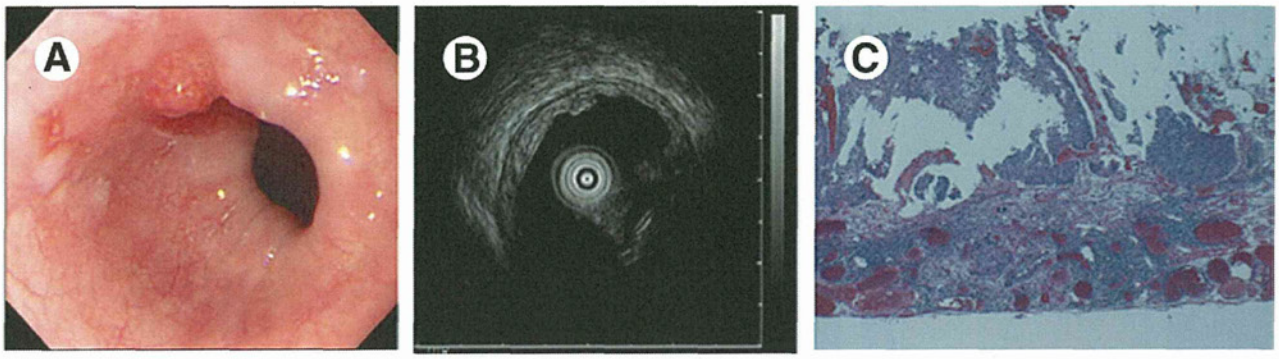

Figure 9 Superficial esophageal carcinoma (squamous cell carcinoma). (A) Conventional white light image shows a reddish area with nodular change. (B) EUS image demonstrates a low echoic mass located in the submucosal layer. (C) This superficial cancer was removed by endoscopic mucosal resection and submucosal 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.

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.

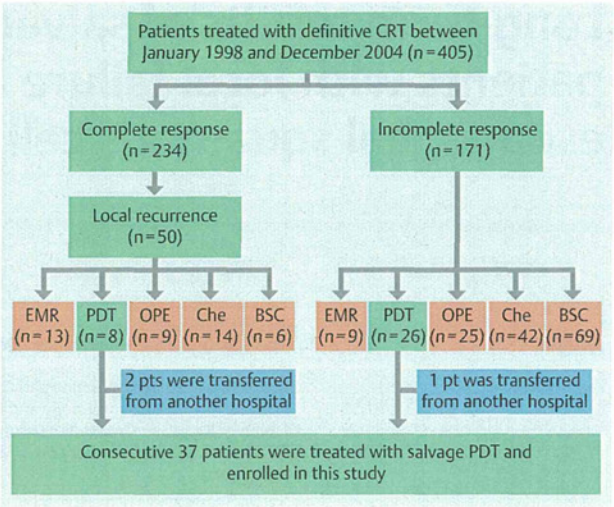

Fig. 1 Flow of patients through the study. CRT, chemoradiotherapy; EMR, endoscopic mucosal resection; PDT, photodynamic therapy; OPE, esophagectomy; Che, chemotherapy; BSC, best supportive care.

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.

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.

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).

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**.

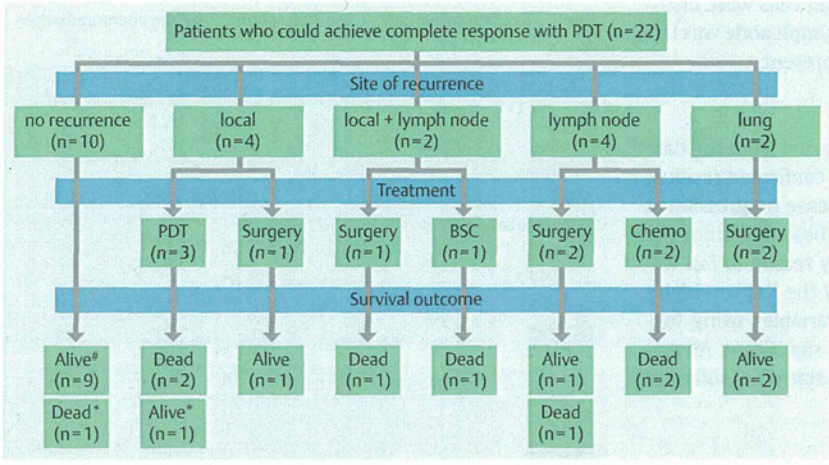

Fig. 2 The clinical flow chart of 22 patients in whom a complete response was achieved with salvage PDT. CR, complete response; PDT, photodynamic therapy; BSC, best supportive care; Chemo, chemotherapy; Dead*, dead from another disease; Alive#, alive with disease.

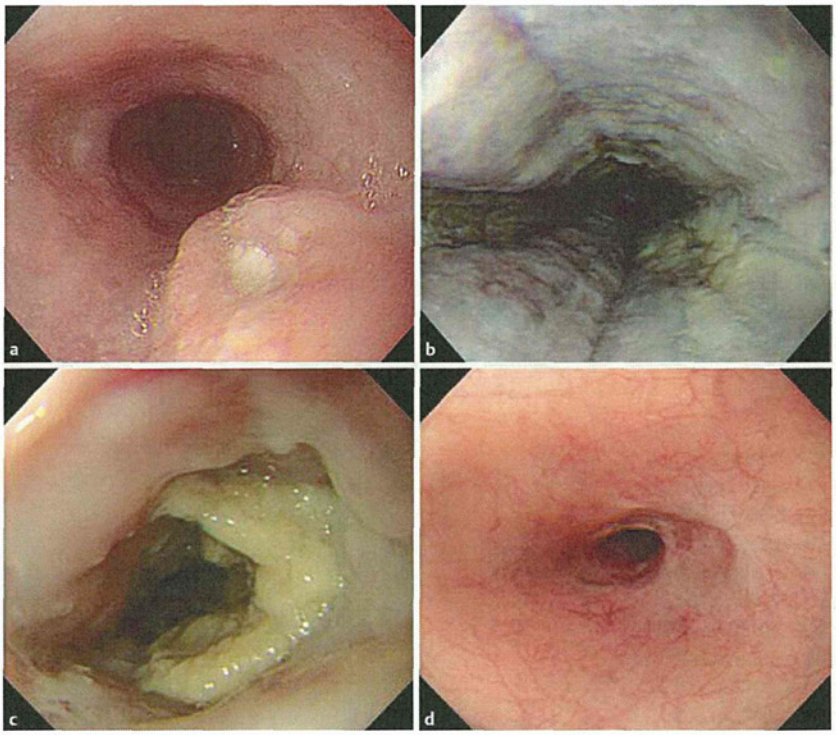

Fig. 3 A patient in whom complete response was achieved with salvage photodynamic therapy (PDT). **a** Local recurrence was detected after chemoradiotherapy and evaluated as uT1 with endoscopic ultrasound. **b** At 3 days after salvage PDT, circumferential ischemic change was observed. **c** At 1 month 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.

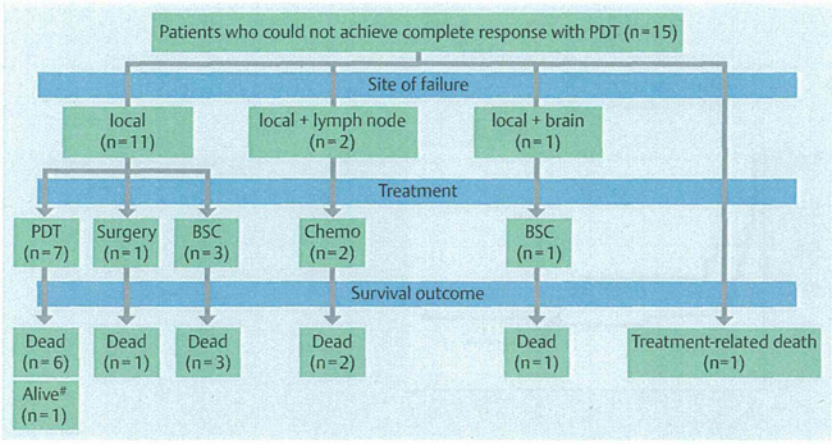

Fig. 4 Clinical flow chart of 15 patients in whom salvage photodynamic therapy did not achieve a complete response. CR, complete response; PDT, photodynamic therapy; BSC, best supportive care; Chemo, chemotherapy; Alive#, alive with disease.

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]; $P = 0.61$).

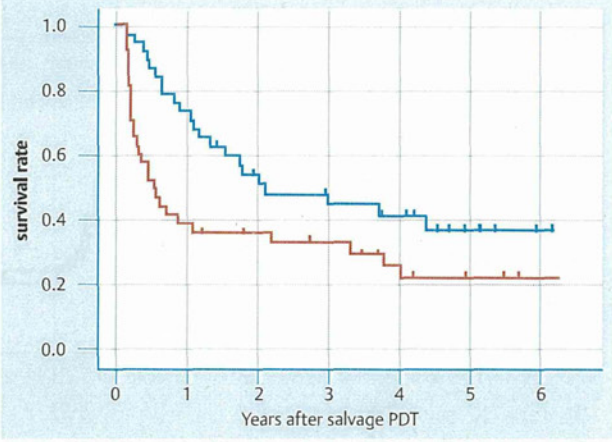

Fig. 5 Overall survival (blue line) and progression-free survival (red dotted line) of all 37 patients from the initiation of salvage photodynamic therapy (PDT).

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

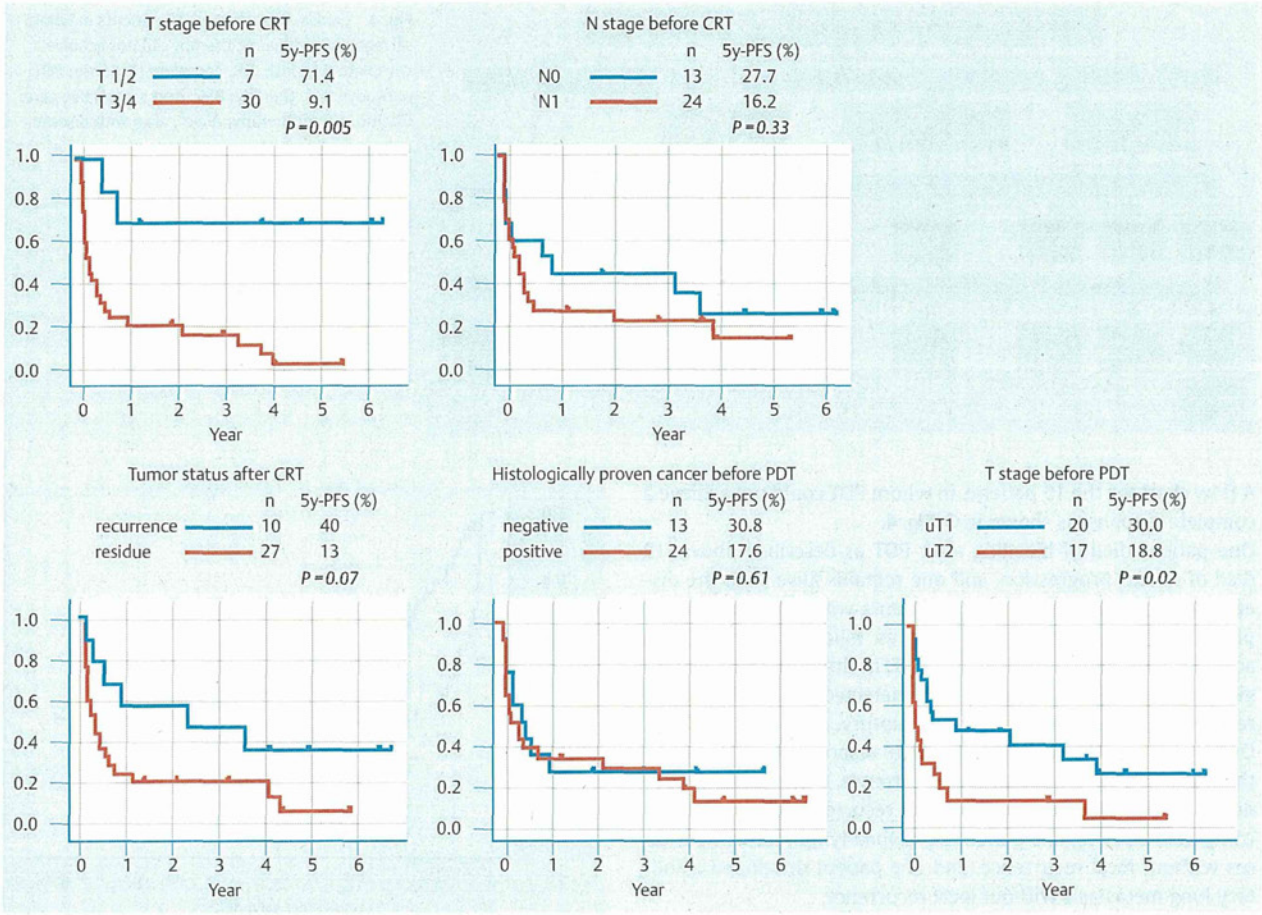

Fig. 6 Comparisons of progression-free survival curves according to various clinical variables before chemoradiotherapy and before photodynamic therapy.

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

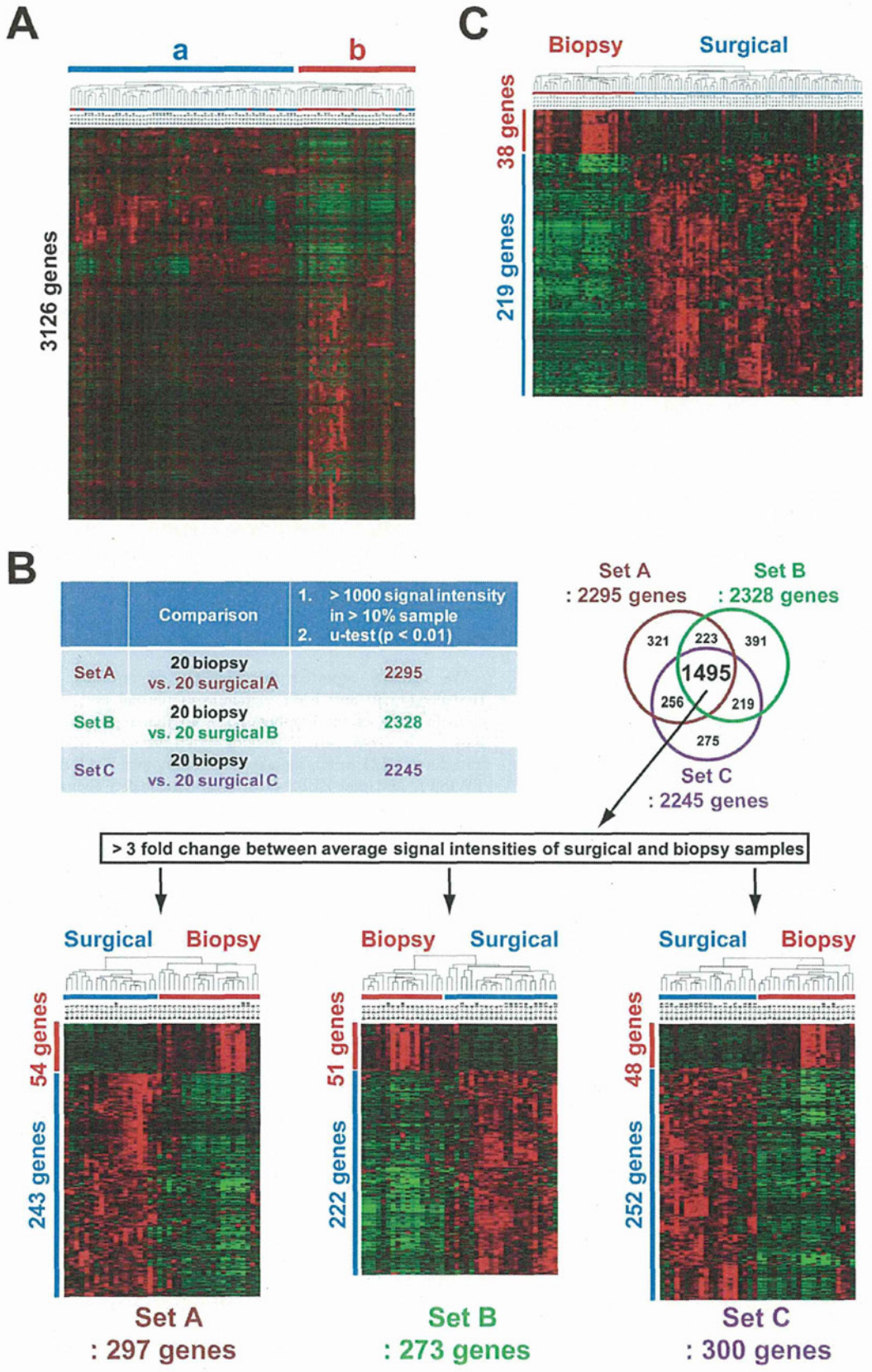
A
a
b
3126 genes
C
Biopsy
Surgical
38 genes
219 genes
B
Comparison
1. > 1000 signal intensity in > 10% sample
2. u-test (p < 0.01)
Set A
20 biopsy vs. 20 surgical A
2295
Set B
20 biopsy vs. 20 surgical B
2328
Set C
20 biopsy vs. 20 surgical C
2245
Set A : 2295 genes
Set B : 2328 genes
Set C : 2245 genes
321
223
391
1495
256
219
275
> 3 fold change between average signal intensities of surgical and biopsy samples
Surgical
Biopsy
54 genes
243 genes
Set A : 297 genes
Biopsy
Surgical
51 genes
222 genes
Set B : 273 genes
Surgical
Biopsy
48 genes
252 genes
Set C : 300 genes

Figure 1. Comparison of expression profiles between biopsy and surgically resected esophageal tumor samples obtained from different cases. (A) Unsupervised clustering with 3,126 processed genes. Surgical (a) and biopsy sample clusters (b) are shown. (B) Comparison of expression profiles among three independent sets (A, B, and C): a randomly selected 20-biopsy sample set versus three surgical sample sets (A, B, and C) containing 20 independent cases. The number of differentially expressed genes selected by u-test ($p<0.01$): 2, 295 in set A, 2,328 in set B, and 2, 245 in set C (Upper). The number of differentially expressed genes with a 3-fold change between two average signal intensities: 297, 273, and 300. Clustering results with these gene sets (Lower). (C) Up-regulated genes in surgical or biopsy samples. By the use of all of the profiles under stringent selection conditions (see Materials and Methods), 38 and 219 genes were identified as up-regulated genes in the biopsy and surgical samples, respectively.
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4 hours after resection revealed that mouse *Zeb1*, *Zeb2*, *Vim*, and *Fn* were induced 4 hours after resection (Figure 6B, lower). Quantitative real-time RT-PCR confirmed over-expression of *Zeb1*, *Zeb2*, *Vim*, and *Fn* after resection (Figure 6C). Since overall sensitivity of mouse affymetrix arrays is known to be lower than in humans, and the use of a small amount of RNA such as laser-captured subjects is also known to reduce microarray sensitivity, *Twist1* mRNA itself could not be detected in this mouse experiment (data not shown).

To investigate whether aiEMT in the mRNA levels affects IHC, we performed IHC on a typical mesenchymal marker vimentin 8 hours after resection. Here we determined conditions under which normal epithelial cell layers are stained. In all of the 3 independent samples examined, normal epithelial cell layers were not found to be stained highly 8 hours after resection (Figures 7A–C). The discrepancy between the mRNA level and protein level can be explained by the two following reasons: 1) although undifferentiated layers (basal and parabasal) have been reported to express EMT-related genes including *VIM* [4], their expression levels were much lower than tumor (Figure 5B). 2) it may also be difficult to show the approximate 2-fold change in the mRNA level (Figures 6B, C) as in the protein level by IHC, because IHC is inferior to RT-PCR in both sensitivity and quantification.

All of the results suggest that EMT, especially in the mRNA level, is induced artificially in both normal and malignant epithelial cells by surgical resection-related events (ischemia-induced hypoxia and hyponutrition, and hypoxia-induced inflammation, etc.).

Artificially Induced EMT (aiEMT) by Surgical Resection Prevents Microarray-based Subgroup Identification

Identification of clinically significant subgroups is very important for personalized medicine and for drug development against intractable cases. When we used the expression profiles of the 35 biopsy samples obtained from patients treated by chemoradiotherapy [8], unsupervised clustering with 5,570 processed genes (Materials and Methods) identified a good responder group consisting of 9 patients (7/9, 78% showing complete response to chemoradiotherapy) from the 35 (Figure 8A, left). However, when the profiles of the 66 surgical samples were used, unsupervised clustering with 2,016 processed genes could not identify any subgroup (Figure 8A, right). Thus, biopsy sample expression profiles seemed to be more effective in subgroup identification than those of the surgical samples. In fact, we previously reported that biopsy sample expression profiles could distinguish long-term or short-term survivors by definitive chemoradiotherapy [8]; however, surgical sample expression profiles never identified poor prognostic subgroups with extensive lymph node metastasis [10]. Moreover, in the surgical samples, EMT was accelerated in 36 (85.7%) out of 42 esophageal cancers [9]. This high percentage seems to be caused by aiEMT.

To address the reason why subgrouping is difficult in surgical samples, we compared the number and distribution of each of the processed genes, which were used for unsupervised clustering. We first selected genes with a signal intensity of more than 1,000 in more than 10% of the samples. From 35 biopsy and 66 surgical samples, 6,551 and 4,797 genes, respectively, were selected. From these genes, we finally selected more than 3-fold changed genes by comparing the average signal intensity of each gene in more than 10% of the samples. In the 35 biopsy samples, 85% (5,570) of 6,551 first processed genes remained, whereas the number of final processed genes decreased from 4,797 first processed genes to 2,016 (42%) (Figure 8B, upper). Of the 2,016 finally processed genes in the surgical samples, 1,724 (86%) were included in the 5,570 finally processed genes in the biopsy samples; however, 3,846 (69%) of 5,570 genes were unique to the biopsy samples (Figure 8B, lower). Moreover, frequency distribution (for percentage of samples) of these two finally processed-gene sets shows that approximately 60% of the 2,016 processed genes in the surgical samples express in only a limited number of cases (0–10%) (Figure 8C). Accordingly, aiEMT in surgical samples may diminish the number of processed genes useful for subgroup identification.

Discussion

We recently reported the presence of crosstalk between Hedgehog (Hh) and EMT signaling in normal and malignant epithelial cells of the esophagus [9]. In that report, *ZEB2* was shown to be a downstream gene of both a primary transcriptional transducer GLI1 in Hh signaling and of another EMT regulator, TWIST1, and that ZEB2 further up-regulated 5 chemokine or growth factor receptors, *PDGFRA*, *EDNRA*, *CXCR4*, *VEGFR2*, and *TRKB* (Figure S4). The Hh signal block inhibited esophageal keratinocyte differentiation and cancer cell invasion and growth. Accordingly, over-expression of *ZEB2* and *TWIST1* in surgical samples of both normal and tumor tissues can induce EMT, resulting in over-expression of representative EMT markers *VIM*, *FN*, and *COLs* (Figures 2, 4, 6, S2, and S3) and membrane signal transducers *IL8*, *CXCL4*, *CCL5*, *CXCR4*, *PDGFRB*, and *TLR2* (Figure 2). Over-expression of the membrane signal transducers can activate further down-stream cascades. This is a major reason for the large difference of expression profiles between biopsy and surgical samples (Figures 1 and 3).

Extensive contamination of normal mesenchymal portions in surgically resected tumor tissues can also explain the over-expression of those EMT regulators and EMT-related genes, even though trained pathologists carefully excised bulk tissue samples from the main tumor, leaving a clear margin from the surrounding normal tissue (Materials and Methods). However, the over-expression was also observed in surgically resected normal tissue and mouse epithelial cell layers 4 hours after resection (Figure 6). Therefore, we concluded that artificially induced EMT, termed aiEMT, occurred in both normal and malignant epithelial cells by the surgical resection-related events (ischemia-induced hypoxia, ischemia-induced hyponutrition, and hypoxia-induced inflammation, etc.) (Figure S1).

Recently, the hypoxia-inducible factors (HIF-1A or HIF-2A) have been reported to directly regulate TWIST1 [13, 14] and

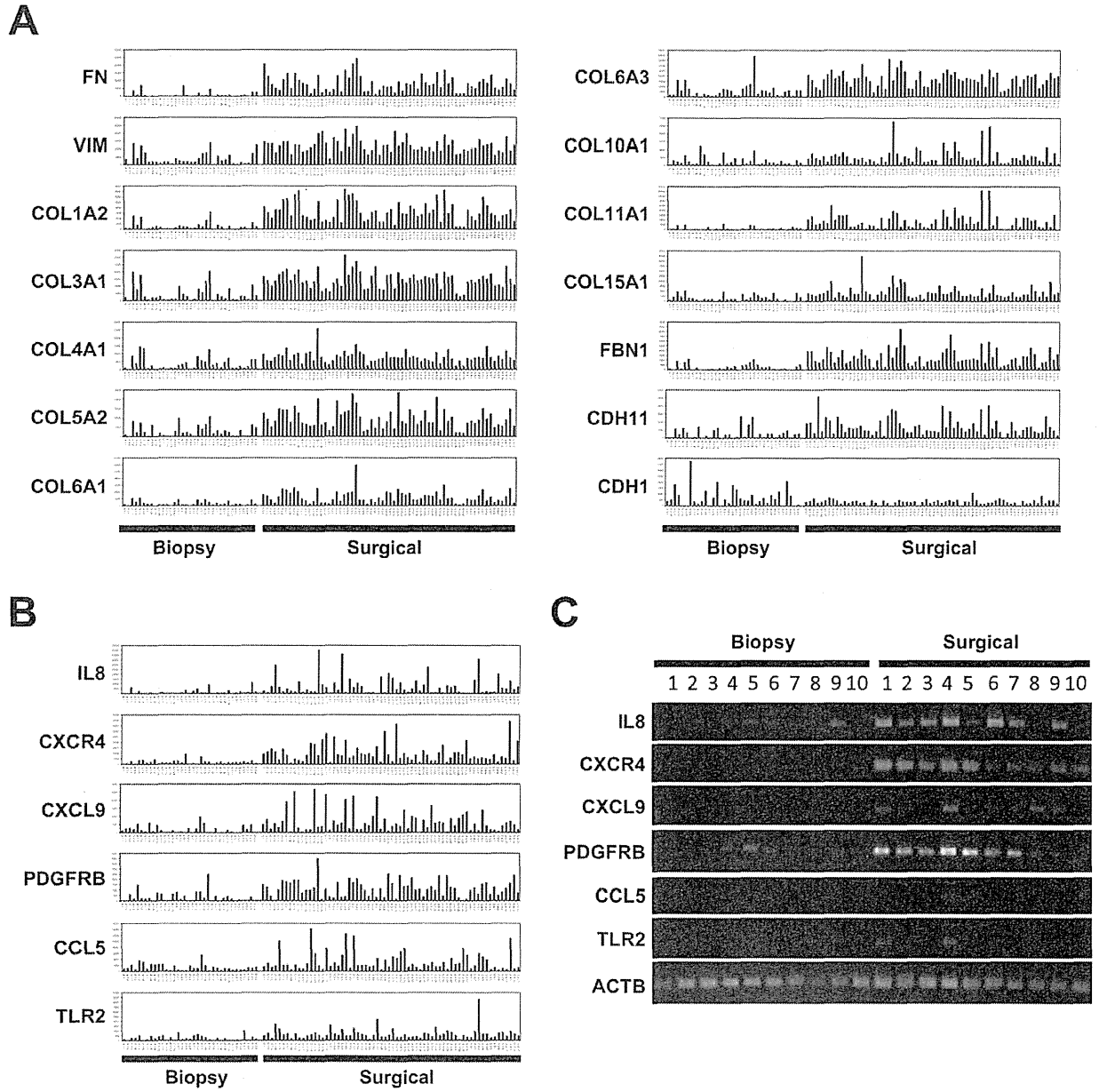

Figure 2. Representative EMT related genes over-expressed in surgically resected esophageal tumor samples. (A) Expression patterns of an epithelial cell marker E-cadherin (*CDH1*) and typical EMT markers including fibronectin (*FN*), vimentin (*VIM*), and collagens (*COLs*). (B) Expression patterns of 6 membrane signal transducers: a cytokine (*IL8*), two chemokines (*CXCL9* and *CCL5*), and three membrane type receptors (*CXCR4*, *PDGFRB*, and *TLR2*). (C) Semi-quantitative RT-PCR results of these 6 membrane transducers in representative samples.
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LOXL2, which reportedly stabilized an EMT regulator, SNAI1/SNAIL, through physical interaction on the SLUG domain and Snail's lysine residues K98 and K137 [15]. The SNAI1 binding site was also found in the 5' promoter region of *ZEB2* [16]. Over-expression of both *HIF1A* and *LOXL2* was observed only in the surgically resected tumor tissues obtained from different cases (Figure S5). Moreover, other *HIF1* families (*HIF1B* and *HIF2A*) were never over-expressed in any of the surgical samples. Therefore, elucidation of the molecular mechanisms of aiEMT in surgical samples remains for future studies. However, we noted that ischemia-induced hypoxia and/or inflammation has been reported to release repression of NFκB [17], which regulates *ZEB1*, *ZEB2*, and *TWIST1* [18, 19] and that TGF-β signaling may be involved in aiEMT, because over-expression of *NFKB1* and *TGFBR2* was found in surgical samples (Figure S6).

As mentioned in the Introduction, surgical samples have been used as important subjects for clinical and basic cancer research for many years. Therefore, aiEMT in surgical samples may have possibly interfered with or prevented not only microarray- or immunohistochemistry-based clinical research (diagnostic marker

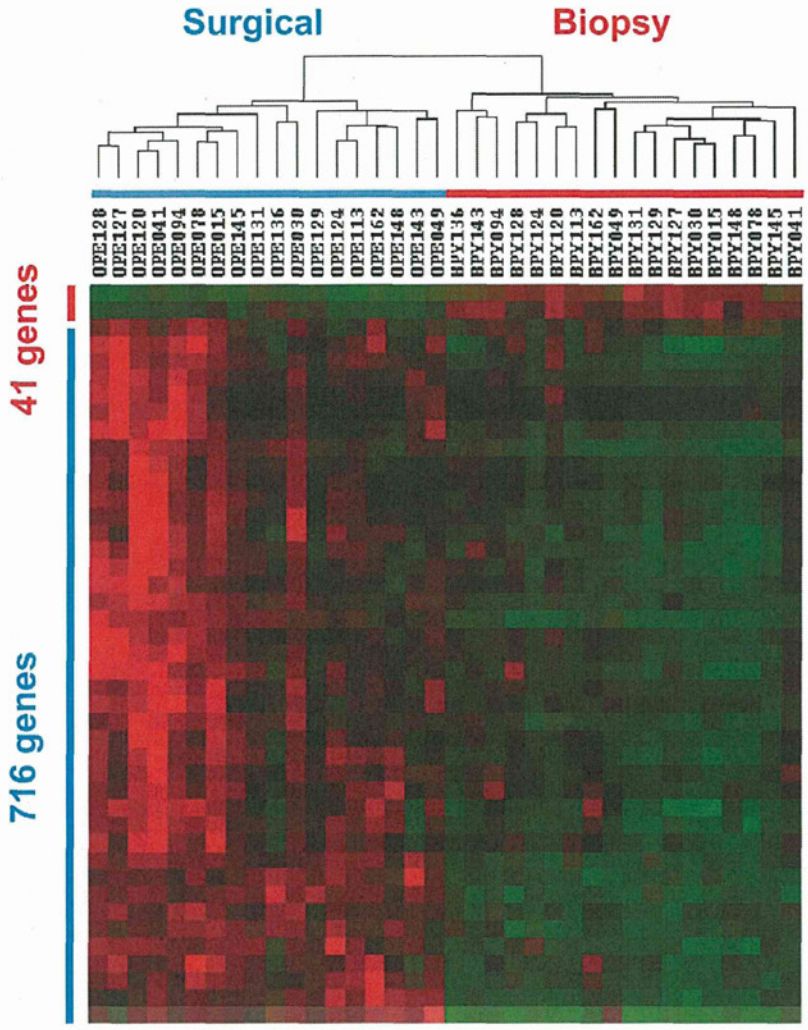

Figure 3. Up-regulated genes in biopsy and surgically resected esophageal tumor samples obtained from identical cases. By stringent selection (see Materials and Methods), 41 and 716 genes were identified as up-regulated genes in the biopsy and surgical samples, respectively.
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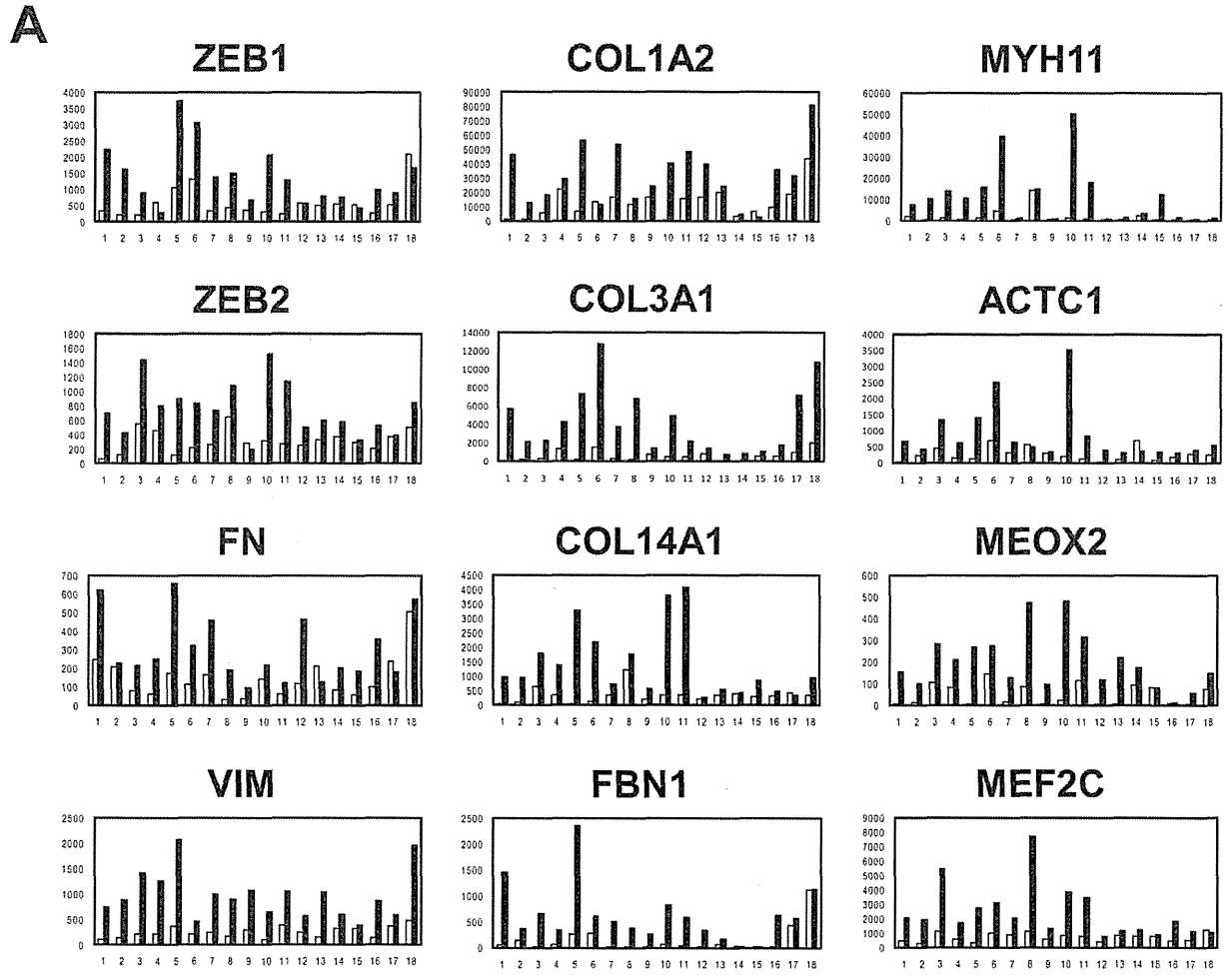
identification, subgrouping, making predictors, and prognosis evaluation, etc.) but also basic research (making a signal pathway map, therapeutic target identification, etc.). This study will likely evoke fundamental misinterpretation including underestimation of the prognostic evaluation power of markers by overestimation of EMT in past cancer research, and will provide some advice for the near future as follows: 1) Understanding how long the tissues were under an ischemic condition (from start of resection to stock or RNA preparation). The total amount of time should never exceed 4 hours. 2) Prevalence of biopsy samples for *in vivo* expression profiling with low biases on basic and clinical research; for example, for clinical outcome prediction of not only neoadjuvant but also adjuvant chemotherapy, radiotherapy, and chemoradiotherapy such as in previous reports [8, 20–23]. 3) Checking cancer cell contents and normal- or necrotic-tissue contamination in biopsy samples for the prevalence. In sampling by a needle biopsy, tumor portions (2mm X 2mm) should be obtained from a margin (periphery) of the tumor by exclusion of central necrotic lesions under endoscopy. If necrotic lesions were severely contaminated in the samples, those samples should be excluded by quantifying and qualifying RNA. If the samples contained extensive normal lesions, such samples can be excluded by the expression profile-based scoring method using normal and/or tumor specific genes.

Materials and Methods

Tissue Samples

All esophageal cancer (squamous cell carcinomas) and non-cancerous tissues were provided by the Central Hospital or East Hospital at the National Cancer Center after obtaining written informed consent from each patient and approval by the Center's Ethics Committee.

All surgical samples were obtained from patients without neoadjuvant therapy, and all biopsy samples were obtained before treatment. For the surgical samples, trained pathologists carefully excised bulk tissue samples from the main tumor, leaving a clear


A
ZEB1
COL1A2
MYH11
ZEB2
COL3A1
ACTC1
FN
COL14A1
MEOX2
VIM
FBN1
MEF2C

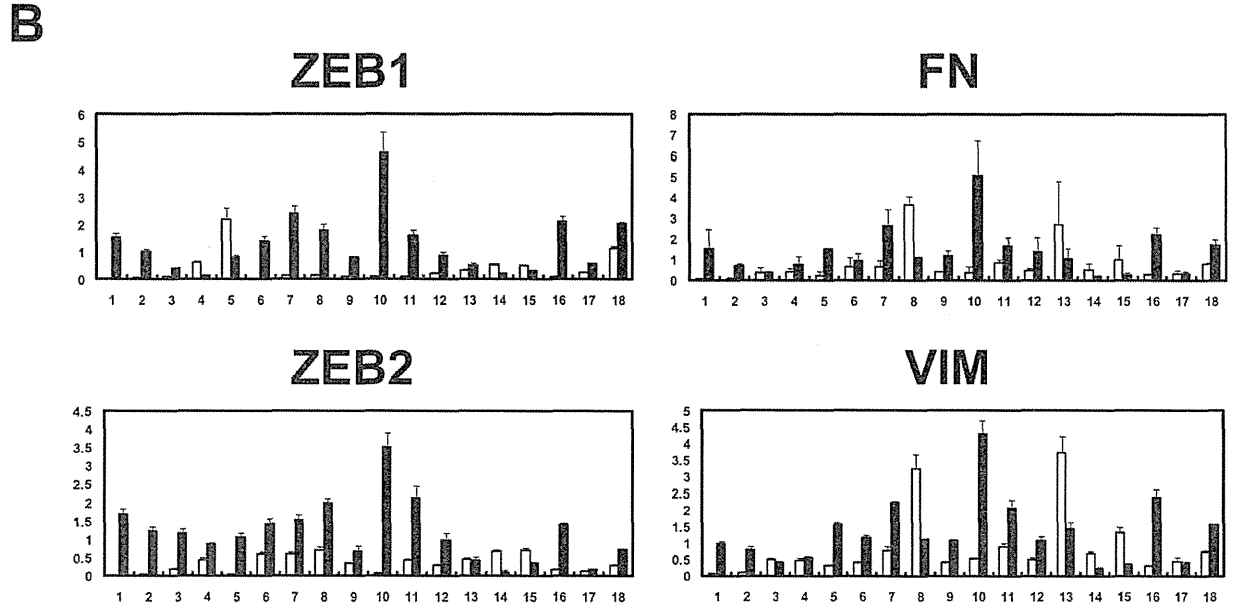

B
ZEB1
FN
ZEB2
VIM

Figure 4. Representative EMT related genes also over-expressed in surgically resected esophageal tumor samples obtained from identical cases. (A) Expression patterns of 2 representative EMT regulators (*ZEB1* and *ZEB2*), 8 typical EMT markers including fibronectin (*FN*), vimentin (*VIM*), 3 collagens (*COL1A2*, *COL3A1*, and *COL14A1*), *FBN1*, *MYH11*, and *ACTC1*, and 2 EMT-related myogenic transcription factors (*MEOX2* and *MEF2C*). (B) Quantitative real-time RT-PCR results of *ZEB1, ZEB2, FN, and VIM*. Closed box: surgical sample; Open box: biopsy sample.
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margin from the surrounding normal tissue. Thus, we obtained surgical samples from a margin (periphery) of the tumor. For the needle biopsy samples, tumor portions (2 mm X 2 mm) were obtained under endoscopy from a margin of the tumor by exclusion of any central necrotic lesions. If the samples were severely contaminated by necrotic lesions, those samples were excluded by quantifying and qualifying RNA. If the samples contained extensive normal lesions, we excluded such samples by

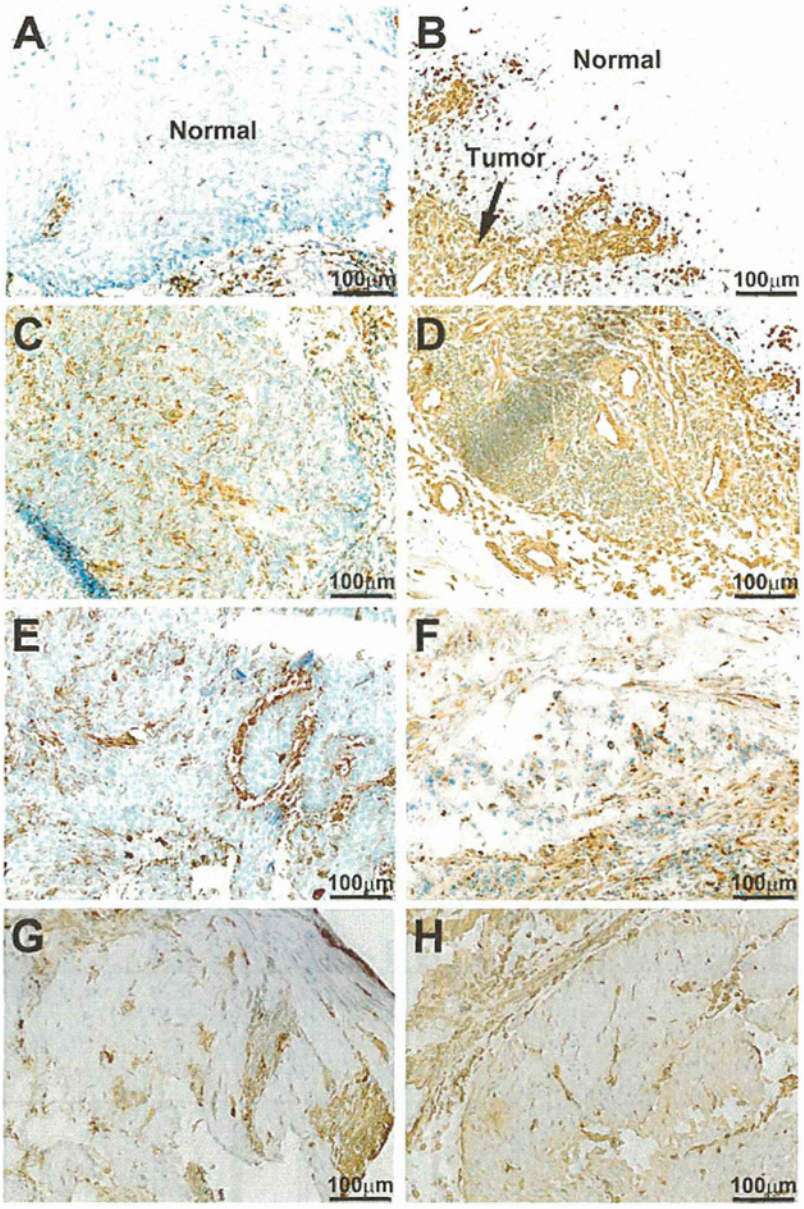


Figure 5. Immunohistochemistry (IHC) of a typical EMT marker vimentin in biopsy and surgically resected tissues. IHC of vimentin in an additional surgical sample, which contained normal portions, showed that normal esophageal epithelial cells were not stained, but invasive tumor cells were (A, B). In 3 out of 5 pairs of biopsy and surgical samples, over-expression of vimentin was observed in the surgical samples (biopsy: C, E, G; surgical: D, F, H).
doi:10.1371/journal.pone.0018196.g005

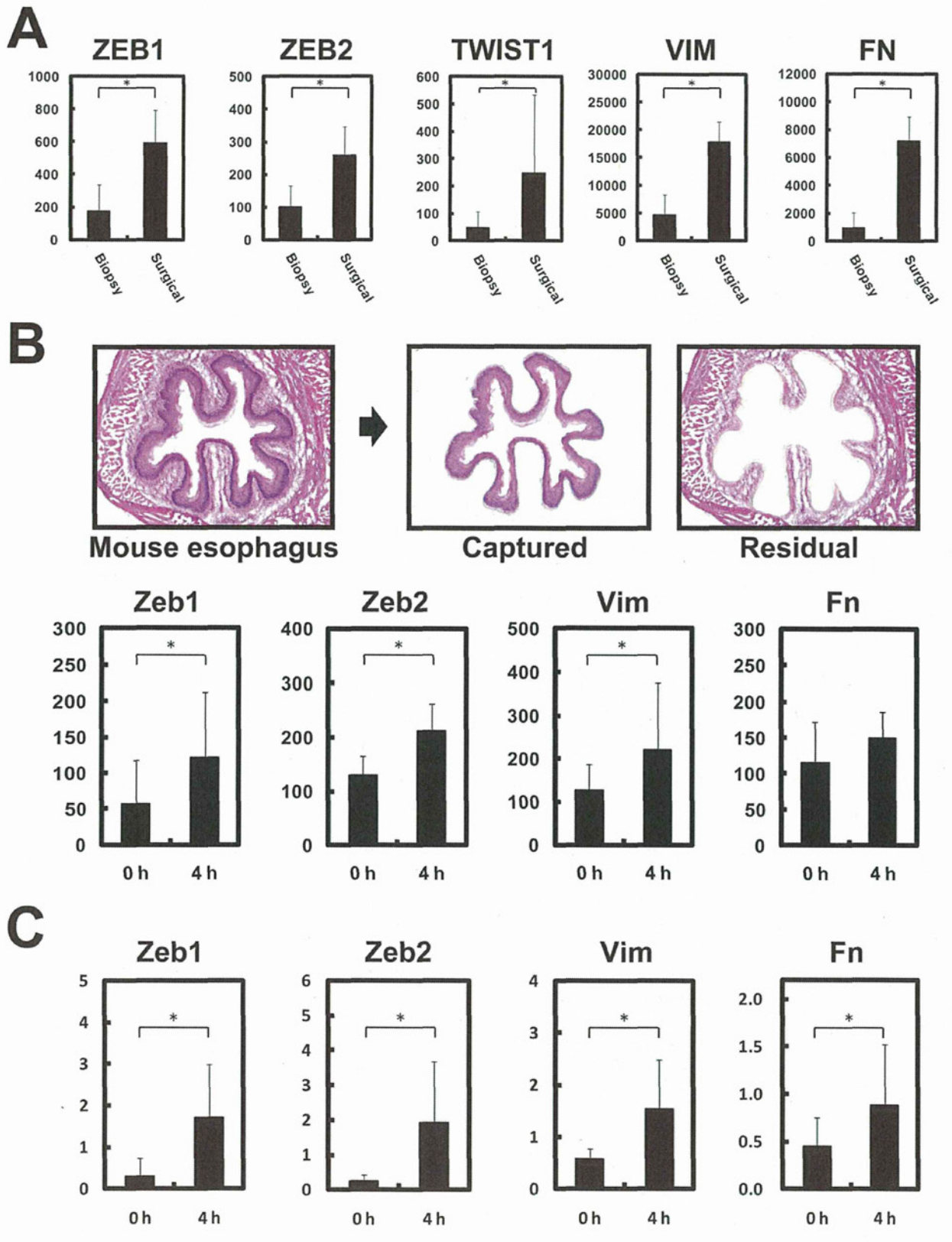

A
ZEB1
ZEB2
TWIST1
VIM
FN
Biopsy
Surgical
B
Mouse esophagus
Captured
Residual
Zeb1
Zeb2
Vim
Fn
0 h
4 h
C
Zeb1
Zeb2
Vim
Fn
0 h
4 h

Figure 6. Over-expression of EMT regulators and markers in surgically resected normal tissues. (A) Over-expression of EMT-regulators (*ZEB1*, *ZEB2*, and *TWIST1*) and EMT-markers (*VIM* and *FN*) in surgically resected normal esophagus mucosa. (B) Induction of mouse *Zeb1*, *Zeb2*, *Vim*, and *Fn* under ischemic condition. After resection of mouse esophagus, we placed it on PBS for 0 or 4 hours at room temperature (under an ischemic condition), immediately made frozen sections, captured the epithelial cell layer (upper) by laser microdissection, amplified mRNA by TALPAT [24–28], and obtained expression profiles using Mouse Expression Array 430 2.0 (Affymetrix, Santa Clara, CA). Experiments were performed on 3 mice. The *Zeb1*, *Zeb2*, *Vim*, and *Fn* genes are induced 4 hours after resection (Lower). $*P<0.05$. (C) Quantitative real time RT-PCR of *Zeb1*, *Zeb2*, *Vim*, and *Fn*. Over-expression of *Zeb1*, *Zeb2*, *Vim*, and *Fn*, shown by microarray, was confirmed.
doi:10.1371/journal.pone.0018196.g006

the expression profile-based scoring method using normal and/or tumor specific genes (in preparation).

The overall process of an esophageal cancer operation requires much time. Therefore, surgical samples were excised from a margin of the tumor by trained pathologists after exposure for 4–7 hours under an ischemic condition, and were immediately frozen at −80°C until use. On the contrary, needle biopsy samples resected under endoscopy were immediately frozen at −80°C until use. Clinicopathological information is given in Tables S3, S4, S5.

Laser Microdissection followed by RNA Extraction and Amplification

Cryostat sections (8μm) of frozen mouse esophageal samples were laser-microdissected with the mmi CellCut system (MMI Inc., Rockledge, FL). Total RNA was isolated by suspending the cells in an ISOGEN lysis buffer (Nippon Gene, Toyama, Japan) followed by precipitation with isopropanol. RNA was amplified by an efficient method of high-fidelity mRNA amplification, called TALPAT (T7 RNA polymerase promoter-attached, adaptor ligation-mediated, and PCR amplification followed by *in vitro* T7-transcription) [24–28].

Microarray Analysis

Gene expression profiles were obtained from 166 samples: tumor sets (different cases) of independent 35 and 20 biopsy samples and 66 surgical samples, another tumor set (identical case) of 18 biopsy samples and 18 surgical samples, a normal set of 4 biopsy samples and 5 surgical samples. Total RNAs extracted from the bulk tissue samples were biotin-labeled and hybridized to high-

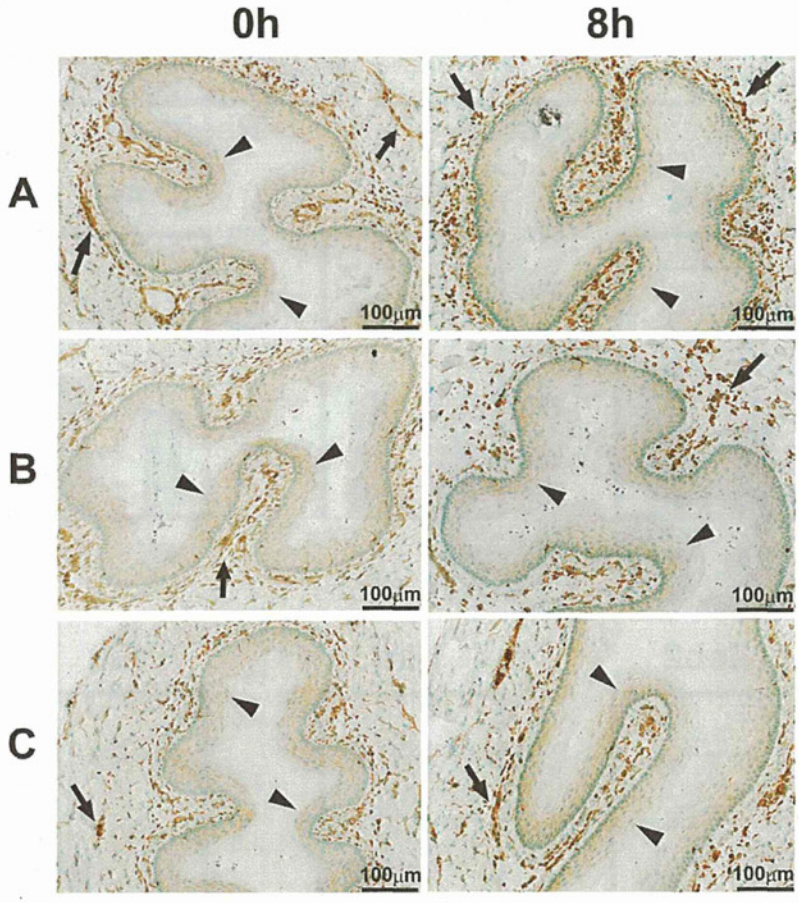
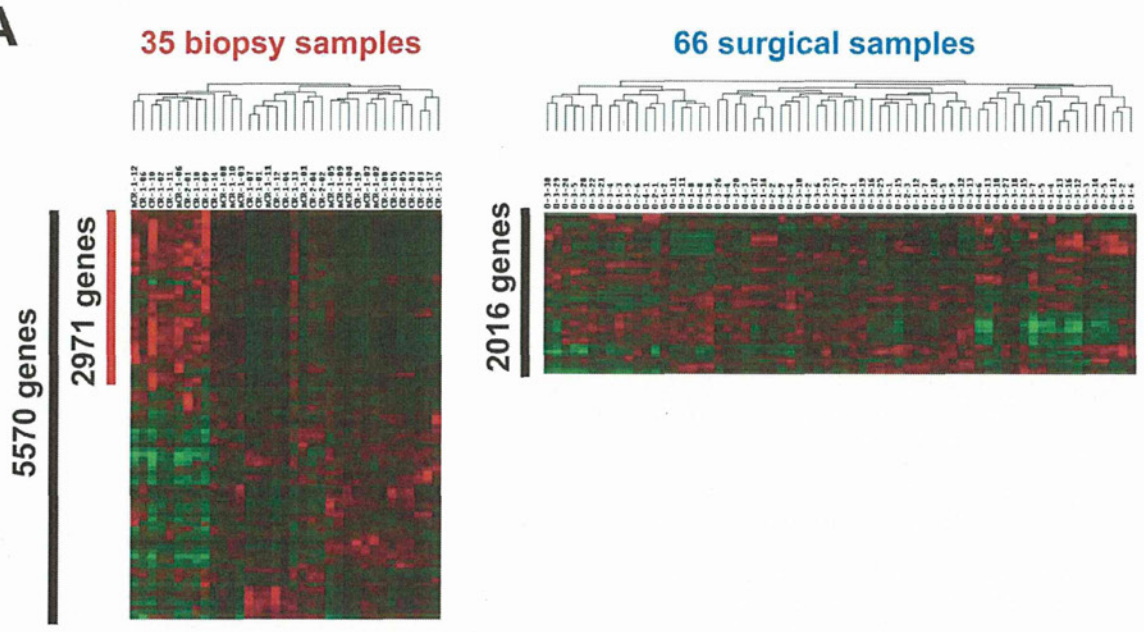


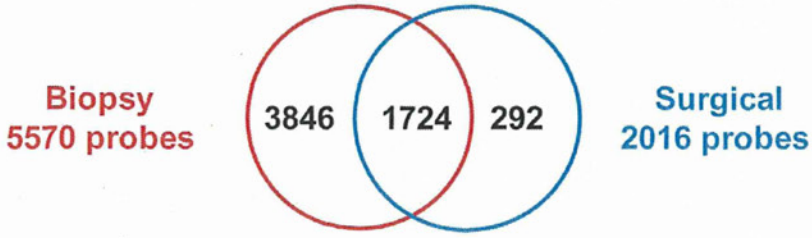
Figure 7. Immunohistochemistry (IHC) of a typical EMT marker vimentin in mouse esophagus. After resection of mouse esophagus, we placed it on PBS for 0, 4, 8 hours at room temperature (under an ischemic condition), immediately made frozen sections, and IHC of vimentin was performed under more sensitive conditions compared with Figure 5. Experiments were performed on 3 mice (A–C). Over-expression of vimentin in mouse esophageal epithelium was not observed even after 8 hours of exposure under an ischemic condition. Arrow: vimentin-positive smooth muscle, arrow head: mouse stratified esophageal epithelial cell layers.
doi:10.1371/journal.pone.0018196.g007

A



B

U95Av2 microarray data	1. > 1000 signal intensity in > 10% sample	1. > 1000 signal intensity in > 10% sample 2. > 3 fold change from Ave. intensity in > 10% sample
Biopsy 35 samples	6551	5570 (85%)
Surgical 66 samples	4797	2016 (42%)



C

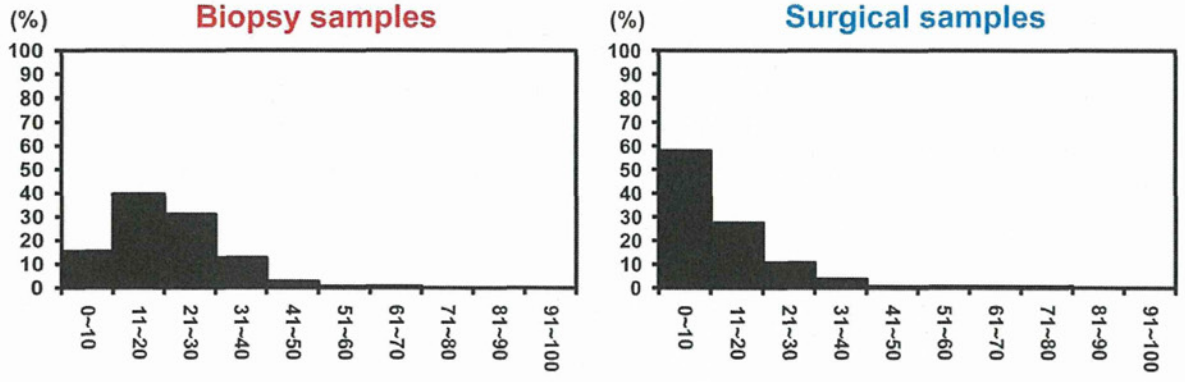


Figure 8. Artificially induced EMT prevents microarray-based subgroup identification. (A) Unsupervised clustering of 35 biopsy and 66 surgically resected esophageal tumor samples with 5,570 and 2,016 processed genes, respectively. A sample cluster with 2,971 genes appears only in the biopsy samples. (B) Comparison of the number of processed genes for unsupervised clustering between biopsy and surgical samples. The number of processed genes and commonly selected genes is indicated. (C) Frequency distribution for percentage of samples of finally processed-gene sets. Each distribution of 5, 570 genes in biopsy samples (Left) and 2,016 genes in surgical samples (Right) is indicated.
doi:10.1371/journal.pone.0018196.g008

density oligonucleotide microarrays (Human Genome U95Av2 or U133PLUS2.0 Array, Affymetrix, Santa Clara, CA, USA) in accordance with the manufacturer's instructions. For laser-captured mouse esophageal epithelial cell layers, Mouse Genome 430 2.0 Array was used. The scanned data of the arrays were processed by Affymetrix Microarray Suite version 4.0 or 5.0, which scaled the average intensity of all the genes on each array to a target signal of 1,000 to reliably compare variable multiple arrays. All the microarray data have been deposited in a MIAME compliant database, GEO; the accession number SuperSeries GSE22954.

Gene Selection from Microarray Data and Hierarchical Clustering

Hierarchical clustering is widely used as one of the unsupervised learning methods. Hierarchical clustering of microarray data was performed by the use of GeneSpring (Agilent Technologies Ltd., CA, USA), Microsoft EXCEL, and Cluster & TreeView software [29].For unsupervised clustering (Figures 1A and 8A), we first selected genes with a signal intensity of more than 1,000 in more than 10% of the samples, and from these genes, we finally selected more than 3-fold changed genes by comparing the average signal intensity of each gene in more than 10% of the samples. For overexpressed genes in the surgical or biopsy samples, we first selected genes by u-test ($p<0.01$), permutation test, and 2- or 3-fold change between the average signal intensities of the two sets of samples, and from the first selected genes we finally selected genes with more than 1,000 in average signal intensity.

Semi-quantitative and Quantitative RT-PCR

Total RNA was isolated by suspending the cells in Isogen lysis buffer (Nippon Gene, Toyama, Japan) followed by precipitation with isopropanol. RT-PCR was carried out using primer sets designed for detecting the 3′ side of cDNA of each human gene: for *IL8*, 5′- TGCCAAGGAGTGCTAAAG -3′ and 5′- CTCCACAACCCTCTGCAC-3′, for *CXCR4*, 5′-TGTATGTCTCGTGGTAGGAC-3′ and 5′-AGACTGTACACTGTAGGTGC-3′, for CXCL9, 5′-ACAAAGAAAATATTTCAAATTACAAGG-3′ and 5′-GGGAACGGTGAAGTACTAAGC-3′, for *PDGFRB*, 5′-ACTGCCCAGACCTAGCAGTG-3′ and 5′-CAGGGAAGTAAGGTGCCAAC-3′, for CCL5, 5′-CCCCGTGCCCACATCAAGGAGTATTT-3′ and 5′-CGTCCAGCCTGGGGAAGGTTTTTGTA-3′, for *TLR2*, 5′-CCAGCAGGAACATCTGCTAT-3′ and 5′-TCCAGGTAGGTCTTGGTGTT-3′, for *ZEB1*, 5′-CGTCTCTTTCAGCATCACCA-3′ and 5′-ATGGGAGACACCAAACCAAC-3′, for *ZEB2*, 5′-CATGACGTTGATCATTTGGGC-3′ and 5′-CGAGCATGGTCATTTTCAAAAG-3′, for *FN*, 5′-CGGGGGAAATAATTCCTGTG-3′ and 5′-CCTTGCAGGCAATCTCTTTG-3′, for *VIM*, 5′-GCTTTCAAGTGCCTTTCTGC-3′ and 5′-GTTGGTTGGATACTTGCTGG-3′, and for *ACTB* (β-actin), 5′-TCATCACCATTGGCAATGAG-3′ and 5′-CACTGTGTTGGCGTACAGGT-3′. Primer sets for detecting each mouse gene were also designed: for *Zeb1*, 5′-TAACATTTATACTTGCCTCC-3′ and 5′-GCTAAGGGAATGAGTTATGG-3′, for *Zeb2*, 5′-ACCAAATCAGACCACGAGGA-3′ and 5′-GCCCCTTCTGTCCCTCTCTA-3′, for *Fn*, 5′-CCGTGGGATGTTTTGAGACT-3′ and 5′-GGCAAAAGAAAGCAGAGGTG-3′, for *Vim*, 5′-ACGGTTGAGACCAGAGATGG-3′ and 5′-CGTCTTTTGGGGTGTCAGTT-3′ , and for *ActB*, 5′-GCTCTTTTCCAGCCTTCCTT-3′ and 5′-GTACTTGCGCTCAGGAGGAG-3′. For semi-quantitative RT-PCR, we showed data within linear range by performing 25–35 cycles of PCR. Quantitative real-time PCR was performed by a Bio-Rad iCycler with iQ Syber Green Supermix (Bio-Rad, Hercules, CA, USA) as directed by the manufacturer. The value of 1/2N (N: the number of PCR cycles corresponding to the onset of the linear amplification of each gene product) was calculated as a relative mRNA expression level of each gene normalized to *ACTB*. The data from 2 independent analyses for each sample were averaged.

Immunohistochemistry

For immunohistochemical staining of frozen sections of human and murine esophagus, specimens that were embedded in a TissueTek OCT medium (VWR Scientific Products, West Chester, CA) and stocked at −80°C until use were cut into 8μm sections, which were then left for 30 min at room temperature followed by fixing in 4% paraformaldehyde for 20 min at room temperature. Endogenous peroxidase activity was inhibited with 3% H_2O_2 in methanol for 30 min. Blocking was carried out with Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA) for 30 min at room temperature. Sections were incubated for 60 min at room temperature with diluted mouse monoclonal antibody directed against human vimentin (N1521, DAKO, Carpinteria, CA) or rabbit polyclonal antibody directed against mouse vimentin (#3932, Cell Signaling Technology Japan, Tokyo, Japan). After washing sections with PBS, biotinylated secondary antibodies were applied for 30 min at room temperature. Detection was carried out by using Vectastain ABC Elite Kit (Vector Laboratories) and the DAB system (DAKO, Tokyo), and the sections were counter-stained with 1% Methyl Green. (Sigma, Saint Luis, MO)

Supporting Information

Figure S1 Schema of artificial factors during surgical resection and sample transportation. Biopsy samples are small, much fresher, with low contamination of normal portions compared to surgical samples, whereas some artificial factors such as ischemia, hypoxia, hyponutrition, and cold stress possibly occur during surgical resection and sample transportation.
(TIF)

Figure S2 Expression levels of *ZEB1* and *ZEB2* in two sets of biopsy and surgical samples (different and identical cases). Over-expression of both genes is observed in surgically resected esophageal tumors, except *ZEB2* in the different cases. $*P<0.05$.
(TIF)

Figure S3 Expression levels of *TWIST1* in two sets of biopsy and surgical samples (different and identical cases). Over-expression of *TWIST1* is observed in surgically resected esophageal tumors. $*P<0.05$.
(TIF)