

Effects of photodynamic therapy for superficial esophageal squamous cell carcinoma *in vivo* and *in vitro*

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Abstract. Photodynamic therapy (PDT) is an ablative treatment leading to intracellular photoexcitation and injury. A total of 15 patients with superficial esophageal squamous cell carcinoma (ESCC) without metastasis underwent PDT and 48-72 h after intravenous Photofrin, the patients were treated with a 630-nm excimer dye laser. A total of 13 patients had local tumor recurrence after definitive chemoradiotherapy (CRT) consisting of 5-fluorouracil (5-FU) and cisplatin (CDDP). Of 6 patients, 5 had submucosal ESCC and were treated with S-1. Complete response was achieved by 11 patients with initial PDT, but 2 had recurrences. The recurrent/residual tumors were successfully treated with repeated PDT. Two patients with intramucosal ESCC succumbed due to metastatic disease, but 11 patients were disease-free. The 5 patients treated with S-1 remained alive despite submucosal ESCC. PDT was applied to human ESCC cells *in vitro* in the presence or absence of 5-FU or CDDP. The combination of PDT with 5-FU or CDDP resulted in enhanced cytotoxic effects, thereby reducing the effective dosage of each drug. PDT is a promising treatment option for selected ESCC cases, particularly for local recurrence following CRT. Our experience suggests that PDT is more effective when combined with chemotherapy.

Introduction

Definitive chemoradiotherapy (CRT) is considered to be an alternative, standard, non-surgical treatment for esophageal cancer, since it shows comparable clinical outcomes to esophagectomy

(1,2). However, persistent or recurrent locoregional disease commonly occurs after CRT and remains an unresolved issue (1,2). The survival of patients who do not achieve complete response (CR) is dismal (3). Therefore, improvement of local control is one of the major factors for better survival of esophageal cancer patients who are treated with CRT. Salvage esophagectomy has been a curative treatment of choice, but it is a more difficult procedure than primary esophagectomy, and the postoperative mortality rates are relatively higher (4). Moreover, there are no curative chemotherapy protocols currently available for the treatment of residual esophageal tumors. Again, salvage endoscopic mucosal resection (EMR) is used for locoregional disease without distant metastasis primarily or following CRT failure (5). Nevertheless, EMR has limitations with respect to resection size and is difficult to perform in cases with fibrous scar lesions (6,7). Endoscopic submucosal dissection (ESD) allows for the removal of larger gastrointestinal tumors *en bloc*, but advanced skill is required. ESD is also associated with a substantial risk of complications (8,9).

Photodynamic therapy (PDT) is an ablative treatment for rapidly proliferating tissues, including dysplastic and malignant lesions (1). It involves administration of a photosensitizing drug followed by the application of a specific wavelength of light, leading to intracellular photoexcitation and injury (11). Theoretically, PDT can cure gastrointestinal neoplasms contained within the submucosal layer (10,12,13). It is a highly effective, painless, and usually well-tolerated procedure that is simple to perform (10,13). Unlike resection, PDT has the advantage of preserving the integrity of the esophagus. This study aimed to investigate PDT as definitive management for non-metastatic superficial esophageal squamous cell carcinoma (ESCC). In addition, the *in vitro* study and our case series suggest that PDT is more effective for ESCC when combined with chemotherapy, such as fluorouracil.

Patients and methods

Clinical setting of PDT. Between April 2007 and March 2010, 15 patients with ESCC were treated by PDT at Nagasaki

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University Hospital. Although all persistent or recurrent tumors were surgically resectable, the decision to undergo non-surgical treatment was based on the patients' refusal of surgery or severe concomitant disease. The criteria for PDT were: i) lack of detection of lymph node or distant metastases; ii) the ESCC tumor invasion was within the mucosal and/or submucosal layer on endoscopic ultrasonography; iii) other non-surgical treatments including EMR and ESD were not indicated for reasons of difficulty or non-curability; and iv) written informed consent was obtained from each patient. A total of 13 patients had CRs with CRT, but the tumor was recurrent at the primary site. In addition, there were 2 naïve cases of ESCC. The CRT consisted of 60 Gy irradiation, along with 2 cycles of continuous infusion with 5-fluorouracil (5-FU) and cisplatin (CDDP). 5-FU (700 mg/m², 24-h intravenous infusion) was administered on days 1 to 4. CDDP (70 mg/m², 2-h intravenous infusion) was administered with hydration on day 1. This schedule was repeated twice, every 4 weeks. Radiotherapy was initiated concurrently on the first day of the first and second course of chemotherapy and was delivered in 30 fractions of 2 Gy for a total of 60 Gy. In addition, 2 courses of the same chemotherapy were added. The definition of CR after CRT was: i) disappearance of the tumor lesion or ulcer of the primary site with confirmed cancer-negative histology, and ii) disappearance of measurable or assessable metastatic lesions confirmed on computed tomography (CT) (14).

The PDT procedure began with an intravenous administration of 2 mg/kg of Photofrin (Wyeth, Tokyo, Japan), followed by dye laser irradiation. The 630-nm wavelength laser beam was provided by an excimer dye laser (EDL-1; Hamamatsu Photonics, Hamamatsu, Japan). The laser treatment was performed 48 and/or 72 h after injection of the photosensitizer. The laser was delivered via a free-cut fiber introduced into the operative channel of the fiberscope. The distal tip of the fiber was kept ~1 cm from the surface of the lesion. The total light density was 80 J/cm², with a 4-mJ/pulse maximum pulse energy and a 40-Hz pulse frequency. All of the patients were instructed to avoid direct exposure to sunlight for 4 weeks after the injection. Endoscopic examination with biopsy was repeated 7 days later, at 1, 3, 6 and 12 months after PDT, and then annually. The effectiveness of PDT was classified as CR when there was no macroscopic or microscopic evidence of ESCC, or non-CR when a tumor was observed at endoscopy and confirmed histologically. Local recurrence was defined as a relapse after achieving CR (14). Cervical/thoracic/abdominal CT was performed at 3, 6 and 12 months after PDT and then annually. Blood samples were obtained from each patient before and 7 days after PDT for measurement of serum total reactive oxygen species (ROS) to monitor whether the total ROS values could predict the efficacy of PDT (15).

For patients with submucosal ESCC, 50 mg of S-1 (Taiho Pharmaceutical, Tokyo, Japan), an oral fluorouracil, was administered twice daily for 28 consecutive days, followed by 14 days of rest for 12 months. S-1 consists of a 1:0.4:1 molar ratio mixture of tegafur and two modulating substances: gimeracil (5-chloro-2,4-dihydroxypyrimidine; CDHP) and oteracil (potassium oxonate) (16).

In vitro study. The cytotoxic effect of combination treatment with PDT and 5-FU or CDDP on a human ESCC cell line,

OE21, was investigated. OE21 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in RPMI-1640 (Nissui Ceutical, Tokyo, Japan) with 10% fetal bovine serum, glutamine (0.6 mg/ml), penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37°C in a humidified atmosphere of 5% CO₂ in air. OE21 cells were exposed to 5-FU (Sigma Aldrich, St. Louis, MO, USA) or CDDP (Nippon Kayaku, Tokyo, Japan) at various concentrations for 24 h to set the half maximal (50%) inhibitory concentration (IC₅₀) for each chemotherapy drug. Subsequently, the cells were exposed to Photofrin at various concentrations in the presence of CDDP or 5-FU at the selected IC₅₀ for each drug for 24 h, followed by irradiation with an Nd:YAG-pumped dye laser (630 nm, 5.0 J/cm²) (17) (Quanta-Ray® DCR-3 and PDL-2, Spectra Physics, Mountain View, CA, USA).

Cellular survival was assessed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-H-tetrazolium (MTS) assay (Promega, Madison, WI, USA). OE21 cells were plated at a density of 3×10³ cells/well on 96-well plates and grown overnight. The assay was initiated 24 h later by adding 20 µl of MTS solution reagent to 100 µl of culture medium for each well. After incubation for 3 h at 37°C, the plates were read in a microplate autoreader (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 490 nm. The results were expressed as the mean optical density for selected paradigms performed in duplicate. All assays were set up in triplicate, and the results were expressed as the means ± standard deviation (SD). Statistical significance was determined by the unpaired Student's t-test using the statistical package StatView (Abacus Concepts, Inc., Berkeley, CA, USA). P<0.01 was considered to be significant.

Results

Clinical outcomes. Patient baseline characteristics prior to PDT are summarized in Table I. The median age was 71 years (range 54-86). There were 14 males and 1 female. Of the 15 patients, 9 had intramucosal ESCC, and the remaining patients had submucosally invasive ESCC. Clinical outcomes with PDT are summarized in Table II. The median total light dosage delivered was 600 J (range 280-1065). Patient tumors showed a response to PDT. CR was achieved in 11 (73%) of the 15 patients, but 2 patients experienced local recurrence after PDT. Thus, the 6 patients were treated again with PDT, and the recurrent/residual lesions were eliminated in all 6 cases. Initial PDT was successfully performed in 4 of the 6 submucosal ESCC cases (Fig. 1), while CR was achieved in 7 of the 9 mucosal tumor cases. Otherwise, no clinicopathological factors had a significant impact on PDT outcomes. At a median follow-up period of 23 months (range 4-35), 13 patients were alive, and 11 were disease-free. Two patients with intramucosal ESCC succumbed due to metastatic disease despite having no local recurrence in the irradiated esophagus. The 5 patients treated with S-1 were alive, and 3 were disease-free, although the median follow-up period was 8 months. One patient with submucosal ESCC was unable to commence chemotherapy treatment due to sustained bone marrow suppression following CRT.

No significant difference was found in pretreatment serum total ROS levels between the CR (156.3±36.2 units) and non-CR groups (177.0±28.0 units) at the initial PDT. No significant

Table I. Background characteristics of the patients with superficial esophageal squamous carcinoma treated by photodynamic therapy (PDT).

Case no.	Age	Gender	Esophageal location	Endoscopic findings	Invasive depth	Chemoradiation
1	73	M	Thorax	IIc	sm	Recurrence
2	54	M	Thorax	III	sm	Recurrence
3	85	M	Thorax	IIc	m	Recurrence
4	71	M	Cervix	IIb	m	Recurrence
5	75	M	Cervix	IIb	m	Recurrence
6	69	M	Abdomen	IIa	sm	Recurrence
7	59	M	Thorax	IIb	m	Recurrence
8	76	M	Abdomen	IIc	m	Recurrence
9	79	M	Thorax	I	sm	Recurrence
10	69	M	Thorax	IIc	m	Recurrence
11	70	M	Thorax	IIb	m	Recurrence
12	59	M	Thorax	IIb	m	Naïve
13	60	F	Cervix	IIb	m	Recurrence
14	86	M	Thorax	IIc	sm	Recurrence
15	76	M	Thorax	IIc	sm	Naïve

sm, submucosal; m, mucosal; M, male; F, female.

Table II. Clinical outcomes of photodynamic therapy.

Case no.	PDT dose (J)	Complications	Response to initial PDT	Repeated PDT	Chemotherapy with PDT	Follow-up (months)	Prognosis
1	400	High-grade fever, chest pain	CR			35	Alive
2	480	Chest pain	Non-CR	Performed	S-1	33	Alive
3	620	High-grade fever	Non-CR	Performed		33	Alive
4	940	High-grade fever	CR			31	Deceased
5	355	Esophageal stenosis	CR			30	Alive
6	1065	Skin phototoxicity	CR		S-1	28	Alive
7	520	Chest pain, high-grade fever	CR			23	Deceased
8	540	Chest pain, high-grade fever	CR			23	Alive
9	900	Esophageal stenosis	Non-CR	Performed	S-1	9	Alive
10	600	High-grade fever, mediastinitis	CR		S-1	8	Alive
11	400	Esophageal stenosis	Non-CR	Performed		8	Alive
12	1000	None	Recurrence after CR	Performed		7	Alive
13	280	None	CR			4	Alive
14	740	High-grade fever, mediastinitis	Recurrence after CR	Performed		4	Alive
15	860	None	CR		S-1	33	Alive

CR, complete response; PDT, photodynamic therapy.

difference was noted in the 7-day post-treatment total ROS values irrespective of the treatment outcome (205.2 ± 56.5 and 204.7 ± 27.2 units, respectively). When limited to patients who had successful PDT, the serum total ROS values were significantly increased from 156.3 ± 36.2 units before PDT to 205.2 ± 56.5 units after PDT ($p < 0.05$).

In all cases, Photofrin administration was well tolerated. There were no allergic reactions or injection site irritation. As

for acute complications within 7 days after PDT, high fever ($>38.0^\circ\text{C}$) and chest pain that required analgesic treatment were observed in 10 and 4 patients, respectively. A total of 6 patients experienced significant complications: 2 had mediastinitis; 3 had esophageal stenosis that required repeated endoscopic balloon dilation; and 1 had cutaneous phototoxicity. Each complication was successfully managed with medical treatment. No deaths were attributable to the PDT procedure itself.

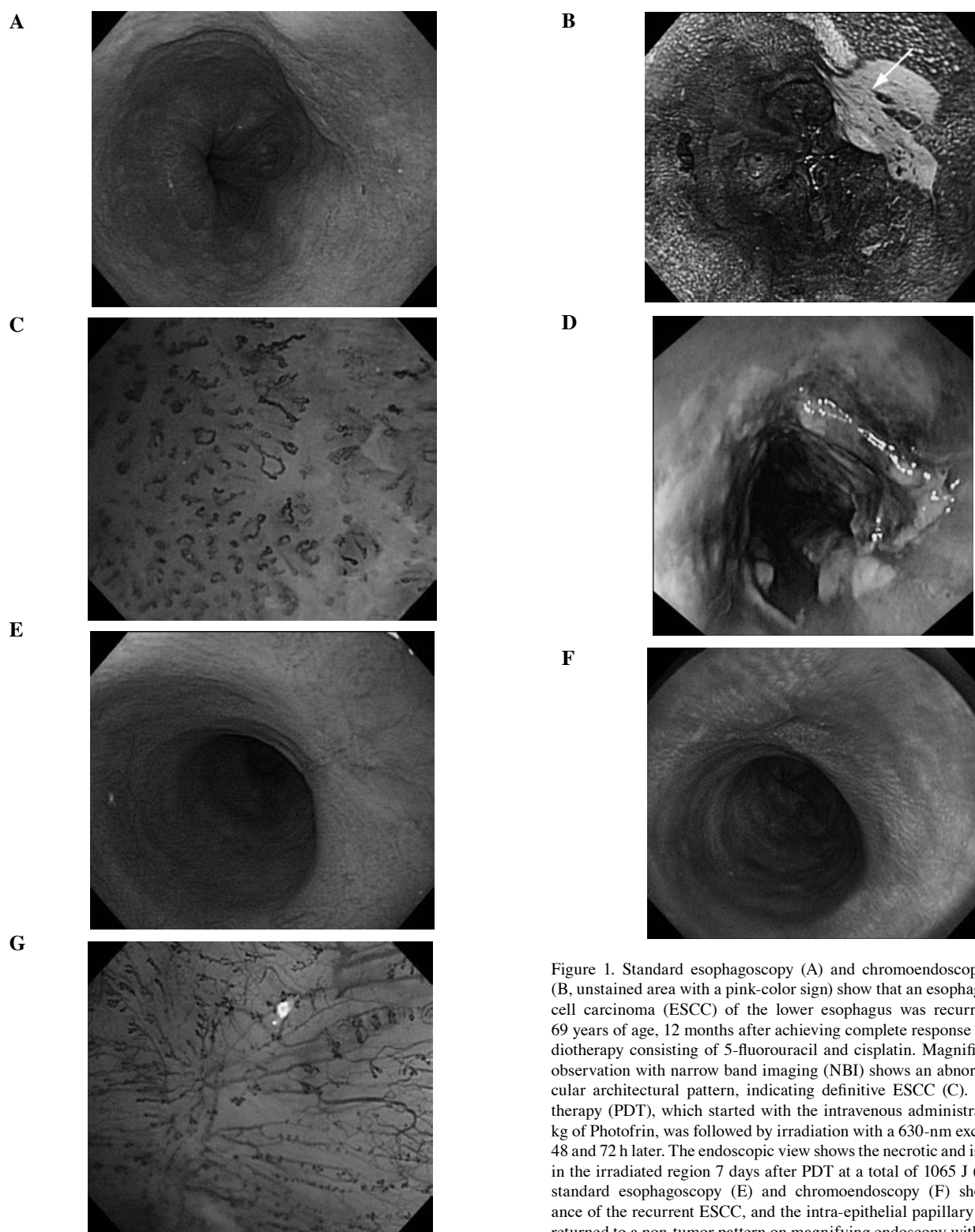


Figure 1. Standard esophagoscopy (A) and chromoendoscopy with iodine (B, unstained area with a pink-color sign) show that an esophageal squamous cell carcinoma (ESCC) of the lower esophagus was recurrent in a male 69 years of age, 12 months after achieving complete response with chemoradiotherapy consisting of 5-fluorouracil and cisplatin. Magnified endoscopic observation with narrow band imaging (NBI) shows an abnormal microvascular architectural pattern, indicating definitive ESCC (C). Photodynamic therapy (PDT), which started with the intravenous administration of 2 mg/kg of Photofrin, was followed by irradiation with a 630-nm excimer dye laser 48 and 72 h later. The endoscopic view shows the necrotic and ischemic injury in the irradiated region 7 days after PDT at a total of 1065 J (D). Follow-up standard esophagoscopy (E) and chromoendoscopy (F) show disappearance of the recurrent ESCC, and the intra-epithelial papillary capillary loop returned to a non-tumor pattern on magnifying endoscopy with NBI (G).

***In vitro* study.** The two chemotherapeutic agents substantially affected survival of the OE21 cells with the IC_{50} of single 5-FU and CDDP set at 3.5 and 9.0 μ M, respectively. PDT showed clear cytotoxic activity against the OE21 cells. To induce IC_{50} , a Photofrin concentration of 10 μ g/ml was required for this ESCC cell line at a laser power of 5 J/cm². Following the combination of 3.5 μ M of 5-FU with PDT using Photofrin at various concentrations, a significant synergistic

effect was observed with the IC_{50} of Photofrin at 0.83 μ g/ml for the same PDT setting. The IC_{50} at a laser power of 5 J/cm² decreased 3-fold (3.3 μ g/ml) in the presence of 9 μ M of CDDP. When PDT was combined with 10 μ g/ml of Photofrin at a laser power of 5 J/cm² with the two chemotherapeutic agents at various concentrations, the IC_{50} s were substantially reduced from 3.5 and 9.0 μ M to 0.75 and 1.2 μ M for CDDP and 5-FU, respectively.

Discussion

In the present study, 12 (80%) of the 15 patients with superficial ESCC achieved CR with Photofrin-mediated PDT. The group consisted of no less than 13 patients with recurrent ESCC after CRT. In a similar setting of ESCC cases, Yano *et al* reported that the CR rate was 62% (8 of 13 patients) by salvage PDT after CRT failure. In their study, the overall survival rate 1 year after salvage PDT was 68.4% (14). In this study, 13 of the 15 patients were alive, and 11 were disease-free at the median follow-up period of 23 months. Two patients succumbed due to metastatic disease although no local recurrence of ESCC was noted after PDT. Hattori *et al* reported that the overall survival rate of patients treated by salvage EMR for locoregional failure after CRT was 56% at 3 years (5). On the other hand, previous data showed that overall 3-year survival for patients with non-CR with definitive CRT was no more than 6% (3). Collectively, the results suggest that local treatment by endoscopic modalities such as EMR and PDT is a treatment option for superficial ESCC without metastasis.

From a technical point of view, PDT appears to be superior to EMR. When the corresponding lesion exhibits ulceration, severe fibrosis, or stenosis, salvage EMR is difficult or impossible to perform (6). Little information is available on clinical outcomes of ESD in the salvage setting, although ESD permits the removal of esophageal epithelial neoplasms *en bloc*, irrespective of size (7). Nevertheless, in ESCC cases with invasion in the submucosal layer, ESD/EMR cannot be used due to its lack of curative potential. Even in such cases, PDT may yield relatively high CR rates, as indicated by the present and previous data (14). Surgical resection has been considered a salvage modality for these patients. However, Swisher *et al* reported that patients treated with salvage esophagectomy had a significantly higher incidence of anastomotic leaks (39 vs. 7%) and a longer hospital stay (29 vs. 18 days) than those treated with planned esophagectomy (4). ESCC cases treated with CRT may occasionally be unresectable due to concomitant diseases (13). Thus, PDT offers an attractive alternative for patients with ESCC tumors without metastasis who would not otherwise be referred to a surgeon for treatment. Moreover, in selected patients with primary superficial ESCC, PDT as definitive therapy may avoid the risks associated with esophageal resection (13).

PDT was performed safely in the present and previous studies, and the majority of complications were manageable with medical treatment (13,14). However, it is of clinical relevance that life-threatening complications are rarely noted. An esophagotracheal fistula may develop with PDT even in naïve early esophageal cancer cases; the reported incidence is 6.5% (14). Similarly, severe mediastinitis and pericardial effusion were previously documented following salvage PDT (13,14). Possible reasons for the complications include radiation-induced esophageal damage and heart disease as well as transmural necrosis potentiated by PDT. Stricture formation at the irradiated esophageal site was common following PDT, occurring in 20-42% of cases. The majority of patients who underwent 3 or more PDT treatments developed a stricture requiring dilation, and the number of attempts varied (range 1-20, mean 2.7) (13).

Nevertheless, the indications for prophylactic pneumatic dilation warrant further evaluation.

Oral S-1, the 5-FU modulating drug, was administered to patients with submucosal ESCC with a potential risk of metastatic disease in the present study. The patients were still alive without significant complications despite their short-term follow-up. Keeley *et al* applied chemotherapy and/or radiation therapy in 16 (32%) of 50 patients with Barrett's high-grade dysplasia and esophageal cancer, varying from T1 to T3 tumor, to supplement primary PDT (13). Of the 16 patients who received concurrent chemoradiation, 6 remained alive at a mean interval of 16 months. Four of the patients who received PDT/chemo/radiation showed no evidence of disease at a mean interval of 13 months, and 2 were alive with disease. Conversely, all 10 patients succumbing to the disease had local recurrence. Due to superior survival and local control, esophagectomy may remain the preferred treatment for patients without physiological impairment. Nevertheless, the preliminary data on chemotherapy using the single fluorouracil along with PDT prompted us to investigate their combined effects *in vitro*. 5-FU and CDDP are widely used in chemotherapy regimens against various types of cancers. Combined chemotherapy consisting of the two key drugs has been a representative standard regimen against ESCC (19). Therefore, we focused on 5-FU and CDDP to enhance the cytotoxicity of Photofrin-mediated PDT in cellular experiments.

Upon activation by the special wavelength light, such Photofrin-derived photosensitizers undergo photochemical reactions to transfer electrons or hydrogen or to form singlet oxygen and generate excessive ROS (11,20). This leads to oxidative damage to proteins, lipids and DNA, resulting in apoptotic or necrotic cell death (11,20,21). In line with this theoretical concept, the patients who achieved CR had a significant increase in circulating ROS levels following PDT, which was not observed in non-CR cases. Our *in vitro* study showed that the combination of Photofrin-mediated PDT and 5-FU or CDDP resulted in a significantly lower cell survival than the single-mode treatment. Notably, with combined treatment, each IC₅₀ dosage was significantly decreased; in particular, much lower concentrations of Photofrin and 5-FU were required to obtain sufficient cell killing and vice versa. Promising results using similar or other chemotherapeutic drugs have been reported in combination with PDT (17,22,23). The synergistic effects of the two treatments may suggest diverse potential mechanisms for cellular death, one directly associated with PDT action and the other associated with each cytotoxic drug (17,22-24). Based on the *in vitro* results and our study, it is evident that more studies are warranted to determine the manner in which chemotherapeutic drugs and PDT interact and can be combined in order to result in increased cell killing with reduced side effects.

In conclusion, PDT demonstrated acceptable short-term outcomes, feasible curative properties, and safety for the treatment of superficial ESCC. Although further long-term follow-up studies are required, PDT is a promising treatment option for selected ESCC cases, particularly local recurrence following CRT. Combination therapy with PDT and 5-FU or CDDP may result in enhanced cytotoxic effects on ESCC, possibly reducing the effective dosage of each drug and decreasing side effects.

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