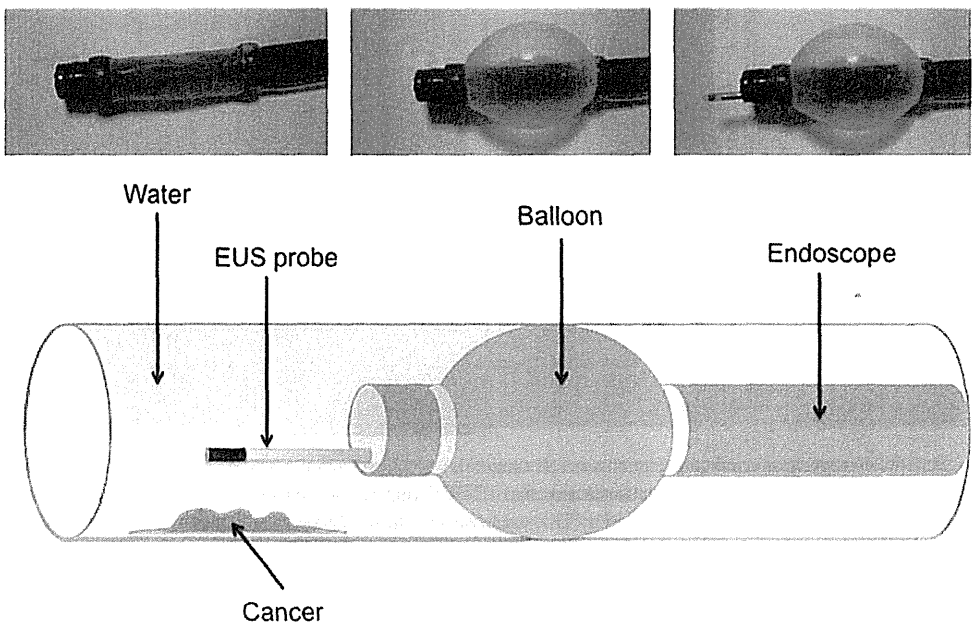

Figure 3 Photographs and scheme of EUS using the balloon method. To keep deaerated water in the esophageal lumen and prevent regurgitation to the pharynx, a balloon should be attached the endoscope itself. After insertion of the endoscope, the tip of endoscope should be directed toward the lesion. At the best position, the balloon should be dilated. With sufficient dilation of the balloon, deaerated water fills the esophageal lumen using the water jet function of the endoscope. Thereafter, scanning of the lesion will be started with a miniature probe. (Color figure is available online at www.techgiendoscopy.com.)

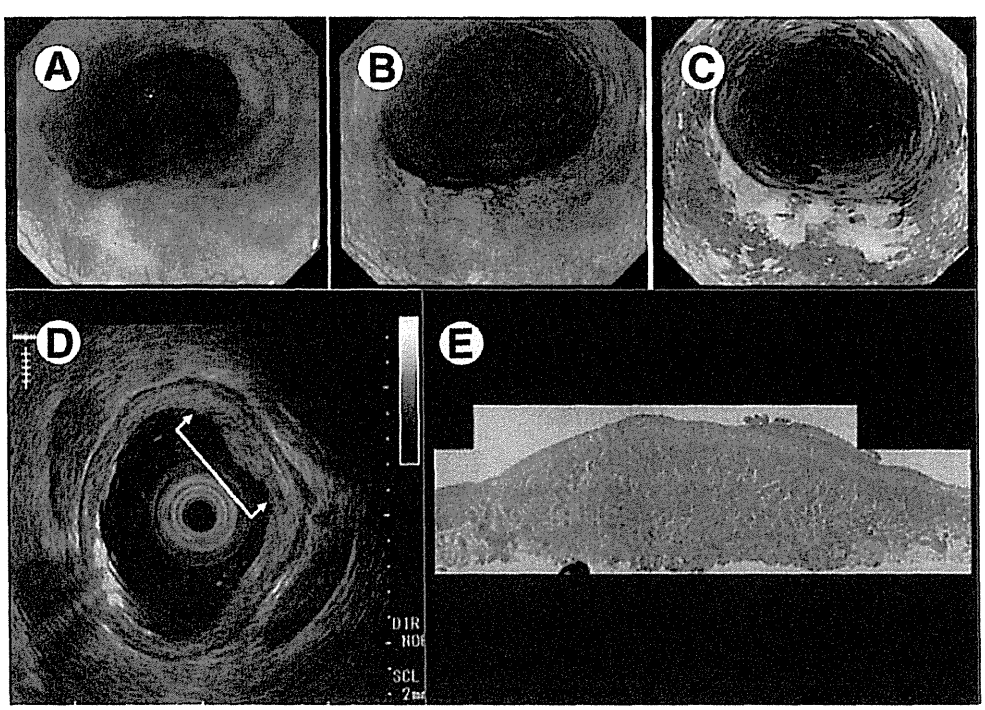

Figure 4 Superficial esophageal carcinoma (squamous cell carcinoma). (A) Conventional white light image shows a reddish area. (B) Narrow-band imaging indicates a well-demarcated brownish area with a white coat. (C) Lugol's staining indicates a well-demarcated unstained area. (D) EUS image demonstrates a low echoic mass located in the submucosal layer (arrow). (E) This superficial cancer was removed by ESD and submucosal invasion of the cancer was confirmed histologically. (Color figure is available online at www.techgiendoscopy.com.)

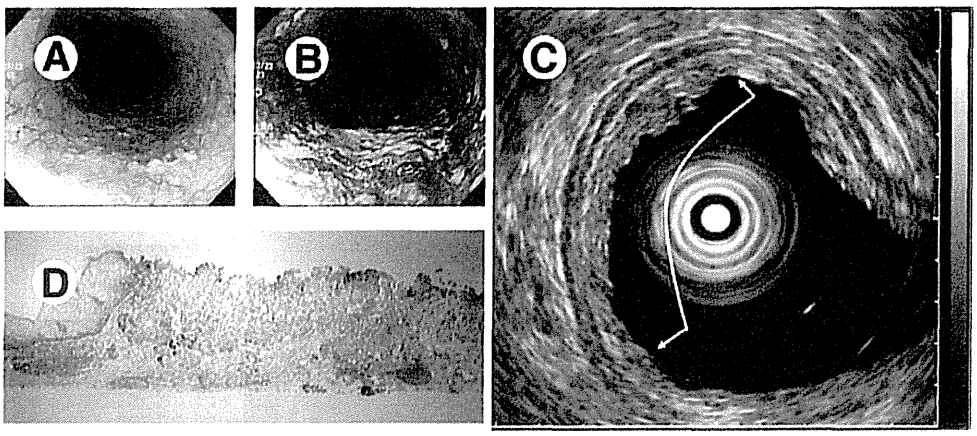

Figure 5 Superficial esophageal carcinoma (squamous cell carcinoma). (A) Conventional white light image indicates a depressed reddish area. (B) Lugol's staining indicates a well-demarcated unstained area. (C) EUS image demonstrates a low echoic mass located in the submucosal layer (arrow). (D) This superficial cancer was removed by ESD and it was confirmed histologically that this tumor invaded to the muscularis mucosae but not into the submucosal layer. This case was suspected to have submucosal invasion clinically; however, the depth of invasion was histologically confirmed as mucosal cancer. (Color figure is available online at www.techgiendoscopy.com.)

Balloon method

The balloon method (Figure 3) is the generalized method. A balloon of a size that fits the endoscope should be selected.

1. To keep deaerated water in the esophageal lumen and prevent regurgitation to the pharynx, a balloon (eg, inner diameter = 10-11 mm) should be attached the endoscope itself (Figure 3A).
2. After insertion of the endoscope, we must direct the tip of the endoscope toward the lesion.
3. At the best position, the balloon should be dilated (Figure 3B).
4. With sufficient dilation of the balloon, we fill the esophageal lumen with deaerated water using the water jet function of the endoscope.
5. Thereafter, we approach the lesion and scan it with the miniature probe (Figure 3C).

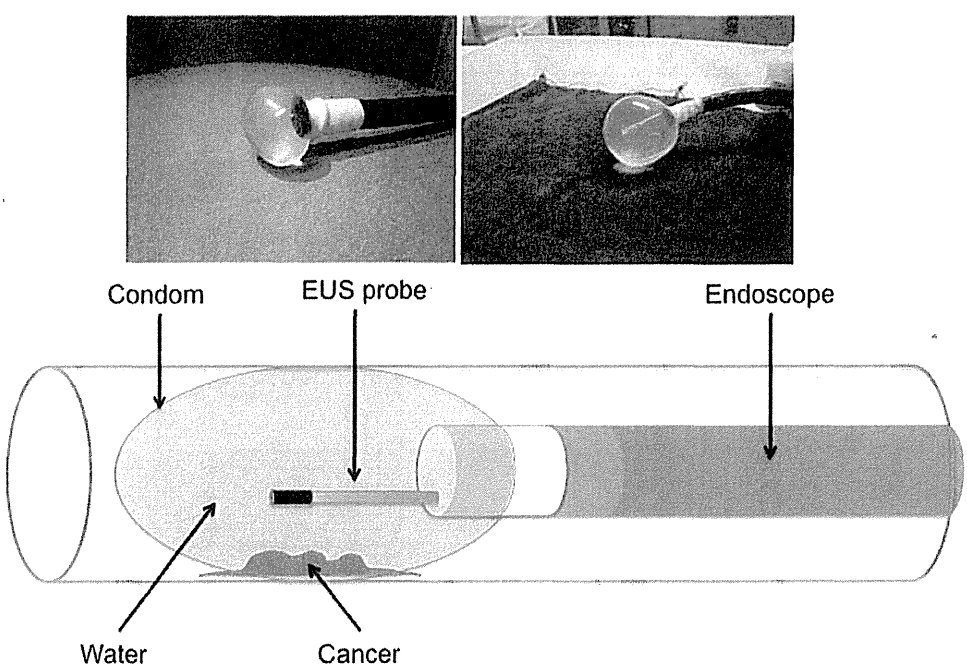

Figure .6 Photo and schema of EUS using the condom method. To keep deaerated water in the condom, a condom for gynecologic ultrasound examination should be attached to the tip of endoscope itself. (Color figure is available online at www.techgiendoscopy.com.)

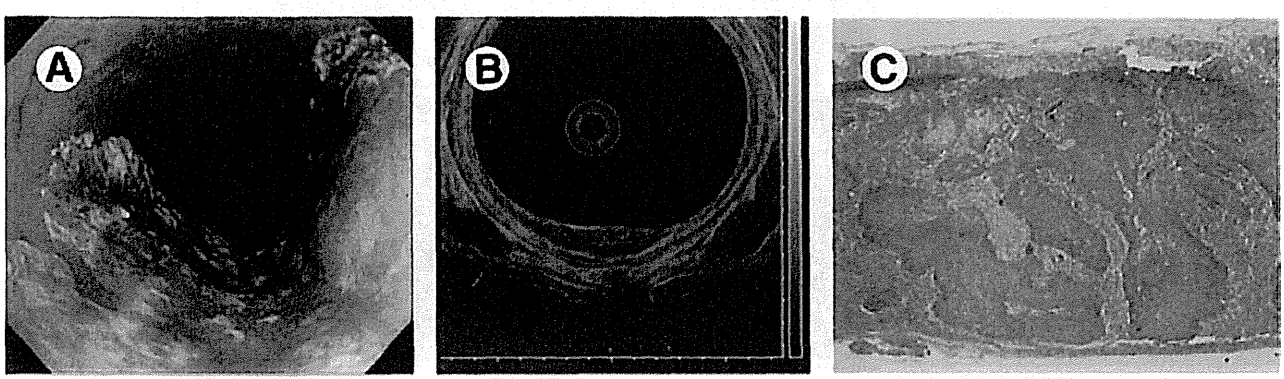

Figure 7 Superficial esophageal carcinoma (squamous cell carcinoma). (A) Conventional white light image indicates a reddish area with an irregular surface. (B) EUS image indicates a low echoic mass located in the submucosal layer. (C) This superficial cancer was removed by ESD and submucosal invasion of the cancer was histologically confirmed. (Color figure is available online at www.techgiendoscopy.com.)

Sample cases are presented in Figures 4 and 5.

Condom method

Similar to the balloon method, the condom method (Figure 6) is useful to keep water in the esophageal lumen because this method can keep water in the condom itself.

A sample case is presented in Figure 7.

Echo jelly method

Echo jelly (Figure 8) is also useful to keep air away from the lesion. A sample case is presented in Figure 9.

Complications

Because the esophageal lumen is filled with deaerated water, caution must be exercised regarding aspiration caused by regurgitation. Excessive balloon expansion is a risk for esophageal injury and hemorrhage. When a patient complains of a chest pain after injection of 15-20 cc of air, the balloon is sufficiently dilated in close contact with the esophageal wall and no more pressure should be applied.

Conclusions

A completely accurate diagnosis of the depth of early esophageal carcinoma can be difficult to attain. Standard

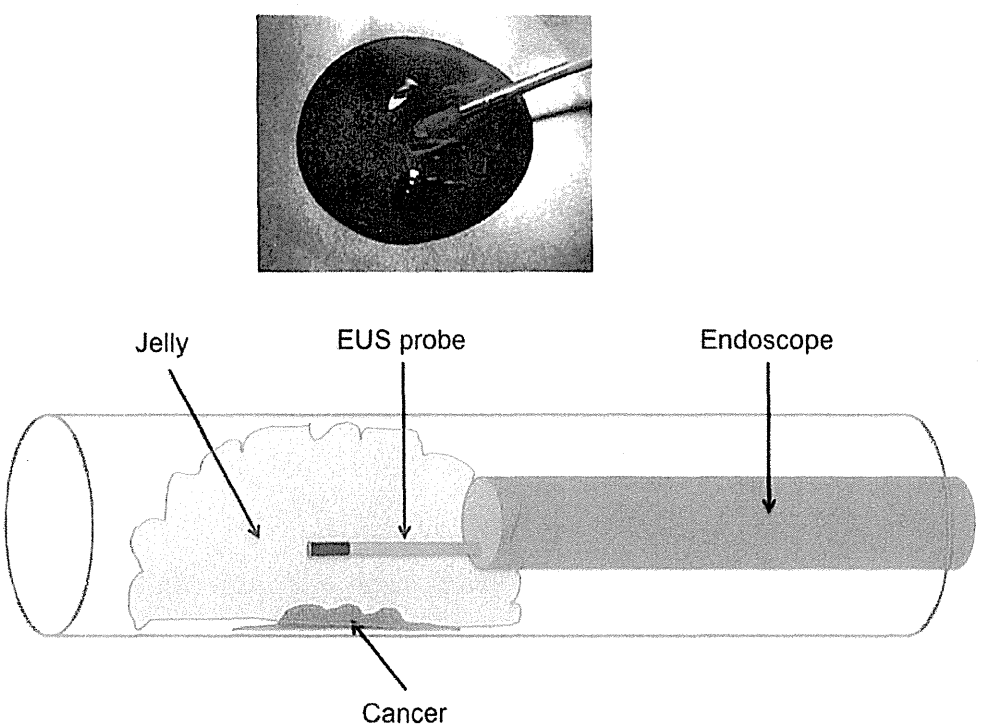

Figure 8 Schema of EUS by the jelly method. (Color figure is available online at www.techgiendoscopy.com.)

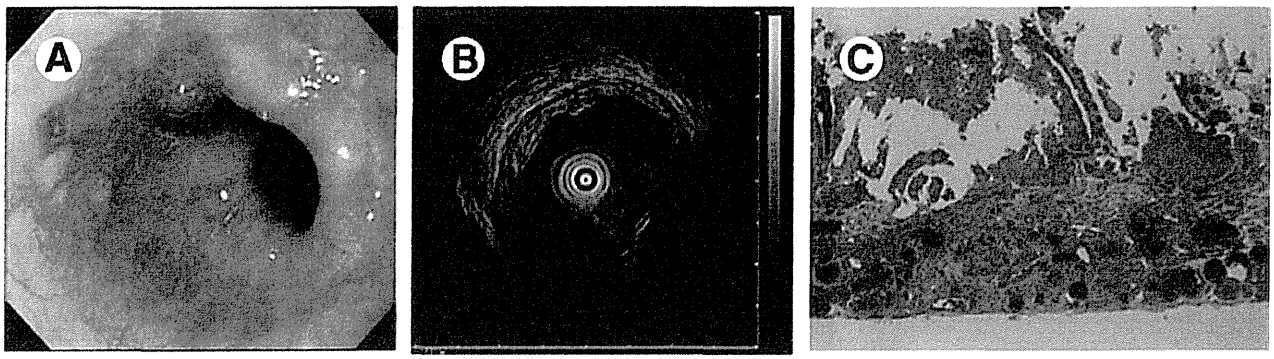

Figure 9 Superficial esophageal carcinoma (squamous cell carcinoma). (A) Conventional white light image shows a reddish area with nodular change. (B) EUS image demonstrates a low echoic mass located in the submucosal layer. (C) This superficial cancer was removed by endoscopic mucosal resection and submucosal invasion of the cancer was confirmed histologically. (Color figure is available online at www.techgiendoscopy.com.)

endoscopy with image enhancement and, if available, with high-magnification combined with EUS is useful in assessing the depth of invasion of lesions being considered for endoscopic resection. In cases treated by endoscopic mucosal resection/ESD, if the depth of invasion was found to have deeper invasion than estimated by pretreatment diagnosis, surgical resection and/or chemoradiotherapy may be necessary as an additional curative treatment. Currently, endoscopic resection is used in cases with shallower invasion. However, given of the risks of lymph node metastasis, informed consent that includes a thorough explanation of all possibilities is required.

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Long-term results of salvage photodynamic therapy for patients with local failure after chemoradiotherapy for esophageal squamous cell carcinoma

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Background and study aims: Local failure after chemoradiotherapy (CRT) remains a major problem for patients with esophageal squamous cell carcinoma (ESCC). The aim of this study was to clarify the long-term results of salvage photodynamic therapy (PDT) for local failure.

Patients and methods: Patients were treated with CRT, consisting of more than 50 Gy irradiation and concurrent chemotherapy. The indications for salvage PDT were as follows: 1) absence of lymph-node or distant metastasis after CRT; 2) failure lesion limited to T2; 3) refusal by patient to undergo salvage esophagectomy; 4) written informed consent. PDT was performed using an excimer dye laser at 48 and 72 hours after administration of Photofrin.

Results: A total of 37 consecutive patients underwent salvage PDT. The baseline stage before CRT was as follows: T1/T2/T3/T4 in 3/4/24/6 and N0/1 in 13/24 patients, respectively. Prior to PDT, 20 patients had a uT1 lesion, and 17 had a uT2 lesion; 24 patients had histologically proven local failure. A complete response was achieved in 22 patients (59.5%) following PDT. Esophageal fistulae, stenosis, and phototoxicity occurred in 4 (10.8%), 20 (54.1%), and 2 (5.4%) patients, respectively. Over a median follow-up period of 55 months, the 5-year progression-free (PFS) and overall survival rates of 37 patients following PDT were 20.7% and 36.1%, respectively. The 5-year PFS and overall survival of 24 patients with proven local failure were 17.6% and 34.6%, respectively.

Conclusion: Salvage PDT is a curative treatment option for patients with local failure after CRT for ESCC.

Introduction

Chemoradiotherapy (CRT) is a curative treatment option for esophageal squamous cell carcinoma (ESCC). However, local failure at the primary site after completion of CRT remains one of the major problems to be overcome for patients with ESCC. Salvage esophagectomy is now indicated for such patients, and it could be curative particularly for patients with T2 or earlier T-stage tumor or for patients without lymph node metastasis [1,2]. However, salvage esophagectomy is still associated with relatively higher morbidity and mortality compared with primary or planned esophagectomy [1–4]. Therefore, the development of curative and safety salvage treatment options for local failure is essential for improving the survival of patients treated with CRT.

We previously reported that patients who achieved complete response with CRT were very unlikely (< 1.0%) to experience a recurrence in locoregional lymph nodes [5]. This may lead to the hypothesis that, in patients who have only local failure after CRT, salvage local treatments such as endoscopic mucosal resection (EMR), and photodynamic therapy (PDT), could have curative potential. In fact, we first introduced EMR as a salvage treatment for local failure after CRT [6,7] and found that the long-term survival could be acceptable [7]. However, the indications for salvage EMR are limited to superficial lesions, and the procedure requires highly skilled endoscopists.

In contrast, PDT is indicated not only for superficial esophageal cancer as a curative treatment [8,9], but also as a palliative treatment for dysphagia due to stenosis of more advanced cancer [10]. Therefore, we consider that PDT could be a more powerful tool for salvage treatment after CRT. We previously reported acceptable short-term results of salvage PDT for local failure after definitive CRT for patients with ESCC [11]. Long-term results, however, have not been reported previously. The aim of the present study was to clarify the long-term survival of consecutive patients who have undergone salvage PDT for local failure after definitive CRT for ESCC.

Patients and methods

Patients

Between January 1998 and December 2004, 405 patients with ESCC were treated with CRT at the National Cancer Center Hospital East, Kashiwa, Japan. CRT consisted of more than 50 Gy external beam irradiation concurrent with two cycles of continuous infusion of 5-fluoruracil and cisplatin. In cases of renal insufficiency or cardiovascular disease, nedaplatin was used instead of cisplatin, because nedaplatin does not require hydration and has shown a low risk of renal toxicity [12].

The indications for salvage PDT were as follows: 1) absence of lymph node or distant metastases by computed tomography (CT) before PDT; 2) residual or recurrent tumor at primary site staging limited to within uT2 by endoscopic ultrasound (EUS); 3) EMR not indicated for reasons of concomitant deep ulceration or severe fibrosis due to radiation or lesion invading the deep submucosal layer; 4) refusal by patient to undergo surgery or physical complications that would have made surgery intolerable and; 5) provision of written informed consent. **Fig. 1** shows the flow of the patients through the study.

Of the 405 patients treated with definitive CRT, a complete response was achieved at the primary lesion in 234; the remaining 171 patients did not show a complete response. Of the 234 patients, 50 developed local recurrence at the primary site and eight patients were indicated for salvage PDT. Two patients with local recurrence were referred from another hospital to receive salvage PDT. Among the 171 patients with an incomplete response following CRT, 26 were indicated for salvage PDT, and one was referred from another hospital to receive salvage PDT. In total, therefore, 37 consecutive patients with local failure after definitive CRT were treated with salvage PDT and enrolled in the study. All information was collected from medical records and provided by the patients' physicians. This retrospective study was performed in accordance with the Declaration of Helsinki.

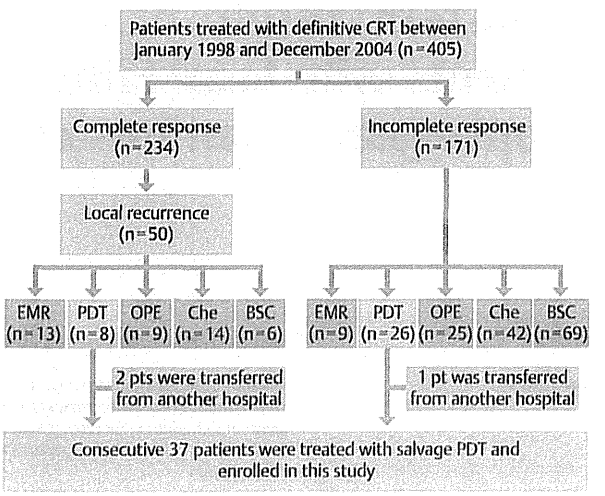

Fig. 1 Flow of patients through the study. CRT, chemoradiotherapy; EMR, endoscopic mucosal resection; PDT, photodynamic therapy; OPE, esophagectomy; Che, chemotherapy; BSC, best supportive care.

Staging

Clinical staging was determined by the TNM classification of the International Union Against Cancer [13]. Clinical T stage was evaluated by endoscopy, EUS, and CT, and clinical N and M stages were evaluated mainly by CT of the neck, chest, and abdomen. In this study, lymph node metastasis was clinically diagnosed if the lymph node was more than 10 mm in diameter on CT. All of the patients who were treated with definitive CRT at our institution are routinely evaluated by endoscopy and CT after completing CRT. Complete response at the primary site was defined as follows: i) disappearance of the tumor lesion and ulceration by endoscopic examination; ii) the absence of cancer cells in biopsy specimens [14]. The complete disappearance of metastatic lesions by CT was defined as complete response.

After confirmation of complete response, follow-up examination with endoscopy and CT was performed every 3 months for 2 years, and every 6 months thereafter. Biopsies of the primary site were routinely obtained at each follow-up endoscopic examination.

Local failures were classified into two groups: residual lesions and recurrent lesions. Residual lesions were defined as lesions that did not achieve complete response immediately after CRT. Recurrent lesions were defined as lesions that relapsed after achieving complete response. If the primary site showed obvious growth or if cancer cells were detected in a biopsy specimen, the lesion was diagnosed as a recurrence. Submucosal tumors or slightly protruding lesions at the primary site were suspected of representing a recurrence and were re-evaluated with EUS.

Before PDT, all patients were evaluated and staged using EUS (EU-M2000; Olympus Co. Ltd., Tokyo, Japan). Lesions were carefully examined with a high-frequency (20 Hz) ultrasound probe. When a hetero-echoic solid component in a submucosal or deeper layer was detected, a diagnosis of local failure lesion was made. The depth of the residual lesions by EUS was divided into either uT1 or uT2. Stage uT1 described lesions whose invasion was limited to the submucosal layer, and uT2 described those invading the muscularis propria layer.

Photodynamic therapy

PDT commenced with intravenous administration of 2 mg/kg of Photofrin (Pfizer Japan Inc.) followed by dye laser irradiation. A 630-nm wavelength laser beam was emitted by an excimer dye laser (EDL-1, Hamamatsu Photonics, Hamamatsu, Japan). The laser treatment was performed in two sessions at 48 and 72 hours after injection of Photofrin. The excimer dye laser was delivered via a microlens-type straight-tip fiber without any light diffuser introduced into the operative channel of the fiberscope (GIF-Q20; Olympus Co., Ltd.) and positioned in the esophagus. The total light density was 75 J/cm^2 with 4 mJ/pulse maximum pulse energy and 40 Hz pulse frequency, and no adaptation of delivered energy to radiotherapy time.

All patients were instructed to avoid direct exposure to sunlight for 1 month after the injection of Photofrin in order to protect them from skin photosensitization. To confirm the ulceration and development of tissue necrosis after PDT, patients were examined endoscopically 1 week after laser irradiation. To evaluate the response and luminal toxicity of PDT, endoscopic examination with biopsy was repeated at least every month until the response was confirmed. CT was used to evaluate the distant organ or lymph node metastasis every 3 months for the first 2 years, and every 6 months thereafter. The response to PDT was classified into two groups: 1) complete response, if there was no macroscopic or microscopic evidence of cancer; 2) incomplete response, if a tumor was seen at endoscopy and confirmed histologically to contain cancer cells. Recurrence after achieving com-

plete response by PDT was defined when cancer cells were histologically confirmed at the primary site, if the lymph node was larger than 10 mm, or if distant metastasis was present.

Statistics

The progression-free survival (PFS) was measured from the date of initial PDT to the first date of histologically confirmed residual lesion at the primary site or recurrence or disease progression at any site or death. The overall survival was measured from the date of initial PDT to the date of death for any reason or last follow-up visit. Survival time was calculated by the Kaplan–Meier method. Survival was compared between variables using log-rank tests. A P value of < 0.05 was considered significant. All statistics were performed by using the Dr SPSS II statistical software package (SPSS Japan Inc., Tokyo, Japan)

Results

Patient characteristics

The baseline characteristics of patients before CRT are summarized in ○ **Table 1**.

The patients consisted of 35 men and two women, with a median age of 64 years (range 50–75 years). No patients had distant organ metastasis, and all lesions were histologically proven to be ESCC before CRT. Lesion characteristics before PDT are summarized in ○ **Table 2**.

Histological confirmation could not be obtained in 13 patients; however, we strongly suspected local failure because the apparent elevation or ulcer formation occurred at the primary site.

Response to salvage PDT

The interval between the last day of radiotherapy and initiation of PDT was 4 months (range 1–85 months) in the entire group of patients, 16 months (range 7–86 months) in 10 patients with local recurrence after achieving a complete response with CRT, and 2.5 months (range 1–17 month) in 27 patients with a residual lesion after CRT. The median total light dose for PDT was 675J (range 300–1000J), and the median hospital stay was 11 days (range 6–33 days). Complete response was attained in 22 of 37 patients with PDT, resulting in a complete response rate of 59.5% for salvage PDT (95% confidence interval [CI] 42.1–75.3). The complete response rate of the 20 patients with uT1 local failure was 75.0% (15/20; 95% CI 50.9–91.3), and that of the 17 patients with uT2 was 41.2% (7/17; 95%CI 18.4–67.1). The median time to confirm a complete response was 102.5 days (range 35–199 days).

Major complications of salvage PDT

Four patients (4/37, 10.8%) developed esophageal fistulae after salvage PDT. Their clinical T stages before CRT were T3 in three patients and T4 in one. All of them had local residual lesions just after CRT, and their T stages before PDT were uT2 in one patient and uT1 in three patients. All of them were treated with ≥ 600J PDT irradiation. In one patient, the fistula closed with conservative treatment, and complete response was achieved without any metastasis. Another patient developed mediastinitis due to esophago-mediastinal fistula. Despite this patient being treated conservatively, by total parenteral nutrition and intravenous administration of antibiotics, she died with bleeding from the primary site at 63 days after PDT. An esophageal-aortic fistula was confirmed at autopsy. The remaining two patients died with cancer progression. Thus, treatment-related death with PDT was 2.7% (1/37).

Table 1 Baseline patient and lesion characteristics before chemoradiotherapy.

Characteristics	No. of patients (n = 37)
Sex	
Male	35
Female	2
Age, median (range), years	64 (50 – 75)
Tumor location	
Upper	6
Middle	24
Lower	7
T-stage	
T1	3
T2	4
T3	24
T4	6
N-stage	
N0	13
N1	24
TNM-stage	
I	2
II	11
III	22
IV	2

Table 2 Lesion characteristics before photodynamic therapy.

Characteristics	No. of patients (n = 37)
Tumor status after chemoradiotherapy	
Recurrent	10
Residual	27
Tumor stage evaluated with EUS	
uT1	20
uT2	17
Ulceration	
Present	17
Absent	20
Circumference of the lesion	
< ¼	4
¼ – < ½	20
½ – < ¾	12
> ¾	1
Histologically proven cancer cells	
Positive	24
Negative	13

EUS, endoscopic ultrasound.

Other complications occurred in 20 patients (20/37, 54.1%) who developed esophageal stenosis requiring balloon dilation. Among them, a complete response could not be achieved in 12 patients following PDT; it is therefore possible that their stenoses might have been caused by progressive refractory tumor as well as by lumen toxicity caused by PDT. Cutaneous phototoxicity requiring medication was experienced in two patients (2/37, 5.4%).

Clinical course after salvage PDT

The median follow-up period of all patients following salvage PDT was 55 months (range 18–75 months). The clinical flow chart of the 22 patients who achieved complete response with salvage PDT is presented in ○ **Fig. 2**.

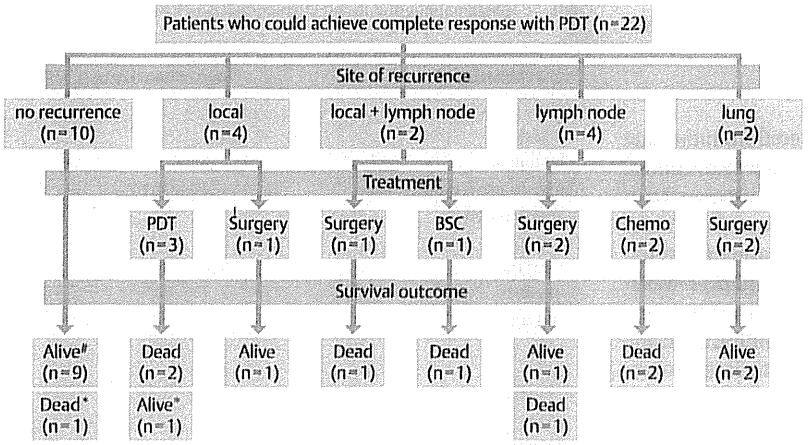

Fig. 2 The clinical flow chart of 22 patients in whom a complete response was achieved with salvage PDT. CR, complete response; PDT, photodynamic therapy; BSC, best supportive care; Chemo, chemotherapy; Dead*, dead from another disease; Alive#, alive with disease.

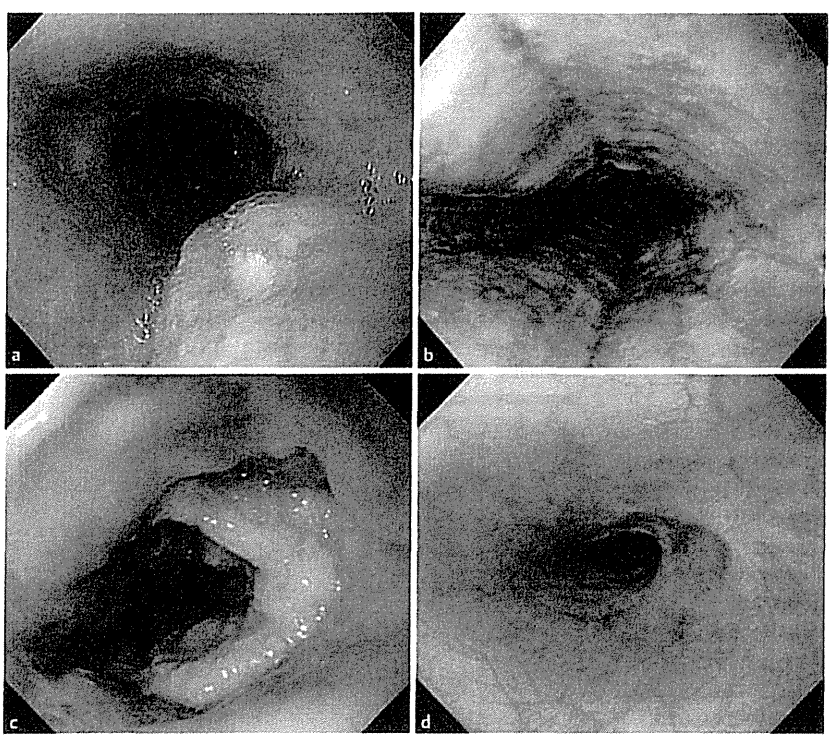

Fig. 3 A patient in whom complete response was achieved with salvage photodynamic therapy (PDT). **a** Local recurrence was detected after chemoradiotherapy and evaluated as uT1 with endoscopic ultrasound. **b** At 3 days after salvage PDT, circumferential ischemic change was observed. **c** At 1 month after salvage PDT, deep ulceration with dense necrotic tissue was observed at the primary site. **d** At 3 years after salvage PDT, treatment was evaluated as a complete response without any recurrence.

Ten patients did not develop any recurrence. Nine of them are still alive, and the tenth died of pneumonia without any esophageal cancer recurrence approximately 4 years after PDT. The details of these 10 patients are as follows: the baseline clinical stages before CRT were T1 (n = 1), T2 (n = 4), T3 (n = 3), and T4 (n = 2); N0 (n = 5) and N1 (n = 5); and stage I (n = 1), stage II (n = 4), stage III (n = 3), and stage IV (n = 2). Lesion characteristics before PDT were uT1 (n = 7) and uT2 (n = 3); six had histologically proven local failure before PDT and the other four had histologically unproven lesions before PDT. Moreover, the baseline tumor stage of five patients, except for the patient who died of pneumonia, with histologically proven local failure who survived without any recurrence before CRT was T1 (n = 1), T2 (n = 4), and all failure lesions were uT1 before PDT.

A representative case of a patient in whom complete response was achieved without any recurrence after salvage PDT is shown in ○ **Fig. 3**.

Local recurrence at the primary site was detected in four patients, one of whom was cured with salvage esophagectomy and is still alive without recurrence. The remaining three patients were treated with a second PDT, but none of them achieved complete response. In two patients, local recurrence and simultaneous lymph node metastasis were detected. One of these was treated with esophagectomy and the other was followed with the best supportive care; however, both died of disease progression. Lymph node metastasis without local recurrence was detected in four patients, of whom two underwent surgery and the other two were treated with systemic chemotherapy. One of the patients who received curative resection for metastatic lymph node is still alive without recurrence; however, the remaining three patients died of cancer progression. Solitary lung metastasis was detected in two patients; both underwent surgery and are still alive without recurrence.

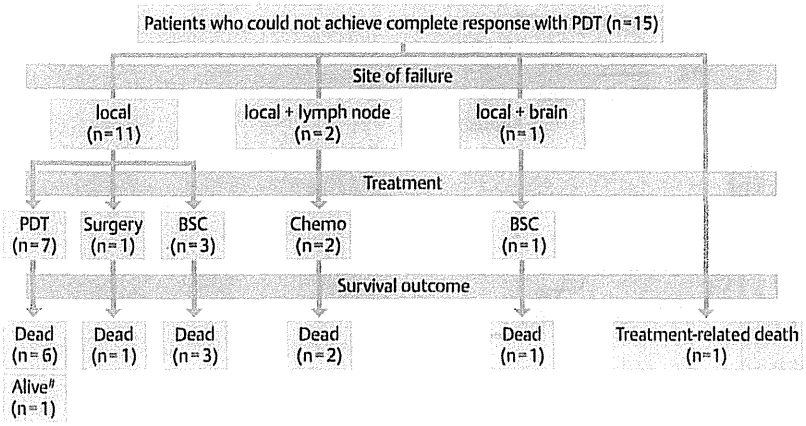

Fig. 4 Clinical flow chart of 15 patients in whom salvage photodynamic therapy did not achieve a complete response. CR, complete response; PDT, photodynamic therapy; BSC, best supportive care; Chemo, chemotherapy; Alive#, alive with disease.

A flow chart for the 15 patients in whom PDT could not achieve a complete response is shown in ◎ **Fig. 4.**

One patient died of bleeding after PDT as described above, 13 died of cancer progression, and one remains alive with the disease. The clinical courses of 13 patients without histologically proven carcinoma before PDT are as follows: nine patients achieved complete response after PDT, in three patients histologically proven residual tumors were detected after PDT, and the remaining patient died with aortic rupture, as described above. Of the nine patients showing complete response for PDT, four of them are still alive without any recurrence, three patients have developed histologically proven local recurrence after achieving complete response, one patient developed lymph node metastases without local recurrence, and one patient developed a solitary lung metastasis without local recurrence.

Survival

The PFS rates at 3 and 5 years from the initiation of salvage PDT were 31.9% (95%CI 16.7–47.1) and 20.7% (95%CI 6.4–30.5), respectively. The overall survival rates at 3 and 5 years from the initiation of salvage PDT were 47.4% (95%CI 30.9–63.8) and 36.1% (95%CI 19.2–53.0), respectively (◎ **Fig. 5**).

In addition, PFS and overall survival of 24 patients at 5 years with histologically proven local failure were 17.6% (95%CI 1.1–34.0) and 34.6% (95%CI 14.5–54.7), respectively. Furthermore, comparisons of PFS according to various clinical variables before CRT and before PDT are presented in ◎ **Fig. 6.**

Patients with clinical T1 or T2 had significantly higher 5-year PFS rates than those with T3 or T4 (T1/2 vs. T3/4 = 71.4% [95%CI 38.0–104.9] vs. 9.1% [95%CI −2.4 to 20.7]; $P = 0.005$), whereas there was no significant difference between patients with N0 and N1 (N0 vs. N1 = 27.7% [95%CI 2.1–53.3] vs. 16.2% [95%CI −1.2 to 33.6]; $P = 0.33$). On the other hand, the 5-year PFS of patients with uT1 before PDT was significantly higher than those with uT2 (uT1 vs. uT2 = 30.0% [95%CI 7.9–52.1] vs. 8.8% [95%CI −0.4 to 24.0]; $P = 0.02$). Patients with recurrence after complete response had a better 5-year PFS rate than patients with residual tumor (recurrent vs. residual = 40.0% [95%CI 9.6–70.4] vs. 13% [95%CI −2.2 to 28.1]; $P = 0.07$), although the difference was not statistically significant. There was no significant difference in progression-free survival between patients with and those without histologically proven cancer cells before PDT (negative vs. positive = 30.8% [95%CI 5.7–55.9] vs. 17.6% [95%CI 1.1–34.0]; $P = 0.61$).

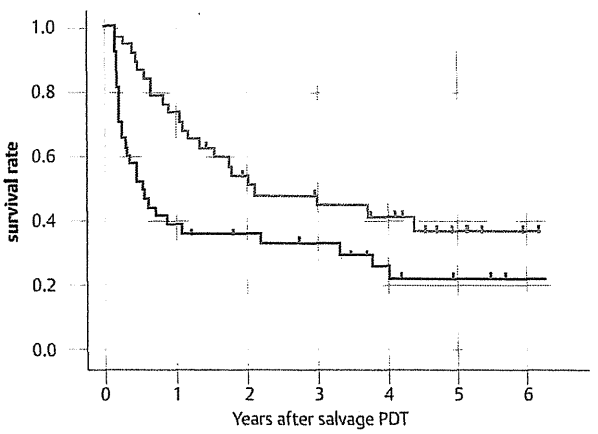

Fig. 5 Overall survival (blue line) and progression-free survival (red dotted line) of all 37 patients from the initiation of salvage photodynamic therapy (PDT).

Discussion

In the present study, salvage PDT for local failure after CRT for ESCC showed a high complete response rate. Moreover, the long-term survival was acceptable, because the prognosis of patients with local failure after CRT is usually quite dismal [14,15]. EMR is a salvage treatment option for local failure after CRT if the failure lesion is superficial. Indeed, we have reported the long-term results for salvage EMR, and the 5-year survival was 49.1% [7]. The difference in 5-year survival between salvage PDT and salvage EMR may depend on both their baseline clinical stage before CRT and clinical stage before salvage treatment. In salvage EMR, more than half of the patients had baseline clinical T1 lesions before CRT, and all of their local failure lesions were within the submucosal layer before EMR [7]. On the other hand, more than 80% (30/37) of patients had baseline clinical T3/4 lesions before CRT, and approximately half (17/37) of failure lesions were uT2 before PDT in the present study. Moreover, salvage EMR is technically quite difficult if the failure lesion has a severe fibrosis after CRT or if there is massive invasion of the submucosal layer. Therefore, PDT might be recommended as a salvage treatment for failure lesions evaluated as uT1 or when EMR is not indicated due to the abovementioned reasons.

The 5-year survival rate after salvage surgery is reported to be approximately 30% [1,2,4]. Most of the patients who achieved

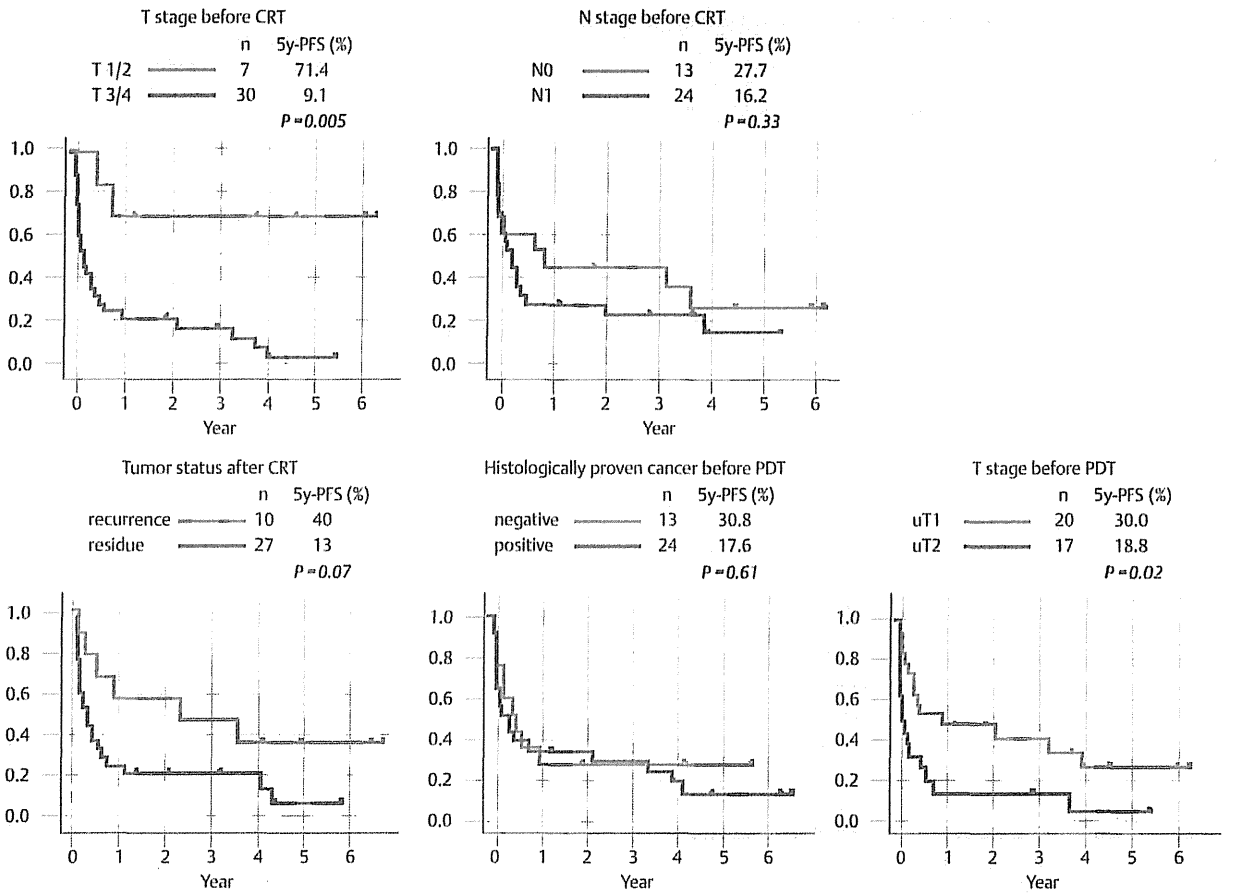

Fig. 6 Comparisons of progression-free survival curves according to various clinical variables before chemoradiotherapy and before photodynamic therapy.

long-term survival after salvage surgery showed T1 or T2 local failures without lymph node metastasis [1,2,4]. Swisher et al. reported that 5-year survival of patients with pathological T1 or T2N0 was 60% in salvage surgery; however no patient with pathological T3, or T4, or N1 survived longer than 7 months [1]. These data suggest that it is difficult to salvage patients with local failure more advanced than T3 and/or lymph node metastasis, even when they are treated with salvage surgery. However, these data cannot be simply compared with the results of salvage PDT, because these reports included patients with deeper local failure or locoregional lymph node metastasis.

The problem with the salvage surgery was a high incidence of complications (15%–39%) and a high treatment-related mortality rate (8%–22%) [1–4,16]. While, we have experienced one case (2.7%) of treatment-related death with salvage PDT in this study, the incidence rate was lower than for salvage surgery and no severe adverse events were associated with PDT. Thus, salvage PDT was a less-invasive treatment option compared with salvage surgery for patients with local failure after CRT. PDT is a treatment option, if local failure after CRT is limited to the muscularis propria layer, especially the submucosal layer without lymph node metastasis, and in patients in whom surgery would be intolerable because of physical complications. Therefore, PDT has a niche role between EMR and surgery in the salvage setting after CRT.

In the present study, 13/37 (35.1%) patients did not have a histologically proven tumor before PDT. We could not deny the possibility that the remarkable 5-year overall survival rate might be influenced by the patients with salvage surgery and by the patients without histologically proven tumor. Actually, of nine patients who are still alive without any recurrence, four patients had histologically unproven local failure before PDT. However, the 13 patients without histologically proven tumor were carefully evaluated by endoscopic examination and EUS and were found to have progressive development of ulceration of the space occupied by the lesion after achieving complete response for CRT. For the purpose of clarifying this disputable situation, we are now evaluating, in a prospective study, the efficacy and safety of salvage PDT only for histologically confirmed local failure after CRT for ESCC.

In the current study, 6 of 37 (16.2%) patients developed lymph node metastasis after PDT. Only one patient without local failure after PDT was cured by lymph node dissection. PDT has no curative potential if there is a high risk of lymph node metastasis. In salvage surgery, more than 30% of the patients developed locoregional or distant metastasis [1,16,17]. This means that the risk of lymph node metastasis is also high even for salvage surgery. Therefore, we have to investigate a more curative strategy for patients with high risk of recurrence even after salvage treatment.

The effect of second-line chemotherapy for patients with refractory or recurrent esophageal cancer after CRT is extremely limited. From the literature, the overall response rate of second-line systemic chemotherapy for previously treated esophageal cancer patients including local failure are low (0–16%), and complete

response is quite difficult to expect (0–6%) [18–21]. Therefore, second-line systemic chemotherapy for failure after CRT is only a palliative treatment. In fact, most of the patients with unresectable failure or distant metastasis were treated with second-line chemotherapy in the current study (© **Fig. 1**). However, among the patients with local failure after CRT, some patients developed only local recurrence and these recurrent or residual lesions could be candidates for salvage PDT and expected to be cured.

As for major complications after salvage PDT, we experienced four cases (10.8%) of esophageal fistulae. Of these, one patient (2.7%) died due to an esophageal-aortic fistula. Esophageal perforation can develop even in patients receiving primary intent PDT for naïve esophageal cancer, as previously reported [8]. However, we cannot deny the possibility that radiation-induced esophageal damage was potentiated by PDT and that the structural damage occurs by transmural necrosis. Lecleire et al. reported a retrospective comparative study of primary intent PDT and salvage PDT after CRT [22]. They found two out of 15 cases (13.3%) of perforation in a salvage setting, whereas no cases (0/25) suffered perforation after primary intent PDT. In the present study, all four patients who developed fistulae had an initial T3 or T4 lesion and had a residual lesion just after CRT, and their total light dose was more than 600 J. Salvage PDT should be carefully performed, particularly in patients in the initial advanced stage and with residual local failure just after CRT. Furthermore, the total laser irradiation dose may correlate with esophageal fistulae.

Patients with baseline T1 or T2 before CRT, and uT1 before PDT tend to achieve long-term survival after PDT. In seven patients with baseline T1 or 2, six patients were evaluated uT1 before PDT. In addition, we could not deny the possibility that patients with more advanced local failure were included in the baseline T3/4 before CRT group, because EUS evaluation is more difficult just after CRT due to radiation esophagitis, especially in advanced cases. From the results of the present study, the treatment efficacy and long-term survival were quite different based on the T stage either before CRT or PDT, and earlier T-stage lesions tended to be cured with PDT, even in the salvage situation. In fact, the baseline tumor stage of five patients with histologically proven local failure who are still alive without any recurrence before CRT was T1 in 1, and T2 in 4, and all their failure lesions were uT1 before PDT. However, caution should be shown when interpreting these survival rates across different variables due to the small sample size.

In conclusion, salvage PDT could be a curative treatment option for patients with local failure after CRT for ESCC when their failure lesions are suspected at stage T2 or earlier without lymph node or distant metastasis.

Competing interests: None

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Artificially Induced Epithelial-Mesenchymal Transition in Surgical Subjects: Its Implications in Clinical and Basic Cancer Research

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Abstract

Background: Surgical samples have long been used as important subjects for cancer research. In accordance with an increase of neoadjuvant therapy, biopsy samples have recently become imperative for cancer transcriptome. On the other hand, both biopsy and surgical samples are available for expression profiling for predicting clinical outcome by adjuvant therapy; however, it is still unclear whether surgical sample expression profiles are useful for prediction via biopsy samples, because little has been done about comparative gene expression profiling between the two kinds of samples.

Methodology and Findings: A total of 166 samples (77 biopsy and 89 surgical) of normal and malignant lesions of the esophagus were analyzed by microarrays. Gene expression profiles were compared between biopsy and surgical samples. Artificially induced epithelial-mesenchymal transition (aiEMT) was found in the surgical samples, and also occurred in mouse esophageal epithelial cell layers under an ischemic condition. Identification of clinically significant subgroups was thought to be disrupted by the disorder of the expression profile through this aiEMT.

Conclusion and Significance: This study will evoke the fundamental misinterpretation including underestimation of the prognostic evaluation power of markers by overestimation of EMT in past cancer research, and will furnish some advice for the near future as follows: 1) Understanding how long the tissues were under an ischemic condition. 2) Prevalence of biopsy samples for *in vivo* expression profiling with low biases on basic and clinical research. 3) Checking cancer cell contents and normal- or necrotic-tissue contamination in biopsy samples for prevalence.

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Introduction

Cancer is a major cause of human deaths in many countries. Gene expression profiles from DNA microarrays are individualized and useful in the diagnosis and prognosis of diseases [1]. Although some artificial factors such as ischemia, hypoxia, hyponutrition, and cold stress possibly occur during surgical resection and sample transportation (Figure S1), surgical samples have long been used as important subjects for clinical and basic cancer research. In accordance with an increase of neoadjuvant therapy (in head and neck, esophageal, lung, pancreatic, prostate, and breast cancers), biopsy samples have recently become imperative for cancer transcriptome. On the other hand, both biopsy and surgical samples are available for expression profiling for predicting clinical outcome by adjuvant therapy (in stomach, colon, liver, bladder, pancreatic, brain, kidney, ovarian, cervical, and breast cancers). The targets for microarray analysis were, for the last ten years, mostly surgical samplesfrom the development and prevalence of two types of microarray: oligonucleotide [2, 3] and cDNA [4, 5]. However, whether a huge number of accumulated surgical sample expression profiles are useful for prediction by the use of biopsy samples from pretreated patients is still unclear, because little has been done about comparative gene expression profiling between the two kinds of samples.

Chemoradiotherapy (CRT) followed by surgery is the standard therapy for esophageal cancer in Western countries. In Japan,

neoadjuvant chemotherapy followed by surgery and definitive CRT are the standard therapies [6], and for locally advanced esophageal cancers (Stage II or III), surgery was the standard therapy there approximately 5 years ago [7]. This enables us to obtain both biopsy and surgical samples from esophageal cancer patients and to compare gene expression profiles between these two kinds of samples. Here we report that artificially induced epithelial-mesenchymal transition (aiEMT) occurs in surgical samples. Its presence there has possibly interfered not only with microarray- or immunohistochemistory-based clinical research but also with basic research.

Results

Comparison of Expression Profiles between Biopsy and Surgically Resected Esophageal Tumor Samples Obtained from Different Cases

We first compared gene expression profiles between 35 fresh biopsy samples containing no necrotic lesion and 66 surgical esophageal tumor samples, which were obtained from a margin of the tumor after exposure for 4–7 hours under an ischemic condition, by unsupervised clustering with 3,126 processed genes (Materials and Methods). There was no significant difference in clinical or pathological stage distribution between these two sets of esophageal cancers because locally advanced tumors (Stage II or III) are major targets of both chemoradiotherapy and surgery [8–10]. Sixty of the 66 surgical samples (90.9%) and 29 of the 35 biopsy samples (82.9%) appeared in a (left) and b (right) sample cluster, respectively (Figure 1A). To investigate the number of differentially expressed genes between these two kinds of samples with reproducibility, we compared expression profiles among three independent sample sets (A, B, and C): another 20 biopsy sample set versus three surgical sample sets (A, B, and C) containing 20 randomly selected cases from the 66 cases (Figure 1B, upper). The number of differentially expressed genes selected by u-test ($p<0.01$) were 2, 295, 2,328, and 2, 245 in sets A, B, and C, respectively. Among these 3 sets, 1,495 genes (65.1% in A, 64.2% in B, and 66.6% in C) were commonly identified (Figure 1B, upper). Therefore, more than 20% (1,495/6,000, 24.9%) of the genes were differentially expressed between biopsy and surgical samples because the average number of detectable genes in each case was approximately 6,000. These results suggested that a large difference exists between the biopsy and surgical samples.

From the 1,495 genes, we further selected differentially expressed genes among the 3 sets that had a 3-fold change between two average signal intensities of each gene between the biopsy and surgical samples. From sets A, B, and C, 297, 273, and 300 genes were identified, respectively (Figure 1B, lower). More than 80% of these genes were over-expressed in the surgical samples, suggesting a preferential presence of artificial factors or a contamination of normal portions.

To address the rationale for the difference, we finally selected genes that expressed preferentially in all the 35 biopsy or 66 surgical samples under stringent conditions with u-test ($p<0.01$), permutation test, and a 2-fold change, etc. (Materials and Methods). By this procedure, 38 and 219 genes were identified as up-regulated genes in the biopsy and surgical samples, respectively (Table S1 and Figure 1C). Interestingly, in the surgical samples, many EMT markers were found to be expressed preferentially and frequently. Microarray results of 13 representative EMT markers including fibronectin (FN), vimentin (VIM) and collagens (COLs) are shown in Figure 2A. Moreover, membrane signal transducers such as cytokine, chemokine, and receptors were also found to be up-regulated in the surgical samples. Representative microarray and RT-PCR results of *IL8*, *CXCR4*, *CXCL9*, *PDGFRB*, *CCL5*, and *TLR2*, respectively are shown in Figures 2B and 2C. In correspondence with EMT, E-cadherin (*CDH1*) was found to be down-regulated in the surgical samples (Figure 2A, right lowest).

Comparison of Expression Profiles between Biopsy Samples and Surgical Resected Esophageal Tumor Samples Obtained from Identical Cases

In the same above way, we compared gene expression profiles between 18 biopsy and 18 surgically resected esophageal tumor samples, and selected 41 and 716 genes that were identified as up-regulated genes in the two kinds of samples, respectively (Table S2 and Figure 3). In accordance with the above results from different cases, many EMT markers and membrane signal transducers were also found to be up-regulated frequently in the surgical samples (Figure 4A). More importantly, two EMT regulators, *ZEB1* and *ZEB2*, and some EMT-related myogenic transcription factors including *MEOX2* and *MEF2C* were able to be selected as up-regulated genes in the surgical samples (Figures 4A). Quantitative real-time RT-PCR confirmed over-expression of *ZEB1*, *ZEB2*, *FN*, *and VIM* in the 18 surgical samples of identical cases (Figure 4B). The over-expression of *ZEB1* and *ZEB2* was also found in the 66 surgical samples of different cases (Figure S2), although these two EMT regulators could not be extracted from expression profiles under the above stringent conditions. SNAI1/Snail, SNAI2/Slug, ZEB1/ZFHX1A, ZEB2/SIP1/ZFHX1B, TWIST1/TWIST, and TWIST2 are representative EMT regulators [11, 12]. Among them, *TWIST1* as well as two *ZEBs* were over-expressed in the two sets of esophageal tumors (Figure S3). To investigate whether aiEMT in the mRNA levels affects immunohistochemistry (IHC), we performed IHC on a typical mesenchymal marker vimentin in biopsy and surgical samples of identical cases. First we determined conditions under which normal epithelial cell layers could not be stained, but tumor cells with EMT could be (Figures 5A, 5B), because undifferentiated layers (basal and parabasal) have been reported to express EMT-related genes including *VIM* [4]. In 3 out of 5 pairs of the samples examined, tumor lesions of a surgical sample were found to be stained more highly than those of a biopsy sample (Figures 5C–H); however, the remaining 2 pairs did not show such difference (data not shown). Therefore, the aiEMT that occurred in the surgical samples in the mRNA level was thought to affect only a subset of surgical samples in the level of EMT-related proteins.

Over-expression of *ZEB1*, *ZEB2*, and *TWIST1* in Surgically Resected Normal Tissues

We obtained 4 biopsy samples and 5 surgical samples of non-cancerous tissues, and compared their expression profiles. In the same manner with the above expression profiles of tumor tissues (Figures 2, 4, S2, and S3), three EMT regulators (*ZEB1*, *ZEB2*, and *TWIST1*) and two typical EMT markers (*VIM* and *FN*) were found to be over-expressed in the 5 surgical samples (Figure 6A). Our previous report showing the involvement of ZEB2 and TWIST1 in the EMT of normal and malignant esophageal epithelial cells [9] supports the presence there of artificially induced EMT.

Finally, to investigate whether these 5 genes are induced in epithelial cells by surgical resection-related ischemia, we resected a mouse esophagus, and placed it on PBS for 0 or 4 hours, and immediately made frozen sections followed by laser-captured microdissection of the epithelial cell layers (Figure 6B, upper). Expression profiles of the mouse epithelial cell layers at 0 or