Table 2
Preoperative laboratory data

Data	Salvage group (n = 27)	Neoadjuvant group (n = 28)	P value
Total protein (g/dL)	6.5 ± 0.47	6.6 ± 0.58	0.3784
Albumin (g/dL)	3.5 ± 0.42	3.8 ± 0.41	0.0330
White cell count (mm^3)	5853 ± 2001	4864 ± 1512	0.0430
Lymphocyte count (mm^3)	781 ± 309	851 ± 366	0.4484
Hemoglobin (g/dL)	11.4 ± 1.5	11.6 ± 1.3	0.5535
Platelets ($\times 10^4/mm^3$)	27.3 ± 10.8	25.5 ± 11.6	0.5546
Pao_2 (mm Hg)*	88 ± 12	94 ± 12	0.0759
$Paco_2$ (mm Hg)†	38 ± 3.7	38 ± 4.2	0.9323
Vital capacity (%)	93 ± 12	104 ± 21	0.0190
Forced expiratory volume 1.0 (%)	76 ± 1.7	78 ± 1.5	0.4231

Values represent mean ± standard deviation.
Pao_2 = arterial partial pressure of oxygen; $Paco_2$ = arterial partial pressure of carbon dioxide.

the mean albumin level in the salvage group was significantly lower than that in the neoadjuvant group ($P = 0.0234$) (Table 2). The mean white blood cell count of the neoadjuvant group was significantly lower than that of the salvage group ($P = 0.0234$), but no differences were found between the two groups regarding the lymphocyte count, hemoglobin level, and platelet count. In lung-function tests, %VC in the salvage group was lower than that in the neoadjuvant group ($P = 0.0190$), but FEV1% did not differ between the two groups. Pao_2 was lower in the salvage group than in the neoadjuvant group, but not significantly so ($P = 0.0759$).

Extended esophagectomy through right thoracotomy with three-field lymph node dissection has been the standard procedure for advanced esophageal cancer in our institution. Because 2 of 14 (14%) patients of the salvage group who underwent extended esophagectomy died of postoperative complications before 1997, less-invasive procedures were performed in 13 patients, excluding 2 with upper thoracic esophageal cancer thereafter. Esophagectomy by way of left thoracotomy and transhiatal esophagectomy was performed in 7 (26%) and 4 (15%) patients from the salvage group, respectively (Table 3). Esophagogastric tube anastomosis in the neck by way of the mediastinal or retrosternal route was the standard procedure for 1-stage reconstruction after esophagectomy. In the salvage group, the subcutaneous route was selected in 17 patients, and 2-stage reconstruction was performed in 4 patients. Reconstruction was performed using a gastric tube in 24 patients from both groups and using the colon or jejunum in the remaining 3 patients in the salvage group and 4 patients in the neoadjuvant group who had already undergone gastrectomy. Surgical time was shorter in the salvage group than in the neoadjuvant group, but the difference was without significance. The blood loss and transfusion requirements of the neoadjuvant group were larger than those of the salvage group, but the difference was not significant.

One patient (3.7%) died of adult respiratory distress syndrome on postoperative day 22, and 1 patient (3.7%) died of anastomotic leakage and pneumonia on postoperative day 62 in the salvage group (Table 4). Both patients underwent extended esophagectomy by way of right thoracotomy with 3-field lymphadectomy before 1997, but no operative mortality or hospital deaths were recorded thereafter. There was no 30-day mortality, but 1 patient (3.6%) in the neoadjuvant group died of pneumonia on postoperative day 122. From 1992 to 2002, 11 (1.7%) of all 660 patients who underwent esophagectomy for esophageal cancer died of postoperative complications within 30 days, and 19 patients (2.9%), including these 11, died at our institution. Anastomotic leakage occurred in 6 patients (22%) from the salvage group compared with 3 patients (11%) from the neoadjuvant group. Three patients had pleural effusion in the salvage group versus only 1 patient after surgery. Only 3 patients (11%) from the salvage group and 7 patients

Table 3
Surgical procedures and operative factors

Operative factors	Salvage group (n = 27)	Neoadjuvant group (n = 28)	P value
Approaches			
Right thoracotomy	16	24	
Left thoracotomy	7	2	0.0809
Transhiatal	4	2	
Lymph node dissection			
Three-field	9	16	
Two-field	12	11	0.1397
Abdominal	6	2	
Reconstruction route			
Subcutaneous	17	11	
Retrosternal	2	8	0.0851
Mediastinal	8	9	
Other operative factors*			
Operation time* (min)	312 ± 106	356 ± 118	0.1442
Blood loss* (mL)	679 ± 414	975 ± 861	0.1125
Blood transfusion* (mL)	571 ± 499	614 ± 915	0.8284

* Values represent mean ± standard deviation.

Table 4
Short outcomes of esophagectomy

Outcomes	Salvage group (n = 27)	Neoadjuvant group (n = 28)	P value
% Mortality (within 30 days)	1 (3.7)		
% Hospital mortality (> 30 days)	1 (3.7)	1 (3.6)	
Mechanical ventilation (days)	3.1 ± 6.4	2.1 ± 2.8	0.4348
Intensive care unit stay (days)	5.9 ± 5.8	5.1 ± 2.8	0.5066
Postoperative hospital stay (days)	39.9 ± 25.4	31.9 ± 22.8	0.2221
Leakage (surgery)	6 (3)	3 (1)	
Pneumonia	3	3	
Wound infection	2	2	
Pleural effusion	3	1	
Residual tumors (%)			
R_0	18 (67)	17 (61)	
$R_{1,2}$	9 (33)	11 (39)	
Pathologic effect (%)			
Complete response	3 (11)	7 (25)	
Partial response	9 (33)	7 (25)	
No response	15 (56)	14 (50)	

(25%) from the neoadjuvant group achieved pathologic complete response. These 3 patients in the salvage group complained of dysphasia caused by stricture of the esophagus within 3 months after definitive CRT. No difference was found between the 2 groups with respect to residual tumor, depth of tumor invasion, lymph-node metastasis, and distant metastasis.

There were no differences in overall postoperative survival between the salvage and neoadjuvant groups (Fig. 1). In the salvage group, the survival of 13 patients with recurrence (>3 months after CRT) was similar to that of 14 patients with residual tumors (<3 months after CRT) (Fig. 2). The survival of 18 patients without residual tumors (R0) was significantly better than that of 9 patients with R1or R2 tumors ($P = 0.0022$) (Fig. 3). The survival of 18 patients who underwent less-invasive esophagectomy was similar to that of 9 patients who underwent 3-field lymph node dissection (Fig. 4). With regard to postoperative recurrence, only 3 patients had distant-organ metastasis in the salvage group versus 6 patients in the neoadjuvant group. The incidence of local recurrence (n = 4), lymph-node metastasis (n = 4), and pleural dissemination (n = 1) in the salvage group was similar to the incidence of local recurrence (n = 3), lymph-node metastasis (n = 3), and pleural dissemination (n = 2) in the neoadjuvant group.

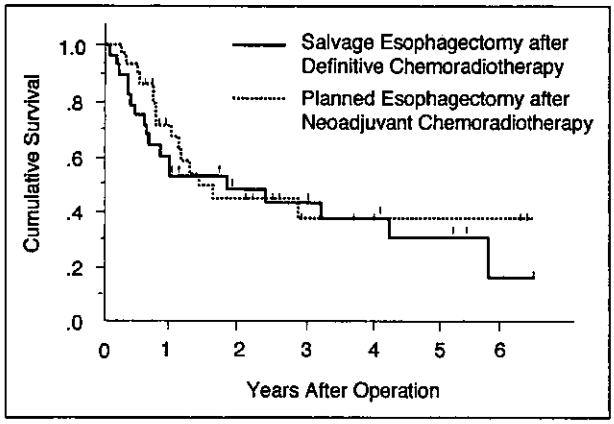

Fig. 1. No difference is shown in cumulative postoperative survival between the salvage and neoadjuvant groups.

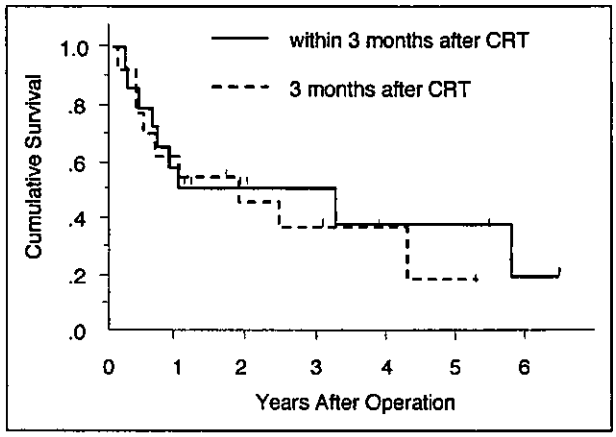

Fig. 2. In the salvage group, no difference was observed in cumulative postoperative survival between patients with recurrence (>3 months after chemoradiotherapy [CRT]) and those with residual tumors (<3 months after CRT).

Comments

The treatment of patients with advanced esophageal cancer remains a challenge for surgeons, medical oncologists, and radiation oncologists. Cisplatin plus 5-FU in combination with CRT has proven to be an effective treatment for squamous-cell carcinoma of the esophagus. Although several studies have compared planned neoadjuvant CRT (30 to 45 Gy) plus esophagectomy with definitive CRT (≥50 Gy), the optimum treatment remains unclear [12,13]. Salvage

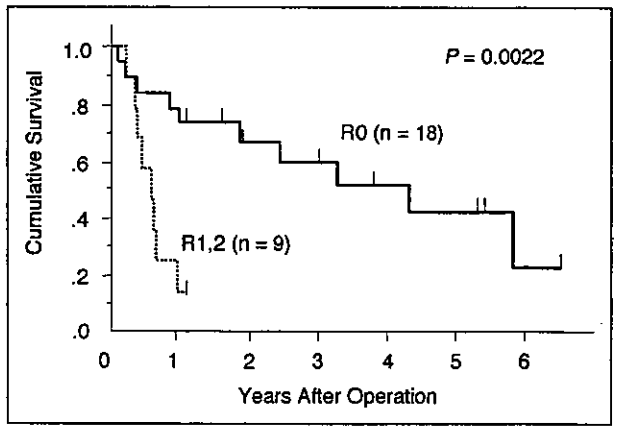

Fig. 3. The survival of patients without residual tumors (R0) was significantly better than that of patients with R1or R2 tumors in the salvage group.

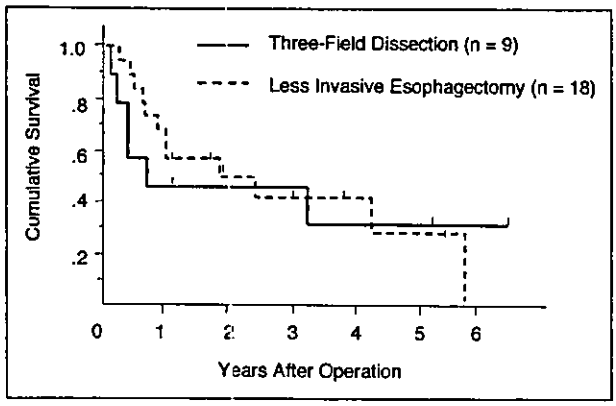

Fig. 4. No difference was observed in cumulative postoperative survival between patients who underwent less-invasive esophagectomy and those who underwent 3-field lymph node-dissection in the salvage group.

esophagectomy could be the best second-line treatment for local and regional recurrence after definitive CRT, but this has not yet been established. The present study showed that the outcome of salvage esophagectomy after definitive CRT was comparable with that of esophagectomy after neoadjuvant CRT.

Although extended esophagectomy with 3-field lymph-node dissection is routinely performed for advanced esophageal cancer, the operative mortality decreased to only 1.7% in this study, the same as that (1.7%) in another Japanese study, because of improved surgical management [1]. However, the 30-day mortality rate of extended esophagectomy with 3-field lymph node dissection was 2.5% (5 of 203), and that of the other less-invasive procedures was 1.3% (6 of 457), during the same periods. Although there was no statistical difference, the mortality rate was twice as high with 3-field lymph node dissection as with the other type. Extended esophagectomy after definitive CRT led to the death of 2 patients who died from postoperative complications before 1997, and we were forced to change the operative procedure to less-invasive methods of esophagectomy. Vogel et al. [6] showed a low mortality rate (5%) and relatively favorable outcome of esophagectomy using less-invasive approaches after neoadjuvant CRT. Neoadjuvant CRT using paclitaxel did not improve outcome of 5-FU–based treatment for locoregionally advanced esophageal cancer [23]. A recent phase III study of definitive CRT showed that outcome of the standard-dose group (50.4 Gy) was relatively superior to that of the high-dose group (64.8 Gy) because of toxicity [24]. These results might suggest that aggressive treatment could guarantee improvement of outcome in patients with advanced esophageal cancer.

Although a previous study of salvage esophagectomy found that preoperative data were similar in both groups [19], %VC and serum albumin level were significantly lower in our salvage group than in our neoadjuvant group. Pretreatment data could not be collected because several patients received definitive CRT at other hospitals, but a lower %VC without change in FEV1% may have been caused by restrictive changes to the lungs as a result of radiation damage [25]. The lungs are usually not affected by neoadjuvant CRT (≤45 Gy) for esophageal cancer using anterior- and posterior-opposed beams, and clinical data have shown no difference in %VC after neoadjuvant CRT [26]. However, more irradiation (>45 Gy) was given obliquely to avoid spinal cord damage, and this may have led to damage to the lung. The low serum albumin level in the salvage group of the present study was possibly related to the long duration of disease. Both %VC and albumin level are important preoperative risk factors for complications after transthoracic esophagectomy [27,28]. Thus, the preoperative risk of salvage surgery could be higher than that of esophagectomy after neoadjuvant CRT.

Anastomotic leakage was frequent (22%) in the salvage group as well as in a previous study (38%) [19]. Irradiation to the cervical esophagus and trachea can influence blood supply, and 1 patient died of tracheal bleeding caused by anastomotic leakage after reconstruction using the mediastinal route. The other 5 patients who had leakage after reconstruction by way of the subcutaneous route did not die, but they needed longer hospital stays. Although we routinely perform 1-stage esophagectomy and reconstruction, 4 patients in the salvage group underwent 2-stage reconstruction to prevent aspiration pneumonia. From 1997 onward, preoperative corticosteroid therapy was routinely given before surgery to prevent pulmonary failure [29]. Enteric nutrition by way of gastrostomy was also used routinely for prolonged pleural effusion in the salvage group.

When compared with patients having residual tumors (<3 months after CRT) or recurrent tumors (>3 months after CRT), no differences were found in the surgical outcome between these groups. Esophagectomy may be unnecessary after complete response, but its diagnosis by imaging is difficult and possible only by esophageal resection [30]. Recently, positron-emission tomography using 2-[18F]-fluoro-2-deoxy-D-glucose has been developed as a tool to assess tumor response to CRT, but it cannot distinguish a complete response from small foci of residual tumors [31]. In this study, cancer cells were detected by endoscopic biopsy specimens in the all patients with locoregional recurrence >3 months after CRT. No difference between the 2 groups was obtained partly because micrometastasis to lymph nodes may have been controlled by CRT, whereas patients with obvious metastasis in distant lymph nodes could not undergo salvage esophagectomy. In either case, to improve the outcome of salvage esophagectomy, patients with residual or recurrent tumors after definitive CRT should be referred immediately to experienced surgical institutions by medical and radiation oncologists.

In conclusion, the outcome of salvage esophagectomy after definitive chemotherapy and radiotherapy was comparable with that of planned neoadjuvant CRT plus esophagectomy. Preoperative risk factors were greater in the salvage esophagectomy group than in the neoadjuvant CRT plus esophagectomy group. Less-invasive surgery and me-

ticulous postoperative care may improve the outcome of patients undergoing salvage esophagectomy.

Acknowledgments

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DISEASES OF THE ESOPHAGUS

Original article

Expression of $p21^{Waf1/Cip1}$ predicts response and survival of esophageal cancer patients treated by chemoradiotherapy

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SUMMARY. **Chemoradiotherapy is a multimodal therapy routinely used as a primary treatment for advanced esophageal cancer. However, it is beneficial only to patients who respond. To identify pretreatment markers predicting response and survival, we examined the expression of cell cycle regulatory molecules, p53, $p21^{Waf1/Cip1}$ cyclin D1, and CDC25B, in biopsy specimens from 76 patients with stage III and stage IV squamous cell carcinoma. Overexpression of p53, p21, cyclin D1 and CDC25B was observed in 58%, 30%, 28%, and 32% of patients, respectively. The expression of p21 correlated significantly with response to chemoradiotherapy ($P = 0.0001$). Survival of patients with p21-expressing tumors was better than that of patients with p21-negative tumors ($P = 0.013$). Expression of other genes was not significantly correlated with treatment response and survival. In patients with p53-negative tumors, survival of those patients with p21-positive tumors was significantly higher than that of those with p21-negative tumors ($P = 0.0452$), but no significant difference was found in patients with p53-positive tumors. Multivariate analysis revealed that p21 expression was an independent variable among pretreatment parameters in predicting survival. These results suggest that p21 expression is potentially useful for predicting the response to chemoradiotherapy and survival of patients with advanced esophageal squamous cell cancer.**

KEY WORDS: **CDC25B, chemoradiotherapy, cyclin D1, p21, p53, squamous cell carcinoma.**

INTRODUCTION

Combined modality therapy, including chemotherapy, is necessary to treat advanced esophageal cancer, which can be widely disseminated at the time of diagnosis.[1] Chemoradiotherapy (CRT) is widely used as an effective therapy for patients with esophageal squamous cell cancer.[2] Combined modality therapy, consisting of CRT and surgery, has been shown to improve the outcome for esophageal cancer.[3,4] In contrast, randomized comparative studies showed no difference in survival between operable patients who received neoadjuvant CRT and those treated with surgery alone.[5,6] The toxicity of CRT is considerable and the operative risk after CRT is increased compared to that without any therapy. If reliable ways to predict the response to CRT could be found, non-responders could be spared the toxicity and the postoperative risk associated with the treatment.[2,4]

Lack of response to CRT can be attributed to the resistance of the carcinoma cells. The presence of functional wild-type p53 is an important determinant of tumor response to chemotherapy and radiotherapy.[7] Although numerous studies have analyzed the association between p53 mutation or expression and anticancer treatment, the results were not consistent in a variety of cancers, including esophageal cancer.[8–11] Cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$, which is transcriptionally activated by p53, is necessary for p53-mediated G1 arrest following irradiation.[12] In rectal and pancreatic cancer,[13,14] p21 expression in relation to p53 can predict the response to CRT or radiotherapy. The other cell cycle-related molecules associated with the response of esophageal cancer to CRT or radiotherapy are cyclin D1 and CDC25B.[15,16] Although these studies reported a correlation with treatment response, the predictive value of these molecules in terms of the response to CRT and patient survival was unclear because of small sample sizes.

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In this study, we examined p53, p21, cyclin D1, and CDC25B expression using immunohistochemistry in pretreatment endoscopic biopsy specimens before CRT and compared these with the response to CRT. To evaluate the prognostic value of expression of these genes among the pretreatment parameters, we used a Cox proportional hazards model in 76 patients with advanced esophageal cancer.

MATERIALS AND METHODS

Patients

Between 1993 and 2001, 108 consecutive patients with advanced esophageal cancer received CRT as their initial treatment. Of these, 11 patients who did not complete the planned CRT and 21 patients whose biopsy specimens could not be examined were excluded from this study. The remaining 76 patients who completed the planned CRT were included. Clinical pretreatment features of the patients are shown in Table 1. The Eastern Cooperative Oncology Group performance status was 0 in 28 patients, and 1 in 48 patients. The patients were staged clinically based on barium swallow X-rays, endoscopy, CT and endoscopic ultrasonography findings according to the TNM classification (UICC).[17] Informed consent for examination was obtained from all patients before enrollment in this study.

Treatment schedule

Patients received CRT according to different chemotherapy regimens; however, the external beam irradiation was over 30 Gy in every case. Standard CRT, consisting of cisplatin (70 mg/m^2, day 1) and 5-fluorouracil (FU) (700 mg/m^2/day, days 1–4) and irradiation given as a continuous i.v. infusion, was given to 38 patients. A low-dose CRT regimen, consisting of cisplatin (3–5 mg/m^2/day), 5-FU (200–300 mg/m^2/day) and irradiation, was given to 14 patients. A different CRT regimen, consisting of nedaplatin, an analog of cisplatin (20 mg/m^2/day for 4 days)[18] and 5-FU (700 mg/m^2/day for 4 days) and irradiation, was given to 24 patients. Radiotherapy (40 Gy) was administered in 23 patients using anterior and posterior opposed equally weighted beams from a 10-MV linear accelerator in 20 fractions of 2 Gy. In the other 53 patients, an additional course of chemotherapy and irradiation of 20–30 Gy (a total of 60–70 Gy) were administered via two parallel oblique fields (definitive CRT).

Table 1 Patient and tumor characteristics

Characteristic	*n* (total 76)
Male : female	65:11
Performance status	
0	28
1	48
Location	
Cervical	7
Upper thoracic	12
Middle thoracic	45
Lower thoracic	12
Depth of invasion	
T3	27
T4	49
Distant lymph node metastasis	
M0	44
M1	32
Differentiation	
Well	8
Moderate	55
Poor	13
Gross type	
Localized type	19
Infiltrative type	57

The mean age of the patients was 63 years (range, 47–79)

Evaluation of response

The clinical response of the tumor was determined in accordance with the criteria for assessment of response to non-surgical treatment of the Japanese Society for Esophageal Diseases.[19] For the primary esophageal lesion, the response was assessed on the basis of the two-dimensional reduction rate and the morphologic changes on barium swallow X-rays and endoscopy. For metastatic lesions, the response was assessed on neck, chest and abdominal CT scans.

Clinical evaluation was carried out by re-evaluation of the images at one month after the initial evaluation of response. In the 32 patients who underwent esophagectomy after CRT, response was evaluated on the basis of histologic examination of the resected specimens according to the histopathologic criteria for assessing the effects of radiation and/or chemotherapy.[19] When no viable cancer cells were evident this was classified as a complete response (CR). When viable cancer cells accounted for less than one-third of the tumor, this was classified as a partial response (PR). Viable cancer cells accounting for one-third or more of the tumor tissue with no discernible therapeutic effect on the tumor led to classification as stable disease (SD). If a new lesion was detected, this was classified as progressive disease (PD).

Immunohistochemistry

Specimens of invasive squamous cell carcinoma were obtained from all 74 patients by endoscopic biopsy before starting CRT. Carcinomas present in the resected specimens were also evaluated in 22 patients who underwent esophagectomy, excluding 10 patients who had a pathological CR.

Written informed consent to examination was obtained from all patients before enrollment in this study.

Specimens were fixed in 10% buffered formalin and embedded in paraffin. Serial sections (3 μm thick) were dewaxed in xylene and rehydrated with graded ethanol solutions. After rinsing with 0.01 M phosphate-buffered saline (PBS), the sections were placed in a plastic container filled with 10 mmol/L citrate buffer (pH 6.0), heated in a microwave oven (500 W) for 15 min, and then left at room temperature for 20 min. Endogenous peroxidase activity was blocked by incubating the sections in 0.3% hydrogen peroxide (H_2O_2) in absolute methanol for 15 min. Immunostaining was performed using the labeled streptavidin-biotin-peroxidase technique (DAKO-LSAB2 system, DAKO, Carpenteria, CA). The primary antibodies were a monoclonal mouse antip53 protein antibody (DO7, DAKO), an anti-Wafl (p21) protein antibody (Clone EA10, Oncogene Research Products, Cambridge, MA), an anticyclin D1/PRAD1 antibody (5D4, Medical & Biological Laboratories, Nagoya, Japan) and an anti-CDC25B antibody (sc-326, Santa Cruz Biotechnology, Santa Cruz, CA). Normal rabbit serum diluted to 1 : 400 was used in place of the primary antibody as a negative control.

After washing with PBS, the sections were incubated with a biotinylated antimouse IgG (DAKO), followed by incubation with peroxidase-conjugated streptavidin (DAKO). The peroxidase reaction was visualized using 0.5 mg/mL diaminobenzidine tetrahydrochloride (DAKO) in 0.03% hydrogen peroxide. The sections were counterstained with hematoxylin, dehydrated and covered with a cover slip. Positive reactivity for p53, p21, cyclin D1 and CDC25B was defined by staining in more than 10% of the cancer cells.

Data analysis

Differences in percentage data were evaluated by the two-sided χ^2 test or Fisher's exact test. Survival was calculated from the first day of the CRT schedule. Survival curves were constructed according to the Kaplan–Meier method and were compared using the log-rank test. Independent prognostic factors for survival were determined by the Cox proportional hazards model. All data were analyzed using JMP version 4 software (SAS Institute, Cary, NC). *P*-values of less than 0.05 were considered to be statistically significant.

RESULTS

Gene expression in biopsy specimens

Expression of p53, p21, cyclin D1 and CDC25B was observed in 44 (58%), 23 (30%), 21 (28%), and 24 (32%) of the pretreatment biopsy specimens, respectively (Fig. 1). No association was found between patients with and without p53, cyclin D1 and CDC25B expression in any pretreatment characteristics. Expression of p21 was inversely correlated with distant metastasis (M0), but without significance ($P = 0.0792$). Four of eight (50%) well-differentiated carcinomas and seven of 19 (37%) localized tumors expressed p21. In the biopsy specimens, p53 expression was inversely correlated with response to CRT, but not to a significant extent ($P = 0.082$) and p21 expression was closely correlated with response to CRT ($P = 0.0001$)

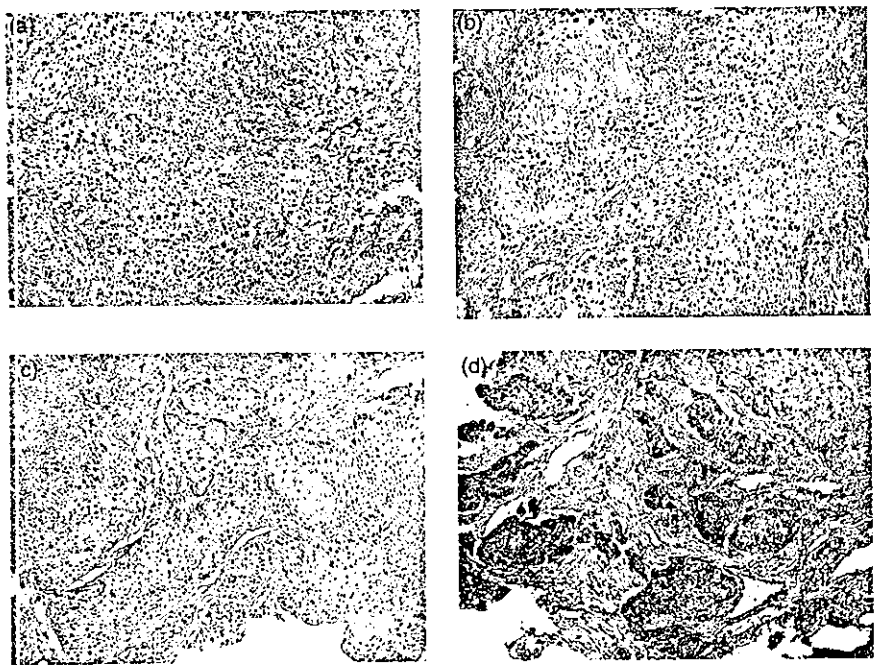

Fig. 1 Results of immunohistochemistry on pretreatment biopsy specimens: (a) p53; (b) $p21^{Waf1/Cip1}$; (c) cyclin D1; and (d) CDC25B. (Original magnification ×100.)

(Table 2). Cyclin D1 expression was not associated with response to CRT. CDC25B expression tended to be correlated with response, but without reaching a significant level.

Gene expression in resected specimens

Tumor specimens were obtained at two time-points during treatment: a biopsy was taken before starting CRT, and tumor specimens were obtained during esophagectomy after CRT in 22 patients (Table 3). No change in p53 expression was shown between the biopsy and resected specimens in 19 of 22 cases (86%). Specimens from 17 of 22 patients (77%) were shown to be p21-negative, both before and after CRT, because only ineffective residual tumors survived and could be investigated in resected specimens. Expression of p21 was found to be induced in three resected tumors, which had been negative at the pre-CRT biopsy. The coincidence rate of cyclin D1 and CDC25B expression was not high.

Survival

Response to CRT was closely correlated with patient survival ($P < 0.0001$). Patients with p53-positive tumors tended to survive for shorter periods of time than those with p53-negative tumors, but the difference was not significant ($P = 0.2323$) (Fig. 2a). The survival of 23 patients with p21-positive tumors was significantly longer than that of 53 patients with p21-negative tumors ($P = 0.013$) (Fig. 2b). No significant difference in survival was observed for cyclin D1 (Fig. 2c) or CDC25B (Fig. 2d) expression. Survival curves divided by the combination of p53 and p21 expression are shown in Fig. 3. In patients with p53-negative tumors, survival of those patients with p21-positive tumors was significantly higher than that of those with p21-negative tumors ($P = 0.0452$), but no significant difference was found in patients with p53-positive tumors ($P = 0.1085$). Pretreatment characteristics were examined by univariate analysis for their value as predictors of survival. M factor and performance

Table 2 Expressions in pretreatment biopsy specimens and response to chemoradiotherapy

	n	p53	$p21^{Waf1/Cip1}$	cyclin D1	CDC25B
Positive cases	76	44 (58%)	23 (30%)	21 (28%)	24 (32%)
Complete response	16	6 (38%)	12 (75%)	4 (25%)	7 (44%)
Partial response	39	24 (62%)	9 (23%)	10 (26%)	12 (31%)
Stable disease	18	13 (72%)	2 (11%)	7 (39%)	4 (22%)
Progressive disease	3	1 (33%)	0	0	1 (33%)

Tumor response to chemoradiotherapy was significantly correlated with $p21^{Waf1/Cip1}$ expression ($P < 0.0001$), but not associated with p53, cyclin D1 or CDC25B expression.

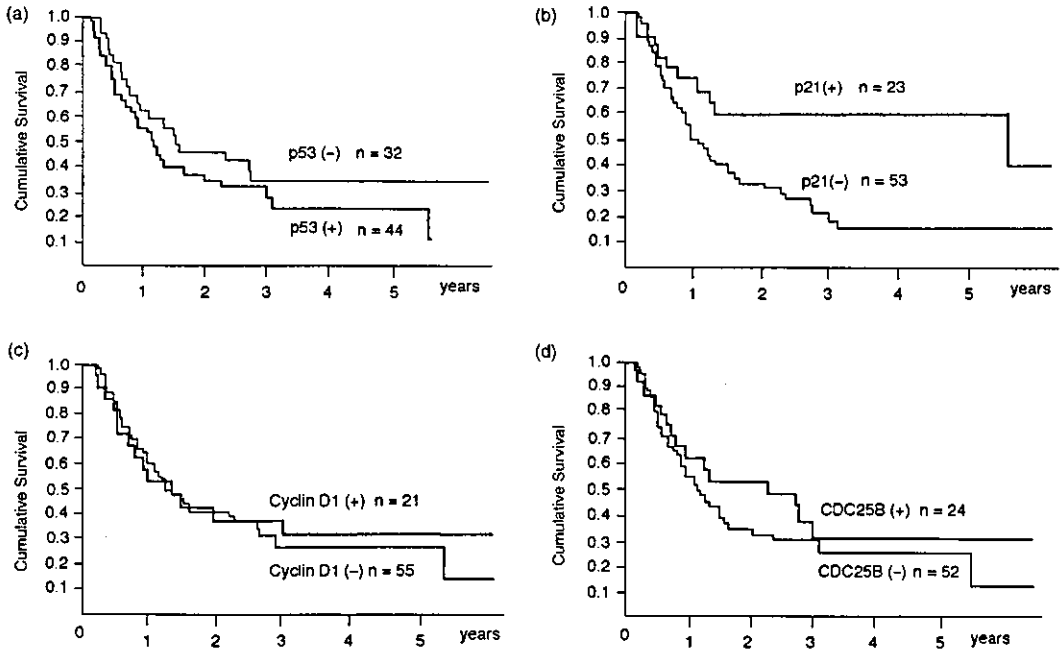

Fig. 2 The probability of survival for the patients was not different by (a) p53; (c) cyclin D1; or (d) CDC25B expression. (b) The probability of survival for patients with $p21^{Waf1/Cip1}$-positive tumors was significantly higher than patients with p21-negative tumors ($P = 0.013$).

status were significant variables in predicting survival, but no differences in survival were found to correlate with differences in gender, location, differentiation or gross type of the tumors. In a Cox proportional hazard model of pretreatment parameters, M factor and p21 expression were independent variables for predicting survival (Table 4).

DISCUSSION

Chemoradiotherapy (CRT) has proven to be an effective treatment for squamous cell carcinoma of the esophagus. The response rate and complete response rate in this study were similar to the rates of 50–70% and 20–30%, respectively, reported in previous studies.[2] Although the patients underwent esophagectomy followed by CRT, the response to CRT was the most significant variable affecting patient survival.[3,6] Given this result, sensitivity to chemoradiotherapy might be an important biological prognostic factor.

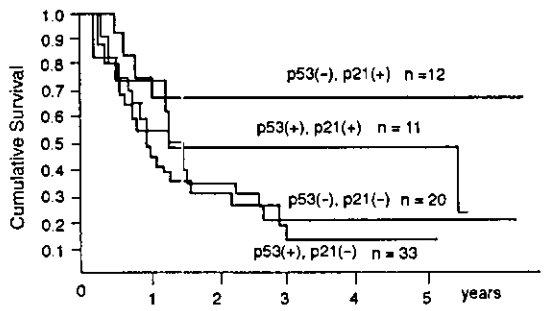

Fig. 3 In patients with p53-negative tumors, the probability of survival for patients with p21-positive tumors was significantly higher than patients with p21-negative ($P = 0.0452$), but no significant difference was found in patients with p53-positive tumors ($P = 0.1085$).

Table 3 Immunohistochemical p53, $p21^{Waf1/Cip1}$ cyclin D1, and CDC25B expression in pretreatment biopsy and resected specimens after chemoradiotherapy

Expression (Before/after)	p53	$p21^{Waf1/Cip1}$	cyclin D1	CDC25B
Positive/positive	13	1	4	3
Negative/negative	6	17	13	12
Positive/negative	3	1	2	2
Negative/positive	0	3	3	5

Wild-type p53 is thought to be involved in the regulation of the cell cycle at the transition from G1 to S phase and in the process leading to apoptosis following irradiation.[7] Although several clinical studies have analyzed the relationship between alternations in p53 and response to CRT, the results for esophageal cancer have been inconsistent.[8–11] In this study, p53 expression correlated with resistance to CRT, although without significance, but was not associated with patient survival. These conflicting results might reflect the multiformity of the genetic changes involved; therefore, investigation of the expression of other genes could be necessary to predict the response to CRT. The p21 gene is transcriptionally activated by p53 and is responsible for the p53-dependent checkpoint that results in G1 arrest after DNA damage.[12] A positive effect of the combined use of cisplatin and radiotherapy will only occur in patients with tumors sensitive to cisplatin.[20] Our previous study showed that p53-positive and p21-negative tumors were resistant to chemotherapy (cisplatin and 5-FU) in esophageal cancer.[21] The p53+/p21-tumors reflect complete abrogation of p53 function, potentially preventing the activation of the apoptotic cascade in response to DNA-damaging drugs.[22] Although most p53+/p21-tumors also showed resistance to CRT, p21 expression was an independent variable in predicting patient survival in this study.

Expression of p21 was a predictor of survival following treatment by radiotherapy or CRT, independent of p53 expression, in rectal and pancreatic cancer.[13,14] An additional mechanism of p21 expression might be associated with a response to CRT in the G2 or M phase. However, recent studies have demonstrated that loss of p21 in colon cancer cells arrested in a G2-like state resulted in apoptosis after irradiation, while a p21-deficient xenograft tumor was cured whereas a case of a tumor with intact p21 genes resulted in no cure after treatment with irradiation.[23] However, either the p53 or the p21 gene-disrupted cells progressed into mitosis and exhibited a G2 DNA content after irradiation because expression of both p21 and p53 is essential to sustain the G2 checkpoint after DNA damage.[24] G2 arrest accompanying irradiation of esophageal squamous cells decreases when p21 protein production is blocked via antisense oligonucleotides.[25] The function of p21 in directing a cell with DNA damage

Table 4 Univariate and multivariate analysis to identify factors predictive for survival of the 76 patients who complete planned chemoradiotherapy

Categories (variables)	Univariate (P)	Multivariate, relative risk (95% CI)	P
Performance status (0 vs 1–2)	0.0176	0.766 (0.567–1.020)	0.0687
T (T3 vs T4)	0.4358	0.942 (0.696–1.259)	0.690
M (Lymph) (M0 vs M1)	0.0152	0.749 (0.567–0.998)	0.0487
$p21^{Waf1/Cip1}$ (negative vs positive)	0.013	2.379 (1.700–3.313)	< 0.0001

to undergo either apoptosis or mitosis at the G2 checkpoint has not been fully elucidated.

In mammalian cells, p21 inhibits not only the activity of each member of the cyclin/cyclin-dependent kinases (CDKs) family, but also, cell proliferation.[26] Expression of p21 was detected frequently in early pathologic stage tumors and was a marker of favorable prognosis in patients with gastric cancer treated by surgery.[27] Expression of p21 was a significant predictor of a favorable outcome of surgery in the p53-negative group but it was not associated with survival in the p53-positive group in esophageal cancer.[28] This prognostic value of p21 expression in the p53-dependent pathway was also found in the patients treated by CRT in this study. Distant metastasis of p21-negative tumors was more frequently detected than that of p21-positive tumors, but this trend did not reach levels of significance. Furthermore, p21 expression was frequently detected in well-differentiated squamous cell cancer and in localized tumors, which were shown to respond well to CRT. Therefore, the comparatively long survival of patients with p21-expressing tumors might partially be due to less aggressive tumors.

Although the immunohistological examination of biopsy specimens may not be reliable enough to use clinically, it has pervaded general hospitals and its cost is not high. Although the reliability of immunohistochemistry can be proven by the continuity of p53 expression, expression of a protein such as p21, which is sensitive to CRT, cannot be verified in the resected specimens after CRT. These results were in concordance with those of an examination of p53 and p21 expression in resected specimens of colorectal cancer after radiation therapy.[29] Evaluating differing expression of cyclin D1 between biopsy and resected specimens might be shown to be an inadequate method of examination of biopsy specimens. In contrast, CDC25B-expressing tumors might be highly sensitive to CRT, because most of these tumors had disappeared in the resected specimens after irradiation.

Cyclin D1 amplification has been demonstrated to be a significant marker of shorter survival and hematogenous metastasis after surgery in esophageal cancer.[30] The previous study also showed that cyclin D1 expression was a marker of resistance to CRT and shorter survival after CRT.[15] In this study, no difference in cyclin D1 expression was found in response to CRT and the length of survival after CRT. These results suggest that CRT is more effective than surgery for patients with cyclin D1-positive tumors. CDC25B phosphatase plays a key role in controlling G2–M progression by dephosphorylating two inhibitory residues of CDC2. Cancer cells that overexpress CDC25B override G2–M arrest by retaining CDC2 kinase activity and these undergo apoptosis after irradiation. Overexpression of CDC25B is associated with a high sensitivity to radiotherapy in esophageal cancer.[13] The present study shows that CDC25B-expressing tumors tend to achieve a response without significance and survival of the patients with CDC25B-positive tumors was slightly better than that of the patients with CDC25B-negative tumors.

In conclusion, p21 expression in pretreatment biopsy specimens can predict CRT response and is an independent variable prognosticator after CRT. Expression levels of p53, cyclin D1 and CDC25B were not valuable as markers for the response to CRT and patient survival. Clearly, the predictive value of p21 expression for the response to CRT and patient survival also needs to be assessed in larger, multicenter trials.

Acknowledgments

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The expressions of p21 and pRB may be good indicators for the sensitivity of esophageal squamous cell cancers to CPT-11: Cell proliferation activity correlates with the effect of CPT-11

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Previously, we demonstrated that CPT-11 is an effective agent against esophageal squamous cell cancers (ESCC), and that the protein level of DNA topoisomerase I can be a predictor for sensitivity to CPT-11 (*Jpn J Cancer Res* 2001; 92: 1335–41). Here, we describe our search for additional predictors of sensitivity to CPT-11, mainly among cell cycle-regulating proteins, because the cytotoxicity of CPT-11 is significantly correlated with the percentage of ESCC cells in S-phase. To this end, we selected and examined the expressions of 5 proteins involved in G_1-S transition, i.e., p53, cyclin D1, p21, p27, and pRB, in 14 ESCC cell lines by western blot analysis. Among these proteins, the expression levels of p21 and pRB showed significant differences that were associated with the IC_{50} values for CPT-11 (P=0.0339 and P=0.0109, respectively). Namely, the expression of p21 or pRB independently could be a good indicator of CPT-11 efficacy in ESCC. In addition, the cell proliferation activities examined by enzyme-linked immunosorbent assay (ELISA) using 5-bromo-2′-deoxyuridine (BrdU) showed a significant correlation with the percentage of total S-phase cells (correlation coefficient=0.568, P=0.0324), and an inverse correlation with the IC_{50} values for CPT-11 (correlation coefficient= −0.601, P=0.0213). Because, as in the case of DNA topoisomerase I, the cell proliferation activity determined using BrdU shows a close relationship with the MIB-1 labeling index, immunohistochemical studies of p21, pRB, and MIB-1 in resected ESCC specimens and/or biopsy samples could make it possible to predict more precisely the sensitivity of ESCC patients to CPT-11 prior to treatment. (Cancer Sci 2004; 95: 464–468)

In recent years, many anti-cancer drugs have been developed that show potent anti-tumor activities against various experimental and clinical cancers. Moreover, combination chemotherapy using several anti-cancer drugs plays an important role in improving therapeutic efficacy. For esophageal squamous cell cancers (ESCC), *cis*-dichlorodiammineplatinum (II) (CDDP) is the key drug used for treatment, with 5-fluorouracil and/or leucovorin mainly used together as biochemical modulators.[1,2] Recently, paclitaxel, docetaxel, and CPT-11 have been selected as therapeutic agents against ESCC, and some clinical trials using these agents together with CDDP[3–5] have indicated them to have a significant clinical impact. Anti-cancer drugs demonstrate excellent therapeutic efficacy if the cancer cells are sensitive to the drugs. However, inappropriate anti-cancer treatments could cause rapid tumor progression in addition to severe side effects. Therefore, it is very important to predict the sensitivity of a cancer to anti-cancer drugs on the basis of molecular biological characteristics to avoid ineffective or harmful treatments, as well as to improve the poor prognosis of ESCC patients.

Previously, we demonstrated that CPT-11 is an effective anti-cancer agent for ESCC, and that the protein level of DNA topoisomerase I can be used as a predictor for sensitivity to CPT-11.[6] In ESCC, several cell cycle-regulating factors, such as p53, p21, cyclin D1, and pRB, exhibit abnormal expressions, and play important roles in tumor invasion, metastatic potential, and the patients' prognosis.[7–13] Because the cytotoxicity of CPT-11 is S-phase-specific,[14] the abnormal expressions of proteins that regulate G_1-S transition through the cell cycle could have important correlations with sensitivity to CPT-11. Therefore, this study was undertaken in an attempt to find additional predictors, mainly among cell cycle-regulating proteins, for sensitivity to CPT-11. The cell proliferation activities of ESCC cell lines were also examined for a correlation with the therapeutic efficacy of CPT-11.

Materials and Methods

Cell lines. Twelve human ESCC cell lines of the TE series[15] were kindly provided by Dr. T. Nishihira of the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. EC-GI-10 was obtained from the Riken Gene Bank, and TT was from the Human Science Research Resources Bank. Ten cell lines (TT, TE-1, TE-2, TE-5, TE-8, TE-10, TE-11, TE-12, TE-13, TE-15) were established from primary ESCC lesions, and the rest were from metastatic lesions.

Cells were grown in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% sterile, filtered fetal bovine serum (Moregate BioTech, Bulimba, QLD, Australia) and 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Antibiotec-Antimycotic, Gibco) at 37°C in a humidified 5% CO_2 atmosphere.

Cell cycle analysis. Cell cycle analysis was performed by fluorescence-activated cell sorting (FACS) as previously described.[16] Briefly, semi-confluent ESCC cells grown in culture dishes were suspended in 1 ml of 0.5% RNase solution at a cell concentration of 1×10^6/ml. Cells were stained with propidium iodide at a final concentration of 50 μg/ml, and analyzed with a FACSCalibur (Becton Dickinson, San Jose, CA). The percentages of total S-phase cells were evaluated using Modfit LT ver. 3.0 for Macintosh (Verity Software House, Inc., Topsham, ME). The percentages of total S-phase cells are expressed as the means of 3 independent experiments.

Antibodies. Anti-p53 antibody (DO-1, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at a dilution of 1:4000, anti-cyclin D1 antibody (DCS-6, DAKO, Glostrup, Denmark) at 1:200, anti-p21 antibody (F-5, Santa Cruz Biotechnology,

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Inc.) at 1:200, anti-p27 antibody (F-8, Santa Cruz Biotechnology, Inc.) at 1:200, and anti-pRB antibody (G3-245, Pharmingen, San Diego, CA) at 1:500. As an internal control, anti-β-actin antibody (AC-15, Sigma, St. Louis, MO) was used at 1:5000 dilution. As the secondary antibody, anti-mouse IgG-AP (Santa Cruz Biotechnology, Inc.) was used at 1:2000 dilution.

Immunoblot analysis. Protein extraction and immunoblotting were performed essentially as described previously.[6] To extract protein from cells, culture dishes containing semi-confluent cells were washed 3 times with ice-cold phosphate-buffered saline (PBS), and then the cells were harvested. Cells were lysed in buffer including 50 μg/ml phenylmethylsulfonyl fluoride (PMSF), 5 μl/ml aprotinin, 5 μg/ml leupeptin, 5 μ*M* NaF, and 0.2 μ*M* sodium orthovanadate in NETN [20 m*M* Tris-HCl (pH 8.0), 100 m*M* NaCl, 1 m*M* EDTA, and 0.5% NP-40], and incubated on ice for 30 min. Protein extracts were obtained after centrifugation at 14,000 rpm for 15 min at 4°C, and quantified by means of the Bradford assay (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA).

The extracted proteins were boiled with an equal volume of 2× SDS sample buffer [2% SDS, 0.1% bromophenol blue, 100 m*M* dithiothreitol, and 10% glycerol in 50 m*M* Tris-HCl (pH 6.8)], resolved by electrophoresis in SDS-polyacrylamide gels, and transferred to PVDF membranes (Hybond-P, Amersham Pharmacia Biotech, Buckinghamshire, UK).

The membranes were blocked with 5% powdered milk in TBST [10 m*M* Tris-HCl (pH 8.0), 150 m*M* NaCl, and 0.075% Tween 20] for 1 h at room temperature, and then incubated overnight with a primary antibody at 4°C. The membranes were then washed 4 times with TBST, and the bound antibodies were detected using alkaline phosphatase-conjugated secondary antibodies, and developed using Immune-Star (Bio-Rad Laboratories) with a lumino-image analyzer (LAS-1000, Fuji Film, Tokyo).

The antibodies were removed by washing the membranes in removal solution [100 m*M* 2-mercaptoethanol, 2% SDS, and 62.5 m*M* Tris-HCl (pH 6.7)] at 60°C for 30 min, and the membranes were re-blotted for β-actin to confirm that equal amounts of protein were loaded.

Cell proliferation assay. The proliferation activities of the ESCC cell lines were analyzed by enzyme-linked immunosorbent assay (ELISA) using a Cell Proliferation ELISA system, version 2 (Amersham Pharmacia Biotech) according to the manufacturer's instructions. In brief, suspensions of each of the 14 ESCC cell lines at 2.5×10^5 cells/ml were seeded in 96-well microplates. After incubation at 37°C in a humidified 5% CO_2 atmosphere for 24 h, the cells were exposed to 10 μ*M* 5-bromo-2′-deoxyuridine (BrdU) solution for 2 h, followed by cell fixation, DNA denaturation and blocking. After incubation with peroxidase-labelled anti-BrdU, the wells were rinsed and 3,3′,5,5-tetramethylbenzidine (TMB) in 15% dimethylsulfoxide (DMSO) was added. The samples were mixed for 10 min, after which 1 *M* sulfuric acid was added to stop the reaction, and the absorbance was measured at 450 nm using a microplate reader (Model 550, Bio-Rad Laboratories). The cell proliferation activity values are expressed as the means of at least 3 independent experiments, each performed in 5 replicates.

Statistical evaluation. Univariate analysis was performed by means of the Mann-Whitney *U* test. The correlation test was performed using Pearson's correlation coefficient. The values of the percentage of total S-phase cells, cell proliferation activity, and IC_{50} (50% inhibition of cell growth of treated cells compared with control cells) are expressed as mean±standard deviation (SD). All statistical evaluations were performed by Stat View 5.0 for Macintosh (HULINKS, Inc., Tokyo). $P<0.05$ was considered statistically significant.

Results

Relationship between sensitivity to CPT-11 and the percentage of total S-phase ESCC cells. Because the cytotoxicity of CPT-11 is S-phase-specific,[14] we hypothesized that tumors with a high percentage of S-phase cells were more likely to be damaged by CPT-11, and that CPT-11 would be effective against such tumors. Therefore, at first, the cell cycles of 14 ESCC cell lines were analyzed by FACS (Fig. 1A). The percentages of total S-phase cells ranged from 13.213 to 45.453 with a mean±SD of 27.558±10.011. The percentages of total S-phase cells in primary tumor cell lines and metastatic tumor cell lines were 29.086±9.299 and 23.739±12.150, respectively, showing no significant difference by the Mann-Whitney *U* test.

To examine the relationship between the therapeutic efficacy of CPT-11 and the percentage of S-phase cells, the IC_{50} values for CPT-11 described previously (Fig. 2)[6] and the percentages of total S-phase cells of 14 ESCC cell lines were analyzed. An inverse correlation was observed between the IC_{50} values for CPT-11 and the percentage of total S-phase cells (correlation coefficient=−0.631, $P=0.0138$: Fig. 1B). This result may indicate that CPT-11 is effective against ESCC tumors with a high percentage of S-phase cells.

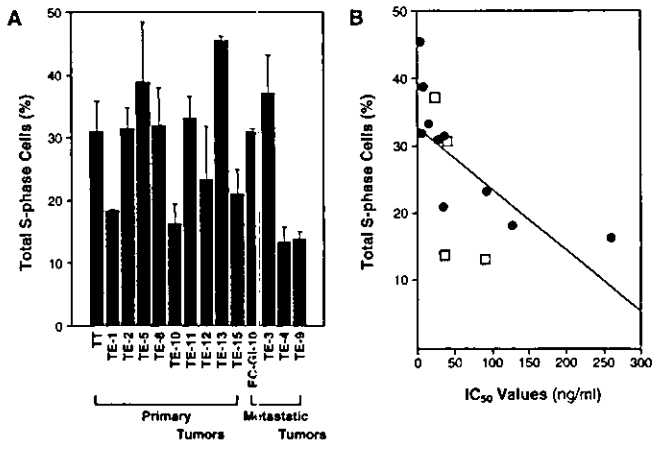

Fig. 1. Cell cycle analysis of 14 ESCC cell lines performed by FACS (A). The percentages of total S-phase cells ranged from 13.213 to 45.453 with a mean±SD of 27.558±10.011. The mean percentages of total S-phase cells in primary tumor cell lines (●) and metastatic tumor cell lines (□) were 29.086±9.299 and 23.739±12.150, respectively (B). The percentages of total S-phase cells in the 14 cell lines show an inverse correlation with the IC_{50} values for SN-38 (correlation coefficient= −0.631, $P=0.0138$).

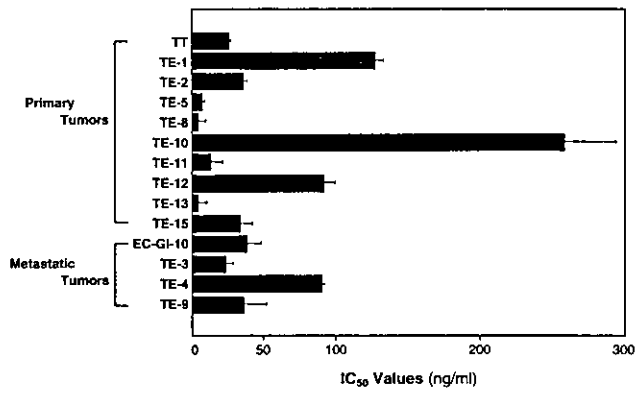

Fig. 2. IC_{50} values of 14 ESCC cell lines for SN-38, an active metabolite of CPT-11, were determined by growth inhibition assay as described before.[6]

Relationship between sensitivity to CPT-11 and the expression of cell cycle-regulating proteins. Because ESCC tumors with a high percentage of S-phase cells may respond to CPT-11, the expression levels of cell cycle-regulating proteins, especially those involved in G_1-S transition, could be good indicators for CPT-11 sensitivity. Therefore, we examined the expressions of 5 cell cycle-regulating proteins (p53, cyclin D1, p21, p27, and pRB) in 14 ESCC cell lines by western blot analysis (Fig. 3). The overexpression of p53 and cyclin D1 was detected in 8 (57.1%) and 5 (35.7%) cell lines, respectively. High levels of p21 and p27 expression were detected in 10 (71.4%) and 6 (42.9%) cell lines, respectively. There was no significant difference in the expression levels of these proteins between primary tumor cell lines and metastatic tumor cell lines (data not shown). As for pRB, 3 cell lines (TE-1, TE-10, and EC-GI-10) showed no expression, and one cell line (TE-4) showed only the hypophosphorylated form. When pRB is hypophosphorylated, the cells are either quiescent or in early G_1-phase. However, the protein samples analyzed were extracted from cycling cells in culture dishes. Therefore, it is unlikely that all TE-4 cells were in G_0 or early G_1 phase. It has been reported that some mutated forms of pRB cannot be phosphorylated, and, as a result, show functional loss.[17-20] The TE-4 protein band detected was considered to represent an aberrant and non-functional pRB. As a result, these 4 cell lines were categorized as abnormal for pRB expression. There was also no significant difference in terms of pRB expression between primary tumor cell lines and metastatic tumor cell lines (data not shown).

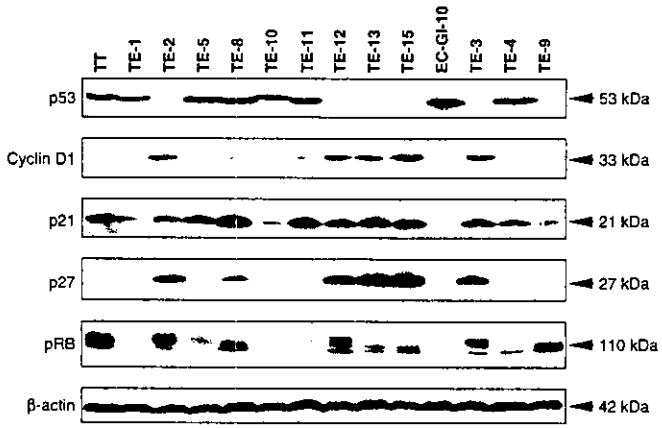

Fig. 3. Western blotting for p53, cyclin D1, p21, p27, pRB, and β-actin in 14 ESCC cell lines. Protein samples extracted from each of 14 ESCC cell lines were separated in SDS-polyacrylamide gels, and transferred to PVDF membranes. The protein bands were detected by chemiluminescence. p53 was detected as a 53 kDa band, cyclin D as a 33 kDa band, p21 as a 21 kDa band, p27 as a 27 kDa band, pRB as a 110 kDa band, and β-actin as a 42 kDa band. The overexpression of p53 and cyclin D1 was detected in 8 (57.1%) and 5 (35.7%) cell lines, respectively. High expression levels of p21 and p27 were detected in 10 (71.4%) and 6 (42.9%) cell lines, respectively. The TE-1, TE-10, and EC-GI-10 cell lines showed no pRB expression, while pRB in TE-4 cells was in a hypophosphorylated state.

Among 5 cell cycle-regulating factors, the expression levels of p21 and pRB showed significant differences in terms of the percentages of total S-phase cells by the Mann-Whitney U test (P=0.0477 and P=0.0339, respectively: Table 1). The percentages of total S-phase cells in the high and low p21 expression groups were 30.670±9.367 and 19.778±7.623, respectively, and the percentages of total S-phase cells showing normal and abnormal pRB expression were 30.719±9.266 and 19.656±7.756, respectively. These results suggest that ESCC cell lines showing a low expression of p21 and/or aberrant expression of pRB have significantly low percentages of total S-phase cells.

The IC_{50} values for CPT-11 in 14 ESCC cell lines have been described before.[6] Using those results, the relationship between the expression levels of cell cycle-regulating proteins and the efficacy of CPT-11 was examined by means of the Mann-Whitney U test (Table 2). As a result, the expression levels of p21 and pRB showed significant differences that were associated with the IC_{50} values for CPT-11 (P=0.0339 and P=0.0109, respectively). In particular, the mean IC_{50} values of the low p21 expression group and the aberrant pRB group were 114.845 ng/ml and 128.560 ng/ml, respectively. These IC_{50} values are remarkably high, and, thus, CPT-11 is expected to be ineffective in cases showing a low expression of p21 and/or abnormal expression of pRB. These results suggest that the expression of p21 or pRB independently can be a good indicator of CPT-11 efficacy in ESCC.

Table 1. Relationship between percentages of total S-phase cells and cell proliferation activity values, and the expressions of cell cycle regulating proteins

	Percentage of total S-phase cells (mean±SD)	*P* value	Cell proliferation activity value (mean±SD)	*P* value
p53		0.6985		0.1213
Normal	28.688±11.606		2.410±0.245	
Overexpression	26.711±9.381		1.836±0.738	
Cyclin D1		0.2571		0.0719
Normal	25.266±9.788		1.866±0.696	
Overexpression	31.684±0.050		2.472±0.215	
p21		0.0477		0.0237
High	30.670±9.367		2.342±0.496	
Low	19.778±7.623		1.432±0.471	
p27		0.1556		0.1556
High	31.724±8.990		2.383±0.291	
Low	24.434±10.118		1.857±0.744	
pRB		0.0339		0.0047
Normal	30.719±9.266		2.426±0.340	
Abnormal	19.656±7.756		1.223±0.153	

Table 2. Relationship between IC_{50} values of CPT-11 and the expressions of cell cycle-regulating proteins

	IC_{50} value (mean±SD)	*P* value
p53		0.6985
Normal	37.200±29.072	
Overexpression	70.718±87.556	
Cyclin D1		0.6407
Normal	66.776±82.750	
Overexpression	37.592±32.486	
p21		0.0339
High	32.956±32.446	
Low	114.845±105.049	
p27		0.2453
High	32.123±31.996	
Low	74.525±84.901	
pRB		0.0109
Normal	27.470±25.633	
Abnormal	128.560±94.214	

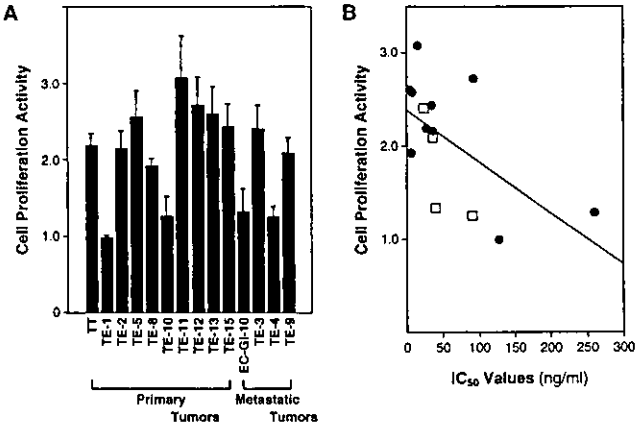

Fig. 4. Cell proliferation activity values of 14 ESCC cell lines measured by ELISA using BrdU (A). Cell proliferation activity values ranged from 0.999 to 3.083 with a mean±SD of 2.082±0.635. The mean cell proliferation activities of primary tumor cell lines (●) and metastatic tumor cell lines (□) were 2.203±0.648 and 1.780±0.565, respectively (B). The cell proliferation activity values of the 14 cell lines show an inverse correlation with the IC_{50} values for SN-38 (correlation coefficient=−0.601, *P*=0.0213).

Relationship between sensitivity to CPT-11 and cell proliferation activity. The cell proliferation activities of 14 ESCC cell lines *in vitro* were determined by ELISA using BrdU (Fig. 4A). The cell proliferation activity values ranged from 0.999 to 3.083 with a mean±SD of 2.082±0.635. The cell proliferation activity values of primary tumor cell lines and metastatic tumor cell lines were 2.203±0.648 and 1.780±0.565, respectively, and there was no significant difference between these 2 groups (Mann-Whitney *U* test).

A significant correlation was found between the cell proliferation activity values and the percentages of total S-phase cells (correlation coefficient=0.568, *P*=0.0324). Because BrdU is incorporated instead of thymidine into the DNA during DNA synthesis in proliferating cells, the cell proliferation activity using BrdU shows the ability to synthesize DNA, and reflects the percentage of total S-phase cells. As in the cell cycle analysis, among the 5 cell cycle-regulating factors, the expressions of p21 and pRB showed significant differences with cell proliferation activity values according to the Mann-Whitney *U* test (*P*=0.0237 and *P*=0.0047, respectively: Table 1). This suggests that ESCC cell lines showing low expression of p21 and/or aberrant expression of pRB have significantly poorer cell proliferation activities. Moreover, there was an inverse correlation observed between the IC_{50} values for CPT-11 and the cell proliferation activity values (correlation coefficient=−0.601, *P*=0.0213: Fig. 4B). This may indicate that high cell proliferation activity leads to an increased chance of ESCC cells entering S-phase, resulting in increased DNA damage by CPT-11; thus, cell proliferation activity could be an important indicator for sensitivity to CPT-11.

Discussion

In the previous study, we described the efficacy of CPT-11 for the treatment of ESCC, and suggested that the protein level of DNA topoisomerase I could be a predicting factor for the effect of CPT-11.[6] In the present study, we have found a significant relationship between the effect of CPT-11 and the percentage of S-phase cells. Following this result, we focused on cell cycle-regulating factors, and examined the relationship between the expression of individual cell cycle-regulating proteins and the therapeutic efficacy of CPT-11. As a result, the expressions of p21 and pRB were found to be predicting factors for sensitivity of ESCC to CPT-11. Namely, ESCC cell lines showing a low expression of p21 and/or aberrant expression of pRB were remarkably unresponsive to CPT-11 treatment.

Moreover, in this study, we examined cell proliferation activity using BrdU, and found a relationship with sensitivity to CPT-11. Because it has been reported that cell proliferation activity as determined using BrdU is closely related to the MIB-1 labeling index,[21-23] immunohistochemical studies of MIB-1 may make it possible to predict sensitivity to CPT-11 before treatment. Namely, our results suggest that immunohistochemical examinations of p21, pRB, and MIB-1, in addition to DNA topoisomerase I, in resected ESCC specimens and/or biopsy samples could be good indicators for sensitivity of ESCC patients to CPT-11.

CDDP is currently the key drug for the treatment of ESCC. However, its therapeutic efficacy is not satisfactory. Recently, CPT-11 has been reported to be effective for the treatment of many solid cancers, and has been selected as a second-line therapy. As for ESCC, CPT-11 is reported to be effective as a second-line chemotherapeutic agent when used with CDDP or docetaxel;[5,24-26] and is a feasible option for use as a second-line chemotherapeutic agent. However, at the same time, CPT-11 causes severe adverse effects such as diarrhea, neutropenia, and nausea. ESCC patients with unresectable or relapsed tumors often develop poor performance status. Therefore, especially for second-line chemotherapy, it is important to predict the chemosensitivity of the cancer prior to treatment to avoid ineffective or harmful results. Our data suggest that examining the protein expressions of p21, pRB, and MIB-1 might be helpful for predicting the sensitivity of ESCC patients to CPT-11.

As for the relationship between ESCC chemosensitivity and cell cycle-regulating proteins, p53,[27,28] cyclin D1,[29] p21,[30-32] and MIB-1[33] are reported to be effective markers. Many anticancer drugs, including CPT-11, demonstrate cytotoxicity by damaging DNA, and have S-phase-specific activity. Therefore, to examine the expressions of p21, pRB, and MIB-1 may help to predict tumor sensitivity to many anti-cancer agents, not only CPT-11.

Recently, various factors, such as p16ink4, NF kappaB, and MDM2, which regulate sensitivity to CPT-11, have been reported in many solid cancers.[34-36] In the future, it will be necessary to clarify the mechanism of the anticancer effect of CPT-11 and to look for more predicting factors for sensitivity to

CPT-11. This will help to identify enhancers of the therapeutic effect of CPT-11, and thus may lead to an improvement in the poor prognosis of ESCC patients.

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根治的化学放射線療法後にsalvage手術を施行した胸部食道癌症例

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症例報告

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近年，局所進行例のほか，切除可能な食道癌に対しても根治的な化学放射線療法（chemoradiotherapy；CRT）が選択され，その結果salvage手術を経験する機会が多くなっている．当施設ではこれまでに根治的CRT後の食道癌に対するsalvage手術を5例経験した．全例臨床病期III期以上の進行症例で，初回治療としてFP療法と放射線治療の同時併用療法を施行し，CR 1例，PR 4例であった．Salvage手術は，3例は局所の再発・再燃に対して行い，2例はCRT後の残存病巣の切除目的にて行った．手術は全例，右開胸開腹食道亜全摘・3領域郭清と大彎側胃管による後縦隔経路再建を施行した．5例中2例に術後合併症を認めたが，縫合不全はなく，また手術関連死もなかった．現在4例が無再発生存中である．当施設においてはsalvage手術により良好な成績が得られているが，今後その意義を明らかにするために，症例の蓄積と検討が必要である．

はじめに

食道癌に対する治療は多様化し，従来外科治療が第1選択とされていた進行度の症例に対しても根治を目的とした化学放射線療法（chemoradiotherapy；以下，CRT）が施行されるようになり，手術と同等の成績が得られるようになってきた[1)~3)]．初回治療として根治的CRTを施行し，その後局所の遺残や再発・再燃を認めた症例に対してsalvage手術を施行する機会も増えてきている．食道癌に対するsalvage手術は，現在の食道外科領域におけるトピックスの1つであるが，その報告例は少ない[4)~7)]．

当施設においては，これまでに根治的CRT後のsalvage手術を5例経験したので，その成績について術後経過を中心に報告する．

症　例

2003年6月までに，食道癌に対する初回治療としての根治的CRT施行後に，salvage手術を施行した5症例を検討した（Table 1）．全例男性で，年齢は46～68歳であった．腫瘍の占居部位は胸部中部食道（Mt）が4例，胸部上部食道（Ut）が1例であった．組織型は全例扁平上皮癌で，分化度は低分化型が3例，中分化型が2例であった．治療前の臨床病期はIII期2例，IVa期2例，IVb期1例であった．また，これは当施設において同期間内に切除可能と判断した食道癌症例に対して根治的CRTを施行した54例中，7.4%に相当し

Table 1　Clinical characteristics of 5 cases of salvage operations

Case	Age/ Gender	Location*1	Histology*2	Clinical Stage
1	46/M	Mt	poorly diff. SCC	T3N2M0-III
2	61/M	Mt	moderately diff. SCC	T4N1M0-IVa
3	61/M	Ut	poorly diff. SCC	T3N4M0-IVa
4	68/M	Mt	moderately diff. SCC	T3N3M1-IVb
5	48/M	Mt	poorly diff. SCC	T4N0M0-III

*1 Ut；upper thoracic esophagus, Mt；middle thoracic esophagus
*2 SCC；squamous cell carcinoma

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た（症例4は臨床病期IVb期と判断しておりこれを除く）．以下，臨床および病理病期は，食道癌取扱い規約第9版[8]に準じて記載した．

1．CRTおよび手術に至る経過

CRTは全例にcisplatin（CDDP）とfluorouracil（5-FU）による化学療法（FP療法）と放射線治療の同時併用療法を施行した．CDDP（40mg/m²；Day 1，8）と5-FU（400mg/m²；Day 1～5，8～12），および放射線照射（2Gy/日；Day 1～5，8～12，15～19）を5週ごとに2コース行うことを基準としているが，2例（症例2，3）で3コース目の化学療法（CDDP 80mg/m²，Day 1と5-FU 800 mg/m²，Day 1～5）を施行し，1例（症例4）は好中球減少のため2コース目の化学療法を中止した．また放射線治療は，4例は頸部から食道胃移行部（esophagogastric junction；EGJ）までのT字型の領域に60Gyを照射し，1例（症例1）は胸部食道からEGJまでのI字型の領域に60.8Gyを照射した．CRTによる有害事象は，Grade 2以上の好中球減少を4例に認め，また1例でGrade 4の腎不全・敗血症を併発した．治療効果は完全寛解（complete response；CR）が1例，部分寛解（partial response；PR）が4例であった．

3例は治療終了後経過観察中に局所再発・再燃をきたしたため手術を行い，2例はCRT後の残存病巣に対して手術を施行した．1例（症例1）は局所の再燃後に患者が化学療法を希望したため，FP療法を1コース施行した．しかし効果なく，その後患者の同意を得て手術を施行した．CRT終了後から手術までの期間は2～19か月（中央値11か月）であった（**Table 2**）．

2．手術および術後経過

手術は全例に右開胸開腹食道亜全摘・3領域郭清，および大彎側胃管による後縦隔経路再建を施行した．手術時間は270～605分，術中出血量は245～1,180mlであった．1例（症例3）で術後呼吸不全に対して4日間人工呼吸器管理を要したが，残る4例は術後手術室にて気管内チューブを抜管した．術後合併症は5例中2例に認めた．1例（症例3）は頸部リンパ漏にて再手術を要し，1例（症例4）は成人呼吸切迫症候群（acute respiratory distress syndrome；ARDS）を併発した（**Table 3**）．

Table 2 Regimen of radical CRT and clinical course

Case	CRT *1		Response	Periods from CRT to Operation (months)	Operative Indication
	Chemotherapy	Radiotherapy			
1	FP 2 cycles	I-shape 60.8 Gy	PR	19	local recurrence
2	FP 3 cycles	T-shape 60 Gy	CR	4	local recurrence
3	FP 3 cycles	T-shape 60 Gy	PR	2	residual tumor after CRT
4	FP 1 cycle *2	T-shape 60 Gy	PR	19	local recurrence
5	FP 2 cycles	T-shape 60 Gy	PR	2	residual tumor after CRT

*1 Regimen of concurrent chemo-radiotherapy
chemotherapy；5-FU（400mg/m²）on Days 1-5, 8-12 and CDDP（40mg/m²）on Days 1,8
radiotherapy；2Gy/f × 5f/w on Days 1-5, 8-12, 15-19
*2 The 2nd cycle of chemotherapy was canceled because of persisted leukopenia.

最終的な病理病期はI期2例，III期2例，IVa期1例であった．1例（症例3）で口側切除断端に癌細胞の浸潤を認めたため，術後にNedaplatin 40 mg/m²（Day 1，8）と5-FU 400mg/m²（Day 1～5，8～12）による化学療法を1コースと，放射線治療（計30Gy）を施行した．また，病理病期IVa期であった症例5には，術後補助療法としてFP療法（CDDP 40mg/m²，Day 1と5-FU 400mg/m²，Day 1～5）を現在施行中である．

3．予後

1例（症例3）は術後10か月目に原病死したが，残る4例は術後3～74か月経過した現在，無再発生存中である．

4．症例提示

症例1

経過：平成7年4月，嚥下困難を主訴に当院内科入院．精査にて，胸部食道癌T3N2M0 stage IIIと診断した．また，食道アカラシアを合併していた．同年4月27日～8月25日にFP療法2コースと放射線治療（計60.8Gy）によるCRTを施行した．1コース目にGrade 4の好中球減少，腎不全，および敗血症を併発したため，2コース目は化学

Table 3 Perioperative factors

Case	Operative Time (minutes)	Blood Loss (ml)	Postoperative Morbidity	Oral Intake (POD*2)	Discharge (POD)	Pathological Stage	Prognosis (months)
1	270	245	(−)	15	55	T1bN0M0-Ⅰ	alive (no recurrence) 74
2	605	1,000	(−)	7	21	T3N2M0-Ⅲ	alive (no recurrence) 30
3	505	700	Lymphorrhea of the neck (re-operation)	26	116*1	T3N2M0-Ⅲ	death 10
4	475	1,180	ARDS	11	28	T1bN0M0-Ⅰ	alive (no recurrence) 14
5	455	750	(−)	7	14	T1N4M0-Ⅳa	alive (no recurrence) 2

Operation method ; transthoracic subtotal esophagectomy with 3-field lymphadenectomy and reconstruction with the stomach through retro-mediastinal route

*1 Adjuvant chemotherapy consisting of 5-FU (40mg/m²) and nedaplatin (40mg/m²) and radiotherapy (30Gy) were administered concurrently because oral resection stump was positive for cancer.

*2 POD ; postoperative day

療法を20%減量して施行した．治療効果はPRであったが，初回治療時の有害事象を考慮して，追加治療は行わず経過観察とした．平成9年1月に局所の再燃を認めた．精査にて新たな再発・転移巣は認めなかった．患者が化学療法による治療を強く希望したため，FP療法（30%減量）を1コース施行した．しかし効果なく，その後患者が手術に同意し，当科紹介となった．

手術：同年3月12日に手術を施行した．胸腔内の組織の線維瘢痕化は軽度で，剥離操作にほとんど影響はなかった．また，麻酔中経過にも問題なく，術後も手術室にて気管内チューブを抜管し，病棟へ帰室した．

術後経過；術後は経過良好で，特に問題はなかった．術後55日目に軽快退院し，術後74か月経過した現在も無再発生存中である．

最終病理組織検査では，低分化扁平上皮癌pT1b (SM) N0M0で，病理病期Ⅰ期であった．また，CRTの病理組織学的な治療効果はGrade 2であった．

症例4

経過：平成12年5月，胸部食道癌にて当院内科に紹介入院となった．両側肺野に散在する小結節影を認め，肺転移と判断，T3N3M1 stage IVbと診断した．同年5月29日～8月7日にFP療法1コースと放射線治療（計60Gy）によるCRTを施行した．Grade 3の好中球減少が遷延したため，2コース目の化学療法は中止し，放射線治療のみ施行した．CRT後，原発巣は消失し，生検においても癌遺残は認めなかった．また，肺腫瘤は画像上変化なく，全体としての治療効果はPRであったが，以後経過観察とした．平成14年1月に局所の再燃を認めた．精査にて肺腫瘤は画像上変化なく，また新たな再発・転移巣も認めなかったため，手術目的にて当科紹介となった．

手術：同年3月1日に手術を施行した．開胸所見より，転移と考えていた肺腫瘤は陳旧性結核結節と判断し，根治手術を施行した．組織の線維瘢痕化が著しく，剥離操作は困難で時間を要したが，麻酔中経過に問題なく，術後も手術室にて気管内チューブを抜管した．また，methylpredonisoloneを術直前に500mgおよび術翌朝に250mg投与した．

術後経過：術後は良好に経過し，術後4日目に