Table 3: Contents and results of radiotherapy (with or without chemotherapy) for postoperative recurrent esophageal cancer. in previous studies. The numbers in parenthesis are a numbers of patients.

author	year	No.	regimen	response rate	median survival time	1-year survuval rate
JL Raoul [13]	1995	24	radiotherapy + chemotherapy	65%	-	47.1%
H Yamanaka [25]	1998	17	radiotherapy + CDGP§ + 5-FU#	76.50%	-	-
K Nemoto [15]	2001	33	radiotherapy alone(21) or radiotherapy + CDDP* + 5-FU (12)	-	7 months	33%
Y Nishimura [12]	2003	13	radiotherapy + CDDP + 5-FU	72%	9.5 months	28%
H Shimada [14]	2003	76	chemoradiotheray(47), chemotherapy alone(17), radiotherapy alone(12)	34%	8 months	31%
K Nemoto [16]	2003	7	radiotherapy + CDGP + 5-FU	100%	-	69%

* CDDP: cisplatin, § CDGP: nedaplatin, # 5-FU: 5-fluorouracil

the patients. Thrombocytopenia of grade 3 or higher was observed in one patient, diarrhea of grade 3 or higher was observed in two patients, and heartburn or mucositis of grade 3 or higher was observed in one patient. These grade 3 hemotoxicities were controllable and transitory, and some patients with grade 3 hemotoxicity were therefore able to complete the regimen without suspension of treatment or reduction of dose in the second cycle of chemotherapy. Grade 1 renal toxicity was observed in one patient, but no patient had renal toxicity of grade 2 or higher. These toxicities were manageable and there was no fatal (grade 5) toxicity in the acute phase (Table 4). However, one patient died 6 months after the protocol due to serious pericardial effusion. There were no patients often than the patient who had grade 3 or higher toxicities in the late phase, although grade 1 or 2 focal pulmonary fibrous change, pericardial effusion and/or pleural effusion were often observed. There was a strong correlation between radiation field (T-shaped or local alone) and acute adverse events (<grade 3 or >grade 3), rate of occurrence of grade 3 or higher adverse events being significantly higher in patients who underwent radiotherapy with a T-shaped field ($p = 0.046$; Pearson's product moment correlation coefficient = -0.367).

In univariate analysis, the difference between survival rate in performance status ($p = 0.033$, Exp (B) = 4.599, 95%CI = 1.131–18.696), age ($p = 0.034$, Exp (B) = 0.909, 95%CI = 0.833–0.993) and recurrent pattern ($p = 0.024$, EXP (B) = 0.261, 95%CI = 0.081–0.836) were statistically significant (Table 5).

Discussion

There have been some studies on the effectiveness of radiotherapy (with or without chemotherapy) for treatment of postoperative recurrent esophageal cancer. In those studies, the median survival periods were 7.0~9.5 months and the 1-year survival rates were 28~69% (Table 3) [12-16,25]. The median survival periods in previous studies on the effectiveness of chemotherapy alone for treatment of recurrent esophageal cancer were similar, 5.0~10.5 months [10,17-19]. None of the results were good. In 2001, we also reported the results of a study on the effectiveness of radiotherapy (with or without concurrent chemotherapy) for treatment of postoperative locoregional recurrent esophageal cancer: the median survival period of patients who did not undergo chemotherapy was 7.0 months, the median survival period of patients who underwent chemotherapy (CDDP and 5-FU) was 9.0 months, and the overall 1-year and 3-year survival rates were 33 and 15%, respectively [15]. In 2003, we reported the results of a study on the effectiveness of radiotherapy combined with CDGP + 5-FU in our institution for treatment of 7 patients with postoperative locoregional recurrent esophageal cancer: the 1-year survival rate was 69% (The median survival period could not be calculated.) [16]. Compared with these results, the results of the present study showing overall median survival period of 39.0 months and 1-year survival rate of 60.6% were excel-

Table 4: Major toxicities related to this regimen (the Common Terminology Criteria for Adverse Events: CTCAE v3.0)

Toxicity	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
neutropenia	16.7%	33.3%	30%	0%	0%
thrombocytopenia	3.3%	0%	0%	3.3%	0%
heartburn or mucositis	50%	3.3%	3.3%	0%	0%
renal toxicity	3.3%	0%	0%	0%	0%
diarrhea	16.7%	20%	3.3%	3.3%	0%

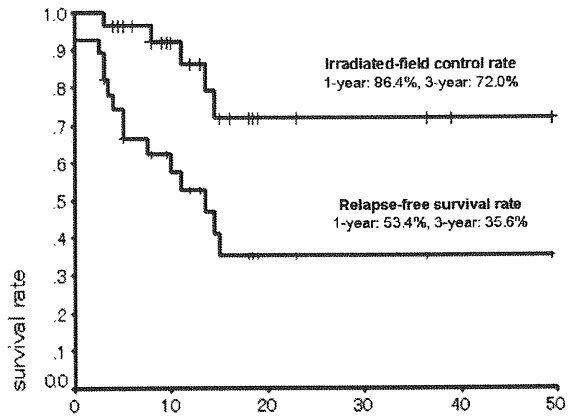

Figure 4
Relapse-free survival and irradiated-field control rates/(Kaplan-Meier method).

lent. Although it seems natural that chemoradiotherapy is more effective than radiotherapy or chemotherapy alone because of their synergistic and/or additive effects, the high rate of completion of the regimen because of less toxicities of CDGP and the high local control rate might have contributed to the better results in our study than those in previous studies on chemoradiotherapy. Although we have no evidence, CDGP or CDGP + 5-FU might have a greater synergy with radiation than CDDP or CDDP + 5-FU. However, we also should consider some biases: for example, the observation period was too short to determine long-term results, patients with performance status of 4 were excluded, patients who had hematogenous metastasis were excluded, all patients had squamous cell carcinoma by pure chance, high dose radiotherapy could be performed easily because of the unused adjuvant nor neoadjuvant therapy in association with initial surgery, and some of the 16 patients who had relapse again were treated with second-line salvage chemotherapy (docetaxel alone, TS-1 alone, or combined with CDDP or CDGP and docetaxel) after relapse again. The median survival period of the 16 patients after relapse again was 9.5 months (95%CI = 2.4–15.6).

However, only 5 of the 30 patients in the present study had relapse again inside the irradiation field. Irradiated-field control rates were 86.4% at 1-year and 72% at 3-year. Eleven of the 30 patients had relapse again in lymph nodes outside the irradiation field or in distant organs. There was no significant difference between survival periods of patients who had relapse again inside the irradiated-field and patients who had no relapse again inside irradiated-field. However, we believe that the high irradiated-field control rate of this protocol contributed to prolongation of survival. Although there was also no significant difference (median survival time; one region vs. multiple regions: 39.0 vs. 6.5 months, p = 0.19), number of recurrent regions also might have no small effect on survival.

Regarding the optimal irradiation field, we have experienced whether we need to irradiate to three-field including the bilateral supraclavicular, mediastinal and celiac regions or just the recurrent region of recurrence. In the present study, a T-shaped field was used in 11 patients. Although there was no significant difference, the median survival period of patients who received irradiation to the recurrent region alone was longer than that of patients who received T-shaped field irradiation (local vs. T-shaped: 39.0 vs. 14.0 months, p = 0.17; log-rank test). It was thought that this difference in the median survival period was due to the fact that a T-shaped field was often used for patients having multiple regional recurrences, although there was no significant correlation. Moreover, the problem of the significantly high rate of adverse events in patients who were treated with a T-shaped field remains, although most of these adverse events were controllable. To our knowledge, there is no report about irradiation fields for postoperative recurrent esophageal cancer. Prospective randomized studies on irradiation fields are needed.

The optimal radiation dose for recurrent esophageal cancer had also not been determined. About 60 Gy of radiotherapy combined with chemotherapy is preferred in Japan. Since TD5/5 (prediction radiation-dose of normal tissue complication probability at 5% within 5 years after radiotherapy) of the stomach is 60 Gy [27] and since one of our patients died of necrosis of the stomach, which had been used for thoracic esophageal substitution, 6 months after the end of 66 Gy radiotherapy, we avoid irradiation to the gastric tube with a total dose of more than 60 Gy. We have no major matter of stomach using 60 Gy/30 fractions/6 weeks radiotherapy. But the tolerance radiation-dose of gastric tube with chemotherapy has not been known. Although patients with primary esophageal cancer in the Intergroup Trial (INT) 0123 (Radiation Therapy Oncology Group (RTOG) 94-05) study were assigned randomly to receive combined-modality therapy consisting of CDDP (75 mg/m^2 bolus day 1) + 5-FU (1000 mg/m^2/24 hours for 4 days) with concurrent 64.8 Gy of radiotherapy or the same chemotherapy schedule but with concurrent 50.4 Gy of radiotherapy, there was no significant difference in median survival, 2-year survival, or local/regional failure and local/regional persistence of disease [26]. Therefore, at present, four cycles of CDDP + 5-FU combined with 50 Gy of radiotherapy is a standard chemoradiotherapy regimen for advanced esophageal cancer in U.S.A.. As for primary esophageal cancer, a total dose 50 Gy radiotherapy with concurrent chemotherapy may be

Table 5: Prognostic factors. Cox's proportional hazards regression model was used for univariate survival analysis.

factor	group	No.	median survival time	log-rank	univariate
			(month)	p	p
performance status	0–1	25	39	0.018	0.033
	2–3	5	8		
age	≥65	12	39	0.017	0.034
	<65	18	10		
preoperative stage (UICC§ 1997)	I – II	9		0.87	0.87
	III – IV	19	39		
number of cycles of chemotherapy	1	5	9	0.50	0.501
	2	25	39		
time interval between surgery and recurrence	>13	14		0.083	0.212
	≤13	16	14		
tumor response (RECIST*)	CR-PR	22	39	0.27	0.277
	SD-PD	8	12		
field	local	19	39	0.17	0.183
	T-shaped	11	14		
relapse again inside irradiated field	+	5		0.57	0.594
	-	25	39		
number of recurrent regions	one	23	39	0.19	0.206
	multiple	7	6.5		
recurrent pattern	local	9	6.0	0.015	0.024
	non-local	21	39.0		

*RECIST: Response Evaluation Criteria in Solid Tumors, §UICC: Union International Contre le Cancer
Three blank columns show that the median survival times could not be calculated.

sufficient for recurrent esophageal cancer. It is necessary to investigate prospectively the optimal radiation dose for postoperative locoregional recurrent esophageal cancer.

CDGP has shown less renal toxicity but an anti-tumor effect similar or superior to that of CDDP in some preclinical and clinical studies [20-23]. Although the optimal doses of CDGP and 5-FU with radiotherapy have not been determined, we used doses of the anti-tumor drugs in the present study based the report by Yoshioka et al. [25]. They administered CDGP at a dose of 80 or 100 mg/m^2/2 hours and 5-FU at a dose of 350 or 500 mg/m^2/24 hours for 5 days and recommended 100 mg CDGP/m^2 and 500 mg 5-FU/m^2. In the present study, we decided to administer CDGP at a 70% dose of their recommendation with consideration of concurrent radiotherapy.

The major toxicities are listed in Table 4. About major toxicities of this protocol in the acute phase, grade 4 toxicity was observed in only two patients. Grade 3 or higher toxicities of neutropenia, thrombocytopenia and esophagitis occurred in 30%, 3.3% and 3.3% of the patients, respectively. However, these grade 3 toxicities were temporary or controllable, and the protocol was performed in 23 patients without suspension or discontinuation and without reduction in the dose of chemotherapy. Therefore, the rate of completion of this regimen was high (76.7%). Compared to results of some clinical studies on chemoradiotherapy, for example, a phase II study by Ohtsu et al. [28], Japan Clinical Oncology Group Trial (JCOG) 9516 [29], a report by Burmeister et al. [30], RTOG 85-01 [31] and INT 0123 [26], which cause a standard regimen for primary esophageal cancer in U.S.A., the rate of grade 4–5 toxicity in this study was low. The chemoradiotherapy protocol used in this study is therefore feasible and safe. The results of several studies, including the present study, indicate that CDGP + 5-FU is no less safe and effective than CDDP + 5-FU [16,17,24,25]. Extensive prospective randomized studies are needed to compare the effectiveness and safety of radiotherapy combined with CDGP + 5-FU and those of radiotherapy combined with CDDP + 5-FU.

As prognostic factors of postoperative recurrent esophageal cancer, PS, age (worse for younger patients) and recurrent pattern (worse for patients with anastomotic recurrence), which had no correlation with others, were significantly associated with survival in univariate analysis in the present study (Table 5). The reason for the poor prognosis of young patients is not known. Tumors in younger patients may be aggressive, although there was no significant correlation between age and the time interval between surgery and relapse in the present study. The reason for the poor prognosis of patients with anastomic recurrence might be because of the significant correlation with PS and pattern of recurrence ($p = 0.002$, Pearson's product moment correlation coefficient = -0.539; the patients with anastomic recurrence were worse PS). We

previously reported that the interval between surgery and relapse was a prognostic factor of recurrent esophageal cancer [15], but this was not selected as a prognostic factor in univariate analysis in the present study.

Conclusion

The present protocol of radiotherapy combined with nedaplatin and 5-fluorouracil is a safe and effective salvage treatment for postoperative locoregional recurrent esophageal cancer.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

KJ and KN drafted the manuscript. KJ and EN performed statistical analysis. KN, YT and SY participated in the study design and coordination. HM, CT, YO and TS performed the chemoradiotherapy and the follow-up. All the authors have read and approved the final manuscript.

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Pre-publication history

The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-2407/6/50/prepub

Tohoku J. Exp. Med., 2006, **208**, 299-306

Predictive Factors for Acute Esophageal Toxicity in Thoracic Radiotherapy

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TAKEDA, K., NEMOTO, K., SAITO, H., OGAWA, Y., TAKAI, Y. and YAMADA, S. *Predictive Factors for Acute Esophageal Toxicity in Thoracic Radiotherapy.* Tohoku J. Exp. Med., 2006, **208** (4), 299-306 —— Acute esophageal toxicity (AET) is a common complication and dose-limiting toxicity in thoracic radiotherapy. Previous studies demonstrated several clinical and dosimetric parameters of AET in patients with lung cancer. However, there are few reports dealing with these variables in intra-thoracic malignancies, including lung cancer and other thoracic malignancy. The purpose of this study was to evaluate the clinical and dosimetric factors associated with AET in patients with intra-thoracic malignancies. We examined 61 patients with intra-thoracic malignancies treated with radiotherapy: 34 patients with non-small-cell lung cancer (55%), 9 cases with small-cell lung cancer (15%), 7 cases with thymic cancer (11%), 4 thymomas (7%), 2 malignant lymphomas (3%), one seminoma (2%), one liposarcoma (2%), and 3 cases of other malignancies (5%). Radiotherapy was performed with a median dose of 60 Gray (Gy) (range 40-67 Gy). AET was graded according to the Radiation Therapy Oncology Group (RTOG) criteria. The following parameters were analyzed with respect to associations with AET by univariate and multivariate analyses: age, gender, thoracic surgery before radiotherapy, concurrent chemotherapy, duration of radiotherapy, maximum esophageal dose, mean esophageal dose, and percentage of esophageal volume receiving from 10 Gy (V10) to 65 Gy (V65), in 5-Gy increments. 43 patients (70%) developed AET: 36 patients (59%) with AET of RTOG Grade 1, 7 patients (11%) with Grade 2, and no patients (0%) with Grade 3 or worse. On multivariate analysis, V35 > 30% was the most statistically significant factor associated with mild AET ($p = 0.013$). Our findings provide a better understanding of the factors related to AET, and might be useful in designing a treatment plan to prevent severe esophageal toxicity. ——— acute esophageal toxicity; radiotherapy; predictions; thoracic neoplasms

Received October 12, 2005; revision accepted for publication February 2, 2006.

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Acute esophageal toxicity (AET) is known to be a significant dose-limiting toxicity (DLT) and one of the main complications of thoracic radiotherapy (TRT) given for lung cancer (Byhardt et al. 1998; Choy et al. 1999; Bruner et al. 2004).

Previous researches reported the following dosimetric predictors for AET: the percentage of esophageal volume receiving > 45 Gy (V45), V50, V60; the length of the esophagus in the treatment field; the maximal esophageal point dose; hyper-fractionated radiotherapy; and the esophageal surface area receiving ≧ 55 Gy (A55) (Werner-Wasik et al. 2000; Hirota et al. 2001a; Singh et al. 2003; Bradley et al. 2004; Patel et al. 2004; Ahn et al. 2005; Kim et al. 2005). Most of these predictors were related to AET Radiation Therapy Oncology Group (RTOG) criteria grade 2-3 or worse (Cox et al. 1995). In contrast, our previous study indicated that V35 was a significant dosimetric predictor for AET of RTOG grade 1-2 (Takeda et al. 2005).

Several authors have noted significant toxicity not only in lung cancer patients, but also in patients with other intra-thoracic malignancy (Hirota et al. 2001b; Perez and Early 2002; Chen et al. 2004). However, there have been few reports dealing with dosimetric parameters for AET in patients with intra-thoracic malignancies, including both lung cancer and other thoracic malignancy treated with TRT. Thus, in the present study, we evaluated clinical and dosimetric parameters associated with AET in intra-thoracic malignancy patients receiving TRT.

Materials and Methods

Patient characteristics

Between February 2000 and April 2005, a total of 61 patients underwent TRT for lung cancer and other intra-thoracic malignancies at the National Hospital Organization Sendai Medical Center and Tohoku University Hospital in Sendai, Japan. All patients were hospitalized during treatment. To be included in this analysis, the 61 patients had to fulfill the following criteria: their charts, hospital computerized data, and radiotherapy datasets for calculation of dose-volume histograms (DVHs) had to be completed and readily available (Sailer 2000) (Fig. 1); they did not have pre-treatment dysphagia, anorexia, or ingestion difficulties; and they were not undergoing palliative treatment. Patient characteristics are shown in Table 1. The study population included 44 men and 17 women with a median age of 68 years (range 26-88 years). The 43 lung cancer patients' histological types were reported as: adenocarcinoma, 16 patients (26%); squamous cell carcinoma, 13 patients (20%); large cell carcinoma, 3 patients (5%); spindle cell carcinoma, 1 patient (2%); small-cell carcinoma, 9 patients (15%); and not otherwise specified, 1 patient (2%). Four patients (6%) had stage I disease, 1 (2%) had stage II, 10 (17%) had stage IIIa, 11 (18%) had stage IIIb, 13 (21%) had stage IV, and 4 (6%) were treated for recurrent disease after surgery. The histology of the

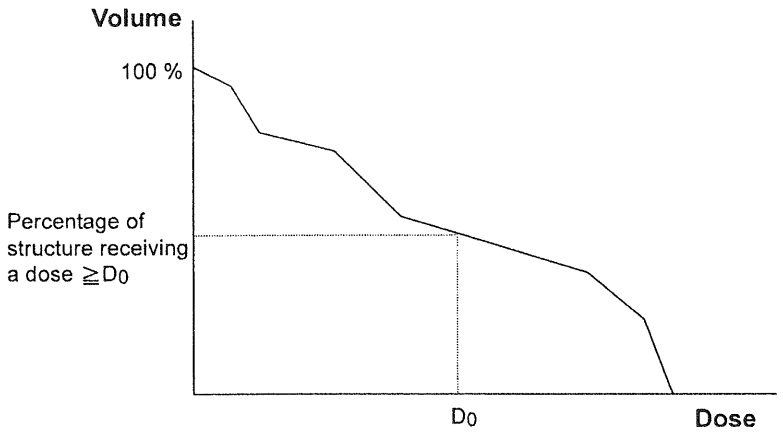

Fig. 1. Dose volume histogram.
The dose-volume histogram (DVH) is one of the fundamental tools used in plan evaluation. At any point on the curve, one is reading the percentage of a volume that receives a dose greater than or equal to the specified dose (Do).

TABLE 1. *Patient characteristics (n = 61)*

Characteristics			*n* (%)
Gender		Men	44 (72)
		Women	17 (28)
Age		Range	26-88
		Median	68
Disease	Lung cancer	Adenocarcinoma	16 (26)
		Squamous-cell carcinoma	13 (20)
		Large-cell carcinoma	3 (5)
		Spindle-cell carcinoma	1 (2)
		Small-cell carcinoma	9 (15)
		Not otherwise specified	1 (2)
	Stage	I	4 (6)
		II	1 (2)
		IIIA	10 (17)
		IIIB	11 (18)
		IV	13 (21)
		Recurrence after surgery	4 (6)
	Other disease	Invasive thymoma	4 (6)
		Thymic well-differentiated cancer	6 (8)
		Thymic undifferentiated cancer	1 (2)
		Heterotopic thyroid cancer	1 (2)
		Diffuse large B-cell lymphoma	1 (2)
		Hodgkin disease	1 (2)
		Seminoma	1 (2)
		Liposarcoma	1 (2)
		Undifferentiated cancer	1 (2)
		Metastatic adenocarcinoma	1 (2)

other diseases was: invasive thymoma, 4 patients (6%); thymic well-differentiated cancer, 6 patients (8%); thymic undifferentiated cancer, 1 patient (2%); heterotopic thyroid papillary carcinoma, 1 patient (2%); diffuse large B-cell type lymphoma, 1 patient (2%); relapsed Hodgkin disease, 1 patient (2%); seminoma, 1 patient (2%); liposarcoma, 1 patient (2%); undifferentiated cancer, 1 patient (2%); and metastatic adenocarcinoma, 1 patient (2%). According to the thymoma staging system of Masaoka et al. (1981), 1 thymoma patient (2%) had stage II, 1 (2%) had stage III, and 2 (3%) had stage IV. Fifteen patients (25%) underwent thoracic surgery before TRT: postoperative prophylactic TRT for lung cancer, 1 patient (2%); invasive thymoma, 3 patients (5%); thymic well-differentiated cancer, 4 patients (6%); heterotopic thyroid papillary carcinoma, 1 patient (2%); seminoma, 1 patient (2%); liposarcoma, 1 patient (2%); and postoperative recurrent lung cancer, 4 patients (6%). We did not include esophageal cancer patients in this study, due to difficulties in evaluating AET in these patients based on clinical and dosimetric analyses. It is very difficult to contour when normal tissue must be distinguished from the malignant portion on computed tomograpy (CT) images in esophageal cancer patients. Furthermore, clinically it is often difficult to clearly determine whether symptoms are due to esophageal cancer or AET.

A total of 41 patients (67%) were treated with concurrent chemoradiotherapy. The regimens of concurrent chemotherapy are summarized in Table 2. Carboplatin was administered at a dose equal to an area under the plasma concentration-time curve of 5-6 every 4 weeks for 2-3 consecutive cycles. Cisplatin (45-80 mg/m^2) was

TABLE 2. *Summary of concurrent chemotherapy*

Chemotherapeutic agents combined with cisplatin or carboplatin	*n* (%)
Paclitaxel	24 (39)
Irinotecan hydrochloride	7 (11)
Vincristine sulfate and etoposide	2 (3)
Vinorelbine ditartrate	1 (2)
Etoposide	5 (8)
Vindesine sulfate and mitomycin C	1 (2)
Docetaxel	1 (2)

given every 3-4 weeks for 3 consecutive cycles. The dose and schedule for the administration of combined agents during the radiotherapy period were: paclitaxel (50-70 mg/m^2) weekly for 3 weeks every 3-4 weeks for 2-3 cycles; irinotecan hydrochloride (45-60 mg/m^2) weekly for 3 weeks every 4 weeks for 3 cycles; vincristine sulfate (1.3 mg/m^2) every 4 weeks for 3-4 cycles; etoposide (60-100 mg/m^2) daily for 3 days every 4 weeks for 3-4 cycles; vinorelbine ditartrate (10 mg/m^2) on days 1, 8, 22, 29, 36, and 44; vindesine sulfate (3 mg/m^2) on days 1, 8, 22, 29, 36, and 44; mitomycin C (8 mg/m^2) every 3 weeks for 3 cycles; and docetaxel (60 mg/m^2) every 3 weeks for 2 cycles.

Treatment planning and treatment

CT-based treatment planning was done in all patients with immobilization devices. Radio-opaque markers were placed on the patient's skin, and the immobilization device was used to assist in positioning. The treatment planning for lung cancer patients was based on gross tumor volume (GTV) that included all the tumors and abnormally enlarged regional lymph nodes greater than 1 cm in diameter seen on CT images (International Commission on Radiation Units and Measurements 1993; Clifford et al. 2002) (Fig. 2). Clinical target volume (CTV) encompassed the GTV, as well as the mediastinal and ipsilateral pulmonary hilar lymph nodes that were regarded as having potential microscopic disease. Planning target volume (PTV) 1 included the CTV and a 1-1.5 cm margin. PTV2 involved the GTV and a 1 cm margin. Radiation was given through the anteroposterior-posteroanterior (AP-PA) portals for PTV1 up to 39.6-40 Gy, followed by off-spinal cord oblique portals for PTV2 in a sequential manner. The treatment planning for patients with other diseases was based on GTV that included all the tumors or residual tumors after surgical resection. CTV included the GTV and a 1-1.5 cm margin, or preoperatively all of the tumors and a 1-2 cm margin. PTV encompassed the CTV and a respiratory movement margin. TRT was directed through the AP-PA portals followed by off-spinal cord oblique portals for PTV sequentially, or two-wedge portals (right and left anterior oblique) for PTV. TRT was delivered by linear accelerators with 4 MV, 6 MV, or 10 MV x-rays using single daily fractions of either 1.8 Gy or 2.0 Gy. The dose to which the spinal cord was exposed was kept below 45 Gy. The median prescription dose to the isocenter was 60 Gy (range 40-67 Gy). Dose calculations were performed to correct for lung in-homogeneity, using superposition algorithm. The median corrected dose for all patients was 60 Gy

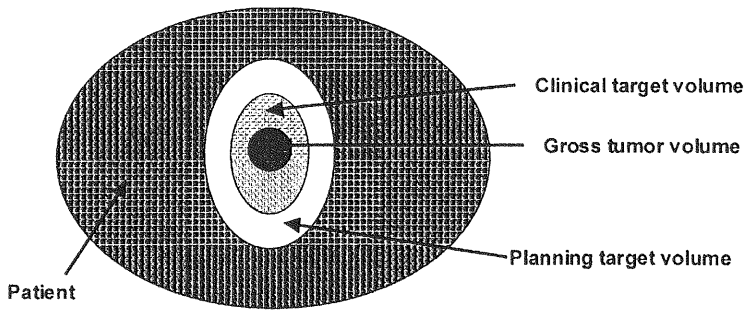

Fig. 2. Volumes of interest.
International Commission on Radiation Units and Measurements Reports No. 50 (ICRU 50) defines the following treatment planning volumes. Gross tumor volume (GTV) encompasses all known gross disease and abnormally enlarged regional lymph nodes. Clinical target volume (CTV) contains the GTV and regions considered to microscopic disease. Planning target volume (PTV) provides margin around CTV to allow for internal target motion, other anatomic motion during treatment, and variations in treatment setup.

TABLE 3. *RTOG scoring criteria for acute esophageal toxicity*

Score	Description
0	No change over baseline
1	Mild dysphagia or odynophagia; may require topical anesthetic, nonnarcotic agents, or soft diet
2	Moderate dysphagia or odynophagia; may require narcotic agents or puree/liquid diet
3	Severe dysphagia or odynophagia with dehydration or weight loss (>15% from pretreatment baseline) requiring nasogastric feeding tube, i.v. fluids, or hyperalimentation
4	Complete obstruction, ulceration, perforation, or fistula
5	Death

RTOG, Radiation Therapy Oncology Group.

(range 40-68 Gy).

Follow-up and evaluation of AET

The follow-up period for all patients was from 2 to 37 months, with a median of 8 months. At least once a week during the course of TRT, patients were evaluated and treated by a radiation oncologist for any complaints. After completion of treatment, patients were followed-up at one-month intervals during the first year and then every 3 to 6 months by their physicians. AET was graded according to RTOG criteria (Cox et al. 1995)(Table 3). The data used to grade the esophagitis reflected the worst grade of toxicity experienced by the patients.

Acquisition of dosimetric data and statistical analysis

The patients' treatment plans were analyzed retrospectively so as to determine the significant factors that could have been involved in causing AET. Our institutional committee did not require ethical approval for this retrospective study. The external surface of the esophagus was contoured uniformly on each 5 mm axial image of the planning CT scan from the level of the lower end of the cricoid cartilage to the gastroesophageal junction by one of the authors. DVHs and dose distributions for the esophagus were calculated. We analyzed the following dosimetric parameters: maximum esophageal dose (Dmax); mean esophageal dose (Dmean); and V10 to V65 in 5-Gy dose increments. Means, medians, and ranges of these parameters are shown in Table 4. Furthermore, we examined the correlation between AET and the following factors: age, gender, thoracic surgery before TRT, concurrent chemotherapy, chemotherapeutic agents, and overall TRT duration. These parameters were analyzed with respect to their relationship with

TABLE 4. *Mean, median, and range of 14 dosimetric parameters*

Parameter	Mean	Median	Range
Dmax (Gy)	53.3	57.8	30.5-68.7
Dmean (Gy)	17.7	17.0	1.9-37.1
V10 (%)	42.0	40.2	5.3-85.7
V15 (%)	39.8	37.1	3.5-83.8
V20 (%)	37.5	33.9	1.6-80.7
V25 (%)	35.2	33.7	0.6-76.1
V30 (%)	31.6	29.9	0.03-74.1
V35 (%)	28.4	28.3	0-70.9
V40 (%)	21.7	21.9	0-66.3
V45 (%)	15.8	11.7	0-59.3
V50 (%)	10.7	5.8	0-54.6
V55 (%)	7.3	1.0	0-42.0
V60 (%)	2.3	0	0-35.9
V65 (%)	0.3	0	0-10.7

Dmax, maximum esophageal dose; Dmean, mean esophageal dose; V10-V65, percentage of esophageal volume receiving 10 Gy to 65 Gy.

Grade 1 or greater esophagitis using Fisher's exact test (two-tailed), chi-square test (two-tailed), and Spearman rank correlation analysis (two-tailed). For multivariate analysis, the stepwise procedure was performed using a logistic regression method containing all variables that achieved univariate statistical significance. Statistical analyses were performed using SPSS (version 11.0 for Windows). Values of $p < 0.05$ were considered statistically significant.

Results

Forty-three of 61 patients (70%) developed AET. The worst AET RTOG grades experienced by the patients were: Grade 1, 36 patients (59%); Grade 2, 7 patients (11%); Grade 3 or worse, 0 patients (0%). In this study, all cases of AET developed during the TRT period, and the patients recovered from AET immediately after TRT completion. All patients had completed TRT without pause due to AET. None of the patients died within 1 month of TRT completion, and none developed severe dehydration.

There were no significant correlations with the following factors: age, $p = 0.868$, correlation coefficient = −0.022 (Spearman's rank correlation); gender, $p = 0.229$ (Fisher's exact test); thoracic surgery before TRT, $p = 0.34$ (Fisher's exact test); concurrent chemotherapy, $p = 0.242$ (Fisher's exact test); chemotherapeutic agents, $p = 0.259$ (Pearson's chi-square test); and overall TRT duration, $p = 0.244$, correlation coefficient = 0.151 (Spearman's rank correlation).

Table 5 shows the results of Spearman's rank correlation for Dmean and V10-V55 to predict AET. On logistic regression analysis, the most statistically significant predictor of AET is V35 ($p = 0.020$). AET developed in 18 of 32 patients (56.3%) with V35 ≦ 30% and in 25 of 29 patients (86.2%) with V35 > 30%, $p = 0.013$ (Fisher's exact test, two tail). AET developed in 6 of 14 patients (42.9%) with V35 ≦ 15% and in 37 of 47 patients (78.7%) with V35 > 15%, $p = 0.018$ (Fisher's exact test, two tail). Furthermore, AET developed in 17 of 44 patients (38.6%) with V35 ≦ 40% and in 16 of 17 patients (94.1%) with V35 > 40%, $p = 0.013$ (Fisher's exact test, two tail). On logistic regression analysis, V35 > 30% was the most significant factor ($p = 0.013$).

TABLE 5. *Correlation between dosimetric parameters and esophagitis*

Parameter	Correlation coefficient	*p*
Dmax (Gy)	0.226	0.080
Dmean (Gy)	0.342	0.007
V10 (%)	0.297	0.020
V15 (%)	0.301	0.018
V20 (%)	0.309	0.016
V25 (%)	0.304	0.017
V30 (%)	0.370	0.003
V35 (%)	0.419	0.001*
V40 (%)	0.405	0.001
V45 (%)	0.357	0.005
V50 (%)	0.333	0.009
V55 (%)	0.259	0.044
V60 (%)	0.134	0.304
V65 (%)	−0.047	0.718

Dmax, maximum esophageal dose; Dmean, mean esophageal dose; V10-V65, percentage of esophageal volume receiving 10 Gy to 65 Gy.

* Highest statistical significance.

Discussion

Previous studies have identified several clinical and dosimetric factors that are related to radiation-induced esophageal toxicity (ET) in patients with lung cancer (Werner-Wasik et al. 2000; Hirota et al. 2001a; Singh et al. 2003; Bradley et al. 2004; Patel et al. 2004; Ahn et al. 2005; Kim et al. 2005; Takeda et al. 2005) (Table 6). However, there have been few studies that have examined these factors for ET in patients with other thoracic malignancies, though several authors have reported on ET in patients with intra-thoracic malignancies (Hirota et al. 2001b; Perez et al. 2002; Chen et al. 2004). According to Chen et al. (2004), Grade 1-2 esophagitis was one of the most common side effects in concurrent chemoradiotherapy for thymic carcinoma. AET occurs not only in lung cancer patients, but also in patients with other intra-thoracic malignancies patients receiving TRT. Therefore, in the present study, we investigated the clinical and dosimetric factors of AET in intra-thoracic malignancies including both lung cancer and other intra-thoracic malignancies.

As shown in Table 6, although most of previous studies indicated parameters of AET of RTOG grade 2 or worse, there are few reports about AET of RTOG grade 2 or less. However, according to previous publications, the incidence of AET of RTOG grade 1 in lung cancer patients is higher

TABLE 6. *Literature review: dose of radiotherapy and dosimetric predictive factors for acute esophageal toxicity*

Study	Dose of radiotherapy	Endpoint	Factors
Werner-Wasik et al. (2000)	45- 69.6 Gy (median, 59.9 Gy) 98 patients with once daily fractions 7 patients with twice daily fractions	Esophagitis index Maximum grade*	CCT CCT, b.i.d RT
Hirota et al. (2001a)	50-60 Gy Single daily fractions of 2 Gy	Grade 2 or worse†	LETT45, V45
Singh et al. (2003)	60-74 Gy (median, 70 Gy) Single daily fractions of 2 Gy	Grade 3 or worse*	Maximal dose ≧ 58 Gy, CCT
Bradley et al. (2004)	60-74 Gy (median, 70 Gy) Single daily fractions of 1.8- 2.1 Gy	Grade 2 or worse*	A55, V60, CCT
Patel et al. (2004)	69.6 Gy Twice daily fractions of 1.2 Gy	Grade 2 or worse*	BMI, V50
Ahn et al. (2005)	30-86.4 Gy (median, 66 Gy) 156 patients with once daily fractions 98 patients with twice daily fractions	Grade 2 or worse* Grade 3 or worse*	Twice daily RT, N stage, age, maximal point dose Twice daily RT, N stage, pre-RT dysphagia
Kim et al. (2005)	54-66 Gy (median, 60 Gy) Single daily fractions of 2 Gy	Grade 3 or worse*	CCT, V60 > 30%
Present study	40-67 Gy (median, 60 Gy) Single daily fractions of 1.8 or 2 Gy	Grade 1-2*	V35 > 30%

CCT, Concurrent chemotherapy; RT, radiotherapy; LETT45, the length of esophagus (total circumference) treated with > 45 Gy; A55, esophageal surface area (cm^2) receiving at least 55 Gy; BMI, body mass index.

* Radiation Therapy Oncology Group scoring criteria for acute esophageal toxicity.

† Modified from National Institutes of Health Common Toxicity Criteria, version 2.0.

than that of severe esophagitis (Werner-Wasik et al. 2000; Ahn et al. 2005). Werner-Wasik et al. (2000) noted 54 patients with RTOG grade 1 esophagitis in their 105 patient series; likewise, Ahn et al. (2005) reported 138 patients with AET of RTOG Grade 1 in their 254 patient series. Therefore, we believe that our result about AET of RTOG grade 2 or less has significance, though it may not be fatal. In the present study, we did not observe any cases of severe AET of RTOG grade 3 or worse, as was the case in our prior report (Takeda et al. 2005). One reason for this may have been that our prescribed doses are lower than those in other studies (Singh et al. 2003; Bradley et al. 2004; Patel et al. 2004; Ahn et al. 2005), or that our study did not use TRT with BID fractionation (Werner-Wasik et al. 2000; Ahn et al. 2005). Another reason may be that concurrent chemotherapy was given consistently in other studies and, therefore, resulted in higher toxicity (Hirota et al. 2001a; Kim et al. 2005).

Although previous authors have noted significant correlations between concurrent chemotherapy and AET in lung cancer treatment (Werner-Wasik et al. 2000; Singh et al. 2003; Bradley et al. 2004; Kim et al. 2005), we did not find a statistically significant relationship between AET and chemotherapy, probably due to the in-

homogeneity of our patients' disease and their treatments, which included various chemotherapeutic regimens, thus lessening the significance of any correlation. Furthermore, as this was a retrospective study, the chemotherapy was not randomly assigned.

In conclusion, the present study has demonstrated that V10-V55 and Dmean are significant parameters associated with mild AET. V35 > 30% appears to be the most critical factor in patients with intra-thoracic malignancies in TRT. Our findings provide new information about AET, and might be useful in designing a treatment plan to prevent severer esophageal toxicity.

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S-1, an oral fluoropyrimidine, enhances radiation response of DLD-1/FU human colon cancer xenografts resistant to 5-FU

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Received February 21, 2006; Accepted April 25, 2006

Abstract. S-1, a novel oral fluoropyrimidine, is increasingly used for the treatment of human cancer including gastrointestinal carcinomas. Using the 5-FU resistant DLD-1/FU human colon cancer cell xenografts, the present study investigated whether S-1 enhances the therapeutic efficacy of radiation and if so what are the underlying mechanisms. Nude mice bearing tumor xenografts were treated with radiation, S-1, or both. Tumor growth delay was the treatments' endpoint. To determine whether S-1 enhances intrinsic cell radiosensitivity, we performed clonogenic cell survival assay. Also we assessed the expression of thymidylate synthase (TS) using immunohistochemistry assay. While S-1 or 5 Gy were only slightly effective as single agents in delaying tumor growth, the combined treatment was highly effective. Clonogenic cell survival showed that S-1 strongly enhanced cell radiosensitivity. Immunohistochemistry showed that the expression of TS was down-regulated in tumors treated by S-1 plus radiation. Combined S-1 plus radiation treatment resulted in a synergistic effect in the therapy of 5-FU resistant human colon carcinoma xenografts (EF=2.06). The effect could be attributed to the ability of S-1 to increase cell radiosensitivity (EF=1.9) and to the down-regulation of TS involved in cellular processes leading to radio- and (or) chemo-resistance.

Introduction

Concurrent chemoradiotherapy, i.e. administering chemotherapeutic agents during the course of radiotherapy, has become a common strategic practice in the therapy of advanced cancer. There exists a solid biological rationale for combining cytotoxic drugs with radiotherapy (1). Because of their systemic cytotoxic action chemotherapeutic drugs reduce the number of cancer cells in the tumor undergoing radiotherapy and act against tumor deposits outside the radiation field. In addition, these drugs may sensitize tumor cells to the cytotoxic effects of radiation by reducing or eliminating intrinsic cellular radioresistance, repair of sublethal and potentially lethal radiation damage, cell cycle related radio-resistance or tumor hypoxia. Also, the drugs can improve tumor radioresponse by counteracting the rapid regeneration of tumor cells during radiotherapy. The improvements in the treatment outcome after concurrent chemotherapy have been observed in terms of increased local tumor control, patient survival and organ preservation rates (2-6). However, concurrent chemoradiotherapy is limited in its application because it is usually associated with a considerable increased normal tissue toxicity, and in spite of achieved advance in treatment outcome the cure rates in the majority of solid tumors still remain poor (2-5). Thus, there is considerable room for improvement of the combined treatment strategies.

This progress in chemoradiotherapy has been achieved mainly by using standard chemotherapeutic agents, which have traditionally been selected for combined treatment based primarily on their known clinical activity in certain disease sites (2-6). 5-Fluorouracil (5-FU) is one of the standard chemotherapeutic drugs commonly prescribed for the treatment of gastrointestinal (GI) tract and head and neck malignancies either alone or in combination with other agents including radiotherapy (2,3,6.7). 5-FU is a pyrimidine analog that interferes with both DNA and RNA synthesis. Its major metabolite, fluorodeoxyuridine monophosphate, inhibits the formation of thymidine by blocking thymidylate synthase. Another metabolite of 5-FU, fluorouridine monophosphate, becomes incorporated into RNA and affects RNA synthesis. Combined with radiation, 5-FU can cause either additive or supra-additive effects (8). Interaction with

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Key words: S-1, radiation, colon cancer

irradiation occurs mainly when the drug is administered within 1 day before irradiation and several hours after irradiation; however, the potentiation of radiation response seems to be greatest when 5-FU is present in cultured cells for ~24 h after irradiation. The exact mechanisms of enhanced cell radiation response are not clear, but they are likely associated with cell cycle redistribution induced by the drug. Clinically, the antitumor efficacy of 5-FU is limited by its toxicity to normal tissues mainly GI toxicity and myelotoxicity, and to its rapid catabolism in the body minimizing its antitumor efficacy (6,7,9).

With respect to 5-FU, a novel fluoropyrimidine derivative, designated S-1, was generated consisting of tegafur (FT), 5-chloro-2, 4-dihydrooxypyridine (CDHP) and potassium oxonate (Oxo) in a molar ratio of 1:0.4:1 (10). FT is a prodrug of 5-FU (11). CDHP and Oxo possess no antitumor activity but modulate certain activities of 5-FU. CDHP competitively inhibits dihydropyrimidine dehydrogenase, which degrades 5-FU, resulting in an increased and prolonged retention of 5-FU in the blood (12). Oxo competitively inhibits pyrimidine phosphoribosyltransferase, which converts 5-FU to 5-fluorouridine-5'-monophosphate (13), and after peroral administration it is distributed mainly within the GI tract leading to reduction in 5-FU-induced GI toxicity (13). S-1 exerts a strong antitumor activity in a variety of rodent tumors and human tumor xenografts (10,14,15). In clinical trials, S-1 has shown considerable antitumor efficacy against a number of common cancers in humans including gastric (16,17), colorectal (18), breast (19), head and neck (20) and lung cancer (21).

Because of its potent antitumor efficacy and good tolerability, S-1 represents a good candidate for combining it with radiotherapy. However, limited information is available on the treatment efficacy when this drug is combined with radiotherapy. Recently, Harada *et al* (22) reported that S-1 increases the *in vivo* radioresponse of tumor xenografts derived from oral cancer cells, and that its active component, 5-FU, has the ability to sensitize the *in vitro* radioresponse of these cells. Using human colorectal carcinoma cell xenografts, sensitive or resistant to 5-FU, the present study investigated whether S-1 enhances cellular radiosensitivity and improves the antitumor efficacy of tumor radiotherapy.

Materials and methods

Mice and tumors. Female BALB/C-nu/nu nude mice, purchased from CLEA Japan (Tokyo, Japan) were 5 to 6 weeks old at the beginning of the experiments and were housed five per cage. The mice were maintained in a specific pathogen-free barrier. Animals used in this study were maintained in facilities approved by the Tohoku university animal facility. Tumor xenografts were derived from the human colorectal carcinoma cell line DLD-1, which is sensitive to 5-FU, and DLD-1/FU, which is resistant to 5-FU. Both cell lines were obtained from the Taiho Pharmaceutical, Co. Ltd (Tokyo, Japan). The 5-FU resistant DLD-1/FU cell line was originally derived from the DLD-1 cell line by continuous *in vitro* exposure of DLD-1 cells to increasing concentrations of 5-FU through a number of successive passages, as described earlier (23). Source tumors were produced by s.c. injection of 10^7 cells into the back of 4-5-weeks-old mice. Tumor cell suspensions were prepared from DLD-1/P or DLD-1/FU cells grown as monolayers *in vitro*. When the source tumors grew to 200-300 mm^3 they were excised and cut into ~2 mm^3 fragments, which were then implanted into the right hind leg of BALB/C-nu/nu mice to generate solitary tumors for the experiments.

Local tumor irradiation. Mice bearing 80-100 mm^3 size tumors in the right hind leg were locally irradiated with a single dose of 5 Gy. A small animal X-ray generator, with a dose rate of 0.72 Gy/min was used. Unanesthetized mice were immobilized on a jig during irradiation. The irradiation was delivered locally to the tumor whereas the remaining body of the mouse was shielded. When S-1 and radiation were combined, S-1 was given orally 1 h before the start of irradiation and continued once daily for 14 days.

S-1 and 5-FU. S-1 is a chemotherapeutic agent prepared by simultaneous mixing of FT, CDHP and Oxo in a molar ratio of 1:0.4:1 and dissolved in 0.5% hydroxypropylmethylcellulose (HPMC). FT, CDHP and Oxo are products of the Taiho Pharmaceutical Co. Ltd. S-1, at a dose of 8 mg/kg, was given to mice orally in a volume of 0.1 ml/10 g body weight daily for 14 consecutive days. The dose of S-1 was expressed as the dose of FT. 5-FU was purchased from Sigma (St. Louis, MO, USA).

Clonogenic cell survival determination. Tumor cells in culture were exposed to 5-FU at IC_{50} doses (inhibition of cell growth 50), 5.8 μM for DLD-1 cells or 350 μM for DLD-1/FU cells, for 2 days. Then the cells were irradiated with graded doses (2, 4, or 6 Gy) of X-rays 0.72 Gy/min. The cells were assayed for colony-forming ability by replating them in specified numbers into 100 mm dishes in a drug-free medium. After 8 days of DLD-1 cell incubation and 14 days of DLD-1/FU cell incubation, the cells were stained with 0.5% crystal violet in absolute ethanol, and colonies with more than 50 cells were counted. Radiation survival curves were plotted after normalizing for the cytotoxicity induced by 5-FU alone. Clonogenic survival curves were constructed from three independent experiments by fitting the average survival levels using least squares regression by the linear quadratic model (24).

Tumor growth delay. Tumor growth delay was the endpoint of treatments (vehicle for the control group, S-1, radiation or both), which were initiated when tumors grew to 80-100 mm^3 in volume [1/2 x (the major axis) x (the minor axis)^2]. To obtain tumor growth curves, two mutually orthogonal tumor diameters were measured with a vernier caliper at 2-3 day intervals and the volumes were calculated. Regression and regrowth of tumors were followed until tumor reached about 1000 mm^3, at which time the mice were sacrificed by cervical dislocation. Tumor growth delay was expressed as the time in days for tumors treated with radiation or S-1 to grow to 4 times their pretreatment volume minus the time in days for untreated tumors to reach the same volume. This is termed as the absolute growth delay (AGD). The effect of the combined TS-1 plus radiation treatment was expressed as

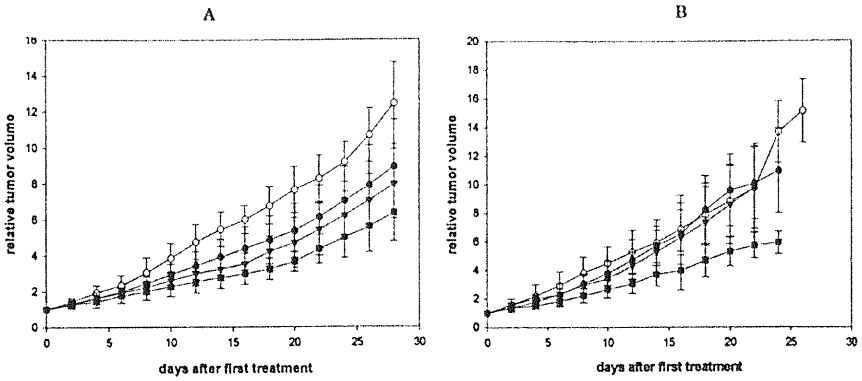

Figure 1. (A) Effect of S-1 on the growth of DLD-1 (sensitive to 5-FU) tumor xenograft. ○, no treatment; ▼, S-1 8 mg/kg; ●, radiation 5 Gy; ■, S-1 8 mg/kg and radiation 5 Gy. Each data point represents the mean volume of 6-9 tumors. Bars, SE. (B) Effect of S-1 on the growth of DLD-1/FU (resistant to 5-FU) tumor xenograft. ○, no treatment; ▼, S-1 8 mg/kg; ●, radiation 5 Gy; ■, S-1 8 mg/kg and radiation 5 Gy. Each data point represents the mean volume of 6-9 tumors. Bars, SE.

the normalized growth delay (NGD), defined as the time for tumors treated with both TS-1 and radiation to grow to 4 times their pretreatment volume minus the time in days for tumors treated with TS-1 alone to reach the same volume. The enhancement factor (EF) was obtained by dividing NGD with the AGD radiation alone. Groups consisted of 8 to 10 mice each.

Thymidylate synthase (TS) immunohistochemical analysis. Tumor xenografts treated with vehicle, 8 mg/kg S-1 for 14 days, 5 Gy, or the combination of the two agents were assessed immunohistochemically for thymidylate synthase (TS) at the end of the treatment with S-1. Using a method described by Miyamoto *et al* (25), the immunoreactivity of TS was examined using an anti-recombinant human TS monoclonal antibody (RTSMA1) (26), kindly supplied by Dr Masakazu Fukushima (Taiho Pharmaceutical Co., Ltd., Tokushima, Japan). All immunohistochemical examinations were performed on tissue sections of formalin-fixed, paraffin-embedded specimens from untreated and treated tumors. Serial 3-μm-thick slices were cut, deparaffinized in xylene, dehydrated with graded ethanol, and then immersed in methanol with 0.3% hydrogen peroxidase for 20 min to inhibit endogenous peroxidase activity. After washing in distilled water, the sections were placed in a 10 mM citrate buffer solution (pH 6.0). For immunohistochemistry, the slides were heated twice at 95°C for 10 min in a microwave oven and cooled for 30 min at room temperature. After washing in phosphate-buffered saline (PBS), non-specific binding was blocked by preincubation with 2% normal swine serum in PBS (blocking buffer) for 60 min at room temperature. All sections were incubated overnight at 4°C with the primary antibodies in blocking buffer at the following concentrations: RTSMA1 1:500 (2 μg/ml). After washing five times in PBS with 0.1% Tween-20 (washing buffer), the slides were incubated with biotinylated second anti-mouse (TS) antibody, diluted 1:200 with blocking buffer for 30 min. After five washes with washing buffer, the sections were incubated with ABC reagent (Vector Laboratories Buringame, CA), and a color reaction was developed using 2% 3-3'-diaminobenzidine in 50 mM Tris buffer (pH 7.6) containing 0.3% hydrogen peroxide, for 5 to 10 min. The sections were counterstained with Meyer's hematoxylin. In the negative controls, the primary antibody solution was replaced by the blocking buffer.

Evaluation of immunostaining. We randomly picked 10 areas in each slide and determined the immunostaining positivity based on a subjective estimation of intensity (0 to 4) in each area. 0, <10% of tumor staining positive; 1, 10-30% of tumor staining positive; 2, 30-50% of tumor staining positive; 3, 50-70% of tumor staining positive; 4, >70% of tumor staining positive. Intensity levels 0 to 2 were considered negative, whereas 2 to 4 staining intensity was considered positive.

Results

Effect of TS-1 on radioresponse of DLD-1 and DLD-1/FU xenografts. Tumor xenografts, generated in the right hind thighs of nude mice by either DLD-1 or DLD-1/FU cells, were 80-100 mm^3 when the treatments were initiated. Mice received a single dose of 5 Gy locally to the tumor, 8 mg/kg S-1 orally daily for 14 days, or both treatments. When the two agents were combined, the first dose of S-1 was administered several hours before tumor irradiation. The relative increase in tumor volume from the start of the treatments is shown in Fig. 1A for the DLD-1 tumor and in Fig. 1B for the DLD-1/FU tumor. S-1, at a dose used here, was more effective than 5 Gy in slowing the growth of the DLD-1 tumor. The combined S-1 plus radiation treatment resulted in greater growth delay than that after the individual treatments, but the effect did not exceed the additive effect by the individual treatments. In comparison to the DLD-1 tumor, the DLD-1/FU tumor responded less well to both radiation and S-1 (Fig. 1B). However, when the two agents were combined, the effect on tumor growth delay was greater than the sum of the effects produced by the individual treatments. To reach 4x the pretreatment volume (Fig. 2), tumors in untreated mice needed 8.7±2.9 days, irradiated tumors 10.7±1.9 days (AGD=2.0 days), tumors in mice treated with TS-1 12±2.2

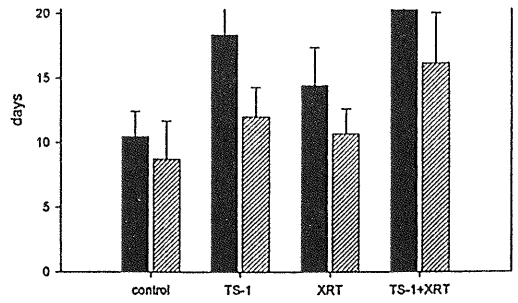

Figure 2. Effect of S-1 on the time taken to increase tumor size 1 to 4 times. Black bar, DLD-1; hatched bar, DLD-1/FU. Each data point represents the mean data of 6-9 tumors. Bars, SE.

days (AGD=3.3 days), and tumors in mice treated with both of these agents needed 16.1±3.8 days (AGD=7.4 days; NGD=4.1 days). This increase in tumor radioresponse was by a factor of 2.1, obtained by dividing NGD of the combined treatment (4.1 days) with the AGD of 2 after radiation alone. This implies that the effect of S-1 plus radiation was synergistic (Table I).

Effect of 5-FU on in vitro cell radiosensitivity. To determine whether the S-1 metabolite, which is 5-FU, affects radiosensitivity of DLD-1 and DLD-1/FU cells, we determined the *in vitro* clonogenic cell survival. DLD-1 cells were exposed to 5.8 μM and DLD-1/FU cells to 350 μM S-1 for two days and then treated with 2 to 6 Gy of X-ray and plated to determine colony formation. After 10 days the DLD-1 cell colonies or after 14 days the DLD-1/FU cell colonies were counted and the survival curves constructed (Fig. 3). Radiation caused a dose-dependent reduction in the cell survival of both cell lines, with DLD-1/FU being more radio-resistant than DLD-1. Treatment with 5-FU only slightly increased the radiation-induced cell killing of DLD-1 cells, but it was strongly effective in increasing the radiation-induced cell killing of DLD-1/FU cells. The enhancement of radiation response of DLD-1/FU cells at the 0.1 cell survival level by a factor of 1.9. Treatment with 5-FU also changed the shape of the radiation cell survival curve by almost completely removing the 'shoulder' region, suggesting that 5-FU may have reduced the ability of tumor cells to repair sublethal radiation damage.

Expression of thymidylate synthase in xenografts. Because thymidylate synthase is an enzyme involved in tumor resistance to both 5-FU (27-29) and radiation (29), it was important to determine whether the levels of this enzyme were affected in DLD-1/FU tumor xenografts treated with S-1, radiation or the combination of the two agents. The doses and schedules of the agents were the same as in the *in vivo* experiment described above. The expression of protein levels of thymidylate synthase was assessed using immunohistochemistry at the end of the chemotherapy treatment, i.e. 14 days after treatment initiation, respectively. Fig. 4 illustrates the thymidylate synthase immunohistochemistry staining of tumor xenografts, untreated or treated with S-1, radiation or both with x200 magnifications. Table II shows the quantitative values for each group. These values were 3.58±0.6 for untreated tumors, 3.25±0.7 for tumors treated with S-1, 3.44±0.7 for tumors treated with radiation and 1.52±0.6 for tumors treated with both S-1 and radiation. Thus, these results show that neither S-1 nor 5 Gy given as single treatments significantly affected the expression of thymidylate synthase, but when combined they strongly down-regulated the expression of this enzyme.

Table I. Enhancement factor.

	Days of 1-4 folds	Absolute growth delay	Normalized growth delay	EF
DLD-1				
control	10,45708	-	-	-
TS-1 8 mg/kg	18,3139	7,85682	-	-
XRT 5 Gy	14,4361	3,97902	-	-
TS-1+XRT	20,2407	9,78362	1,9268	0,48424
DLD-1/FU				
control	8,708717	-	-	-
TS-1 8 mg/kg	12,0044	3,295683	-	-
XRT 5 Gy	10,7099	2,001183	-	-
TS-1+XRT 5 Gy	16,1345	7,425783	4,1301	2,063829

Absolute growth delay (AGD) was defined as tumor growth delay expressed as the time in days for tumors treated with radiation or S-1 to grow to 4x their pretreatment volume minus the time in days for untreated tumors to reach the same volume. Normalized growth delay (NGD), was defined as the time for tumors treated with both S-1 and radiation to grow to 4x their pretreatment volume minus the time in days for tumors treated with S-1 alone to reach the same volume. The enhancement factor (EF) was obtained by dividing NGD with the AGD radiation alone. <1, no effect; 1, additive; >1, synergistic. Groups consisted of 8 to 10 mice each.

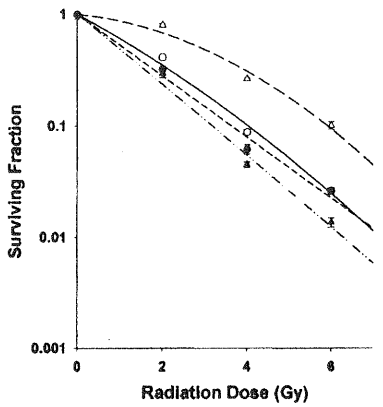

Figure 3. Effect of 5-FU on radiosensitivity of DLD-1 cells and DLD-1/FU cells *in vitro*. Cells were treated with 5-FU (5.8 μM for DLD-1, 350 μM for DLD-1/FU) for 2 days before radiation. ○, DLD-1 control; ●, DLD-1/normal, 5-FU 5.8 μM 2 days; □, DLD-1/FU control; ▼, DLD-1/FU, 5-FU 350 μM 14 days. Values shown are the means ± SE for three independent experiments.

Table II. Evaluation of immunohistochemistry of TS.

	Evaluation of IHC	
	Two weeks after initial treatment	
Control	3.58±0.577	(TS positive)
S-1 8 mg/kg	3.25±0.672	(TS positive)
Radiation 5 Gy	3.44±0.647	(TS positive)
S-1+radiation	1.52±0.624	(TS positive)

Evauation value was determined by the immunostaining positivity based on a subjective estimation of intensity (0 to 4) in each area. 0, <10% of tumor staining positive; 1, 10-30% of tumor staining positive; 2, 30-50% of tumor staining positive; 3, 50-70% of tumor staining positive; 4, >70% of tumor staining positive. Intensity levels 0 to 2 were considered negative, whereas 2 to 4 staining intensity was considered positive.

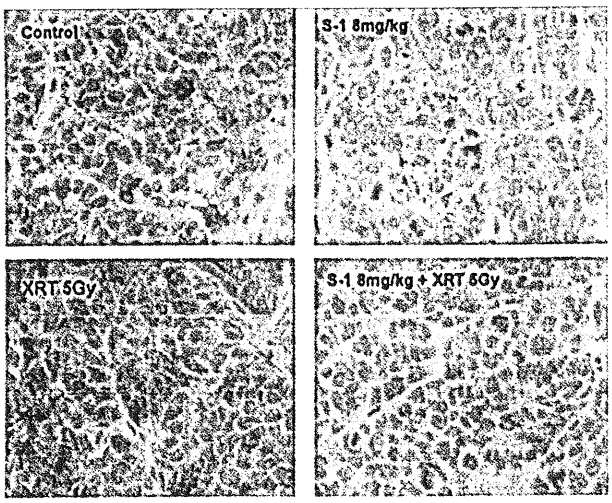

Figure 4. Effect of S-1 on the expression of thymidylate synthase. The thymidylate synthase immunohistochemistry staining of DLD-1/FU tumor xenografts. Upper left, no treatment; lower left, treated with radiation 5 Gy; upper right, treated with S-1 8 mg/kg; lower right, treated with S-1 8 mg/kg plus radiation 5 Gy. TS staining is seen in the cytoplasm area. Original magnification x200.

Discussion

Chemotherapy using 5-FU, or 5-FU-based chemotherapy, in combination with radiotherapy has been a common treatment for many types of human cancer for several decades. This combination treatment improved local tumor control and patient survival rates in many cancers such as head and neck, pancreatic, cervical, esophageal and gastric cancer. It also resulted in improved organ preservation with a good functional outcome in a number of anatomic sites including head and neck and the rectum where it improved sphincter preservation. A variety of administration schedules of 5-FU and radiotherapy has been developed to optimize the antitumor effectiveness and minimize normal tissue toxicity. Although there is no universal schedule, a prolonged continuous infusion of 5-FU has been shown to be superior to dose bolus injection both in terms of tumor response and more acceptable toxicity profile (6). However, poor bioavailability of 5-FU if administered orally, inconvenience with protracted i.v. administration of 5-FU, and a high rate of normal toxicity especially when combined with radiotherapy are issues that have stimulated research on developing oral fluoropyrimidines that would be more effective against tumors and less toxic for normal tissues, either when used alone or in combination with radiotherapy. S-1 was developed with the aim to meet such expectations.

Our present study tested whether S-1 can improve the antitumor efficacy of ionizing radiation, and the effect was assessed using colorectal carcinoma cells, either sensitive (DLD-1 cells) or resistant (DLD-1/FU cells) to 5-FU. The results showed that S-1 increased the *in vivo* radioresponse of tumor xenografts generated by these cells, and that the active component 5-FU enhanced the *in vitro* sensitivity of these cells. However, these effects both *in vitro* and *in vivo* greatly depended on the sensitivity of cells to 5-FU, with S-1 (*in vivo*) and 5-FU (*in vitro*) being strongly effective against DLD-1/FU cells resistant to 5-FU. As shown in Fig. 3, the 5-FU sensitive DLD-1 cells are also more sensitive to ionizing radiation than the 5-FU resistant DLD-1/FU cells. Interestingly, while the 5-FU sensitive cells showed only a slight increase in radiosensitivity when exposed to 5-FU, 5-FU resistant cells were highly radiosensitized, with the enhancement factor of 1.86 at the 0.1 level of cell survival.

S-1 was strongly effective when combined with radiation in the treatment of tumor xenografts. Similarly to the *in vitro* findings, S-1-induced potentiation of the tumor radioresponse differed between DLD-1 and DLD-1/FU tumors. The DLD-1 tumor was sensitive to the treatment with S-1 only. The combined S-1 plus radiation treatment resulted in a greater antitumor efficacy when compared with the efficacy of the individual treatments, but the effects were less than additive. In contrast, although the DLD-1/FU tumor was less responsive to radiation and S-1 as individual treatments, it responded

more than additively when the two agents were combined (Fig. 1B). The enhancement factor was 2.1. Harada *et al* (22) recently reported that S-1 given either 1 h before or 1 h after irradiation, given for 5 consecutive days, improved radioresponse of human head and neck carcinomas, notably B88 squamous cell carcinoma cells derived from a tongue lesion and HSG salivary gland carcinoma cells. These cells were also radiosensitized when treated *in vitro* with 5-FU. However, the magnitude of the increase in radioresponse both *in vitro* and *in vivo* was much higher for DLD-1/FU cells we used in the present study than for head and neck carcinoma cells reported by Harada *et al* (22). In that study, the radiosensitivity of B88 cells was increased by a factor of 1.45, and that of HSG cells by a factor of 1.28.

The mechanisms that underlie the increase in the *in vivo* tumor response to radiation by a chemotherapeutic agent, including S-1, are more complex compared to the *in vitro* induction of radioenhancement. While *in vitro* studies demonstrate a direct interaction on cellular sensitivity, the *in vivo* tumor response depends on many more factors in addition to the direct effect of tumor cell radiosensitivity, including accelerated tumor cell regeneration, tumor angiogenesis and tumor hypoxia (1). An important observation of our study is that the combined S-1 and radiation treatment resulted in significant down-regulation of thymidylate synthase, an enzyme involved in tumor resistance to 5-FU (27-29), and radiation (29). Based on our *in vitro* data, it is likely that increased tumor cell sensitivity to radiation is an important component responsible for the increased radioresponse of *in vivo* tumors. A similar explanation was given for the S-1-induced increase in the radioresponse of human head and neck tumor xenografts, and mechanistically the observed effect was attributed to the induction of apoptosis (22). Because 5-FU constitutes the cytotoxic component of S-1, it is reasonable to assume that both S-1 and 5-FU have similar mechanisms that underlie their radiosensitizing properties. Earlier studies on 5-FU show that this agent enhances tumor cell radio-sensitivity through cell cycle effects and inhibition of the repair of radiation-induced DNA damage (8,30,31). The involvement of this latter mechanism in the observed radioenhancement of DLD-1/FU cells (Fig. 2) is strongly suggested by the shape of the radiation-dose survival curve when the cells were exposed to 5-FU: almost complete loss of the shoulder region fn the cell survival curve.

In conclusion, our results show that treatment with S-1, oral fluoropyrimidine, of tumor DLD-1/FU xenografts derived from human colon carcinoma cells resistant to 5-FU enhances the response of these tumors to local tumor radiotherapy. This response was associated with the down-regulation of thymidylate synthase, an enzyme involved in tumor resistance to 5-FU and radiation. *In vitro* tumor cell survival results showed that S-1 (its active component 5-FU) is a potent enhancer of the radiosensitivity of DLD-1/FU cells, which implies that *in vivo* potentiation of tumor radioresponse is at least partly due to direct interaction between S-1 and radiation on tumor cells. Because of a low toxicity profile of S-1 on normal tissues, these data suggest that S-1 has a potential to improve the therapeutic gain when combined with radiotherapy for colon cancer resistant to 5-FU.

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