survival rate in patients who received surgery alone. The JCOG-9501 trial was designed to compare survival in patients with D2 versus D3 lymph node dissection without adjuvant chemotherapy or RT, while in INT-0116 the majority of patients had a D0 or D1 lymph node dissection and also received chemoRT. In the Japanese trial JCOG-9501 there was a higher proportion of patients with T2 disease than in the INT-0116 trial (49% *v* 26%) and also a lower proportion of patients with T3 disease (44% *v* 62%), respectively. Five-year survival rates were considerably higher (71%) in patients who received surgery only in the Japanese trial versus 42% in patients who received surgery plus chemoRT in the US trial. The Japanese interpretation of these results are that D0 or D1 lymph node dissection plus chemoRT is better than D0 or D1 lymph node dissection alone, but may be worse than D2 surgery alone. Determining whether a D0 or D1 lymph node dissection can replace a D2 lymph node dissection should be evaluated in a randomized, controlled clinical trial; however, D2 lymph node dissection in the United States appears difficult to achieve. Also, whether chemoRT after D2 surgery can improve the results of surgery alone is another unresolved issue.

Several factors should be considered when interpreting the differences in the results of these trials. Because the incidence of gastric cancer is several times higher in Japan than in the United States there are more stringent screening programs in place that may affect the baseline condition of patients accrued onto clinical trials. Moreover, the standard curative resection in the United States is gastrectomy plus D0 or D1 lymphadenectomy, whereas in Japan gastrectomy plus D2 lymphadenectomy with en bloc dissection of the lymph nodes around the common hepatic artery and the splenic artery is used. Japanese surgeons believe that these differences may be because of the additional experience they have acquired due to the higher incidence of gastric cancer in Japan.

The Japanese viewpoint on the use of adjuvant therapy in patients with gastric cancer following curative resection is that the quality of surgery, including diagnostic procedures or pathologic procedures, will be a more important prognostic factor than adjuvant chemotherapy because no survival advantages have been shown in patients with gastrectomy and D2 lymph node dissection in clinical trials. However, standard adjuvant chemotherapy after good local control by surgery (D2 or more) has yet to be established and remains an urgent issue. Also, data from clinical trials indicate that patients with stage 1–2 tumors should be excluded from the target populations of randomized, controlled clinical trials. In the United States and Europe there had been either no or only marginal improvement in OS or disease-free survival for patients receiving adjuvant chemotherapy following gastric cancer resection, until the results of INT-0116 became available, at which time the issue of postoperative chemoRT became the standard treatment for patients with gastric carcinoma. The question as to whether or not chemoRT can improve the results of D2 surgery alone remains unsolved.

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Risk factors for interstitial lung disease and predictive factors for tumor response in patients with advanced non-small cell lung cancer treated with gefitinib

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Summary A high incidence of interstitial lung disease (ILD) has been reported in patients with non-small cell lung cancer (NSCLC) treated with gefitinib in Japan. We retrospectively analyzed 112 patients with advanced NSCLC who received gefitinib monotherapy. Univariate and multivariate analyses were used to identify risk factors for gefitinib-related ILD and predictive factors for tumor response to gefitinib. The incidence of ILD was 5.4%, and it was higher in the patients with pre-existing pulmonary fibrosis (33% versus 2%; $P < 0.001$). The results of a multivariate analysis showed that pulmonary fibrosis was a significant risk factor for ILD (odds ratio: 177, 95% confidence interval: 4.53–6927, $P = 0.006$). The response rate was 33% in the 98 evaluable patients and higher in women (53% versus 23%; $P = 0.003$), patients with adenocarcinoma (38% versus 6%; $P = 0.010$), never-smokers (63% versus 18%; $P < 0.001$), and the patients with no history of thoracic radiotherapy (39% versus 13%; $P = 0.015$). The results of a multivariate analysis showed that the predictors of tumor response were "no history of smoking" and "no history of thoracic radiotherapy". Never-smokers had a significantly longer survival time than smokers ($P = 0.007$). Although gefitinib therapy confers a clinical benefit on patients with advanced NSCLC, especially on women, patients with adenocarcinoma, never-smokers, and patients with no history of thoracic radiotherapy, it also poses a high risk of ILD, especially to patients with pulmonary fibrosis. The risk-benefit ratio must be carefully considered.

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1. Introduction

Gefitinib (Iressa®; AstraZeneca, Osaka, Japan) is an orally available, selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor that displays antitumor activity in patients with previously treated advanced non-small cell lung cancer (NSCLC). The safety and tolerability of gefitinib was established in four open-labeled, multicenter, phase I dose-escalation studies [1–4]. Although diarrhea, skin rash/acne, and nausea were common adverse effects, most of them were mild. Two large-scale, multicenter, randomized phase II studies (IDEAL 1 and 2; Iressa® Dose Evaluation in Advanced Lung Cancer) have demonstrated clinically significant antitumor activity of gefitinib monotherapy in patients with advanced NSCLC who had previously received platinum-based chemotherapy [5,6]. The response rate for gefitinib 250 mg per day in the IDEAL 1 and 2 trials was 18.4 and 11.8%, respectively. These studies also showed that gefitinib monotherapy significantly improved disease-related symptoms and quality of life.

Based on the results of the IDEAL trials, gefitinib was approved in Japan for the treatment of inoperable or recurrent NSCLC on 5 July 2002, and an estimated 28,300 patients had been treated with gefitinib as of April 2003. During the first few months after its approval, many patients demanded to be treated with gefitinib as a "magic bullet" cure; however, when the incidence of interstitial lung disease (ILD) came to light in October 2002, the media reported it in a sensational manner, and as a result patients have become confused by excessive expectations and fear of ILD. The Ministry of Health, Labour and Welfare of Japan reported that the number of gefitinib-related cases of ILD had reached 616 as of 22 April 2003 and that 246 of the patients had died of it. The incidence of ILD and mortality rate from it has been calculated at 2.2 and 0.87%, respectively. Some case reports also suggested a high incidence of gefitinib-related ILD in Japan [7]. In view of this situation, an evidence-based assessment of the risk-benefit of gefitinib for the treatment of NSCLC was urgently needed. However, many questions regarding gefitinib administration remained unanswered, particularly in regard to the risk factors associated with ILD complications. We therefore analyzed a series of cases treated with gefitinib at the National Cancer Center Hospital (NCCH) in Tokyo.

2. Patients and methods

Between July and December 2002, 115 NSCLC patients at the NCCH began taking gefitinib and the 112 of these patients who were followed at the NCCH were retrospectively analyzed in this study. The other three patients were excluded from the analysis because they were followed-up at other hospitals after the first prescription of gefitinib. All the 112 patients had histologically or cytologically confirmed NSCLC. Their disease was locally advanced, recurrent, and/or metastatic. They all received gefitinib monotherapy at a dose of 250 mg per day.

Two independent board-certified diagnostic radiologists (M.K. and U.T.) diagnosed pre-existing pulmonary fibrosis (PF) on the basis of the findings on chest X-rays taken within 1 week of the start of gefitinib therapy. The radiologists had no knowledge of the patients' outcome. The diagnostic criteria for PF were a diffuse linear or honey-comb pattern on chest X-rays that was predominant in the lower zone of the lung.

If a patient had measurable disease, the World Health Organization criteria were used to assess the tumor response. The response rate was calculated as the total percentage of patients with a complete or partial response. Drug-related adverse events were evaluated using the National Cancer Institute-Common Toxicity Criteria (Version 2.0). Chest X-rays were performed periodically to evaluate response and detect pulmonary toxicity, and computed tomography scans of the chest were performed as needed to confirm the response or diagnose ILD. The extent of patients' smoking history was evaluated by using pack-years, which are defined as the average number of cigarettes smoked per day multiplied by the total duration of smoking in years divided by 20. Patients who had smoked for 0, 1–39, and $\geq$40 pack-years were categorized as "never-smokers", "moderate smokers", and "heavy smokers", respectively.

Univariate and multivariate analyses were performed to identify risk factors for ILD and predictive factors for tumor response to gefitinib. The patient characteristics tested as potential risk factors for ILD and predictive factors for tumor response were age ($<$70 versus $\geq$70 years in the univariate analysis and as a continuous variable in the multivariate analysis), sex (female versus male), histological diagnosis (adenocarcinoma versus non-adenocarcinoma), smoking history (never-smokers versus moderate/heavy smokers), performance status (PS 0–1 versus PS 2–3), prior surgery (yes versus no), prior chemotherapy (yes versus no), prior thoracic radiotherapy (yes versus no), and PF (yes versus no). These factors were compared by using a chi-square test in the univariate analysis. Logistic regression analyses were also performed to adjust for each factor. Differences

in time to treatment failure (TTF) and overall survival (OS) among the subgroups were compared by using Kaplan–Meier curves and log-rank tests. TTF was defined as the interval between the start of gefitinib administration and discontinuation of treatment for any reason, confirmed disease progression, or death. All analyses were performed using SPSS statistical package (SPSS version 11.0 for Windows, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Patient characteristics

The patient characteristics are listed in Table 1. All patients were Japanese. Twenty-eight patients (25%) received gefitinib as a first-line treatment; 19 were considered unfit for platinum-based chemotherapy because of poor PS (10 patients) or advanced age (9 patients), and 9 refused platinum-based chemotherapy. The diagnosis of pre-existing PF was almost the same between two radiologists. Although discordance occurred in three cases, 12 patients were finally diagnosed as PF by consensus. All of the 12 patients had computed tomography findings consistent with idiopathic pulmonary fibrosis/usual interstitial pneumonia.

3.2. Interstitial lung disease (ILD) and other toxicities

Among the 112 patients reviewed, ILD developed in 6 (5.4%) during the course of gefitinib therapy, and 4 patients (3.6%) died from ILD. The characteristics of the six patients with ILD are listed in Table 2. All of them had acute onset or exacerbation of respiratory symptoms. In five patients, chest computed tomography scanning revealed new diffuse interstitial changes in both lungs with ground-glass appearances. Because bronchoalveolar lavage or lung biopsy was not performed, we cannot completely exclude lymphangiosis carcinomatosa or other diseases, but the clinical courses and imaging appearances were consistent with drug-induced ILD. Although the other patient (patient 3) died before imaging diagnosis, the autopsy revealed diffuse alveolar damage, and we concluded she died from gefitinib-related ILD.

The results of univariate and multivariate analyses on risk factors for ILD are shown in Table 3. The incidence of ILD was 33% (4/12) among patients with PF and 2.0% (2/100) among the other patients. PF was the only significant risk factor for ILD in the univariate analysis (odds ratio [OR]: 16.7, 95% confidence interval [95% CI]: 3.40–83.3, $P < 0.001$), and this finding was supported by the results of the multivariate analysis (OR: 177, 95% CI: 4.53–6927, $P = 0.006$). Since all of the patients with ILD were smokers, pack-years were analyzed as a continuous variable in the multivariate analysis, and the results of it suggested the association between increased pack-years and a higher risk of ILD ($P = 0.062$). Since all of the ILD cases had a PS score of 1 and had never undergone thoracic radiotherapy, it was impossible to assess the association between poor PS or prior thoracic radiotherapy and ILD in the multivariate analysis.

Table 1 Patient characteristics

	Patients ($n = 112$)	
	No.	%
Age		
Median (range) (years)	63 (29–83)	
<70 years	80	71
≥70 years	32	29
Sex		
Female	35	31
Male	77	69
Histological diagnosis		
Adenocarcinoma	93	83
Squamous cell carcinoma	12	11
Non-small cell carcinoma (not specified)	6	5
Large cell neuroendocrine carcinoma	1	1
Smoking history (pack-years)		
Never-smokers (0)	34	30
Moderate smokers (1–39)	30	27
Heavy smokers (≥40)	48	43
ECOG performance status		
0–1	92	82
2–3	20	18
Stage		
IIIA/IIIB	21	19
IV	58	52
Recurrence after surgery	33	29
Prior chemotherapy		
Yes	84	75
No	28	25
Prior thoracic radiotherapy		
Yes	26	23
No	86	77
Pre-existing pulmonary fibrosis		
Yes	12	11
No	100	89

Table 2 Characteristics of patients who developed interstitial lung disease

	Age (years)	Sex	Histological diagnosis	PS	PY	Stage	Prior chemotherapy		Thoracic radiotherapy	Pre-existing lung disease	Length of treatment (days)	Survival (days)
							First	Second				
1	66	M	Ad	1	44	IIIB	CDDP+VNR	DTX	No	PF	10	22[a]
2	69	M	Ad	1	28	IV	CBDCA+PTX	—	No	PF	32	67[a]
3	52	F	Ad	1	48	IV	CDDP+GEM	—	No	None	42	42[a]
4	71	M	Ad	1	51	IIIB	UFT	—	No	PF	47	123[a]
5	64	M	Sq	1	129	IV	CBDCA+PTX	DTX	No	None	18	237[b]
6	74	M	Ad	1	64	Rec	CBDCA+PTX	—	No	PF	39	400[b]

Ad: adenocarcinoma, Sq: squamous cell carcinoma, PS: performance status, PY: pack-years smoked, Rec: recurrence after surgery, CDDP: cisplatin, CBDCA: carboplatin, VNR: vinorelbine, DTX: docetaxel, PTX: paclitaxel, GEM: gemcitabine, PF: pulmonary fibrosis.

[a] Treatment-related death.

[b] Death from lung cancer.

Table 3 Risk factors for interstitial lung disease ($n = 112$)

	No. of patients	Incidence of ILD (%)	Univariate analysis		Multivariate analysis	
			Odds ratio (95% CI)	*P*-values	Odds ratio (95% CI)	*P*-values
Total	112	5.4				
Age						
<70 years	80	5.0	0.80 (0.15–4.18)	0.791	2.05 (0.46–9.17)	0.347[a]
≥70 years	32	6.3	1			
Sex						
Female	35	2.9	0.44 (0.053–3.62)	0.428	19.1 (0.44–837)	0.126
Male	77	6.5	1		1	
Histological diagnosis						
Adenocarcinoma	93	5.4	1.02 (0.13–8.26)	0.984	0.26 (0.012–5.46)	0.383
Non-adenocarcinoma	19	5.3	1		1	
Smoking history (pack-years)						
Heavy smokers (≥40)	48	10.4	–	0.096[b]	1.50 (0.98–2.29)	0.062[c]
Moderate smokers (1–39)	30	3.3	–			
Never-smokers (0)	34	0.0	1			
PS						
2–3	20	0.0	0	0.240		
0–1	92	6.5	1			
Prior surgery						
Yes (recurrence)	33	3.0	0.48 (0.056–3.94)	0.480	2.48 (0.14–43.2)	0.534
No (advanced disease)	79	6.3	1		1	
Prior chemotherapy						
Yes	84	7.1	–	0.146		
No	28	0.0	1			
Prior thoracic radiotherapy						
Yes	26	0.0	0	0.166		
No	86	7.0	1			
Pulmonary fibrosis						
Yes	12	33	16.7 (3.40–83.3)	<0.001	177 (4.53–6927)	0.006
No	100	2.0	1		1	

CI: confidence interval.

[a] Age was analyzed as a continuous variable in the multivariate analysis. Odds ratio was calculated per 10-year decrease.

[b] Smoking history was analyzed by comparing never-smokers and moderate/heavy smokers in the univariate analysis.

[c] Smoking history (pack-years) was analyzed as a continuous variable in the multivariate analysis. Odds ratio was calculated per 10-pack-year increase.

The incidence of drug-related adverse events is listed in Table 4. Grade 1 or 2 skin rash (81%) and diarrhea (56%) were the most frequent adverse events. Grades 1–3 elevation in glutamic-oxaloacetic transaminase (GOT) and/or glutamic-pyruvic transaminase (GPT) levels was observed in 46% of the patients.

3.3. Efficacy

Of the 112 patients, 98 had measurable disease. Four patients were not evaluated due to early discontinuation. Complete response, partial response, stable disease, and progressive disease were observed in 2, 30, 29, and 33 patients,

Table 4 Toxicity

	No. of patients evaluated	Grade			
		1	2	3	4
Skin rash	109	59	29	0	0
Diarrhea	109	57	4	0	0
GOT/GPT	106	31	8	10	0
Nausea	109	21	5	0	0
Interstitial lung disease (ILD)	112	0	1	1	4[a]

[a] Treatment-related death.

respectively. The response rate was 33% (32/98). The response rates in each subgroup of patients are listed in Table 5. According to the results of the univariate analysis, female gender ($P = 0.003$), adenocarcinoma ($P = 0.010$), no history of smoking ($P < 0.001$), and no history of thoracic radiotherapy ($P = 0.015$) were significant predictors of tumor response to gefitinib. The response rate of male smokers was 14% (8/56), which was lower than both that of female smokers (40%, $P = 0.052$) and that of male never-smokers (70%, $P < 0.001$). When pack-years were analyzed as a continuous variable among the smokers, the association between

Table 5 Response rates among subgroups of patients ($n = 98$)

	No. of patients	Response rate (%)	Univariate analysis		Multivariate analysis	
			Odds ratio (95% CI)	*P*-values	Odds ratio (95% CI)	P values
Total	98	33				
Age						
<70 years	69	36	1.50 (0.76–2.97)	0.244	1.57 (0.96–2.56)	0.071[a]
≥70 years	29	24	1			
Sex						
Female	32	53	2.34 (1.34–4.06)	0.003	1.84 (0.51–6.56)	0.349
Male	66	23	1		1	
Histological diagnosis						
Adenocarcinoma	81	38	6.51 (1.58–26.8)	0.010	4.27 (0.48–37.0)	0.191
Non-adenocarcinoma	17	6	1		1	
Smoking history (pack-years)						
Never-smokers (0)	32	63	3.44 (1.98–5.97)	<0.001[b]	3.92 (1.03–14.9)	0.045[b]
Moderate smokers (1–49)	22	23	1		1	
Heavy smokers (≥50)	44	16				
PS						
0–1	83	31	0.78 (0.38–1.62)	0.510	0.46 (0.10–2.09)	0.314
2–3	15	40	1		1	
Prior surgery						
No (advanced disease)	68	28	0.64 (0.36–1.14)	0.134	1.25 (0.35–4.41)	0.732
Yes (recurrence)	30	43	1		1	
Prior chemotherapy						
No	24	42	1.40 (0.76–2.58)	0.279	1.32 (0.35–4.95)	0.678
Yes	74	30	1		1	
Prior thoracic radiotherapy						
No	74	39	3.14 (1.24–7.90)	0.015	6.76 (1.30–35.7)	0.023
Yes	24	13	1		1	

CI: confidence interval.

[a] Age was analyzed as a continuous variable in the multivariate analysis. The odds ratio was calculated per 10-year decrease.

[b] Smoking history was analyzed by comparing never-smokers and moderate/heavy smokers.

increased pack-years and a lower response rate was also shown (OR per 10-pack-year increase: 0.74, 95% CI: 0.56–0.99, $P = 0.041$).

The results of a multivariate analysis showed that "no history of smoking" ($P = 0.045$) and "no history of thoracic radiotherapy" ($P = 0.023$) were significant predictors of response. It was also suggested that younger patients tended to obtain a higher response rate ($P = 0.071$). Although female gender and adenocarcinoma were not found to be predictive factors in the multivariate analysis, sex and histological diagnosis were significantly associated with smoking history, and these variables may have canceled each other's effect on the dependent variable. The proportion of never-smokers was 69% (22/32) among the women versus 15% (10/66) among the men (correlation coefficient [r] = 0.536, $P < 0.001$), and 67% (54/81) among the patients with adenocarcinoma versus 0% (0/17) among those with non-adenocarcinoma ($r = 0.319$, $P = 0.001$). When a multivariate analysis was performed excluding smoking history as a factor, the OR of the females and patients with adenocarcinoma was 3.81 (95% CI: 1.36–10.7, $P = 0.011$) and 6.45 (95% CI: 0.76–55.6, $P = 0.087$), respectively.

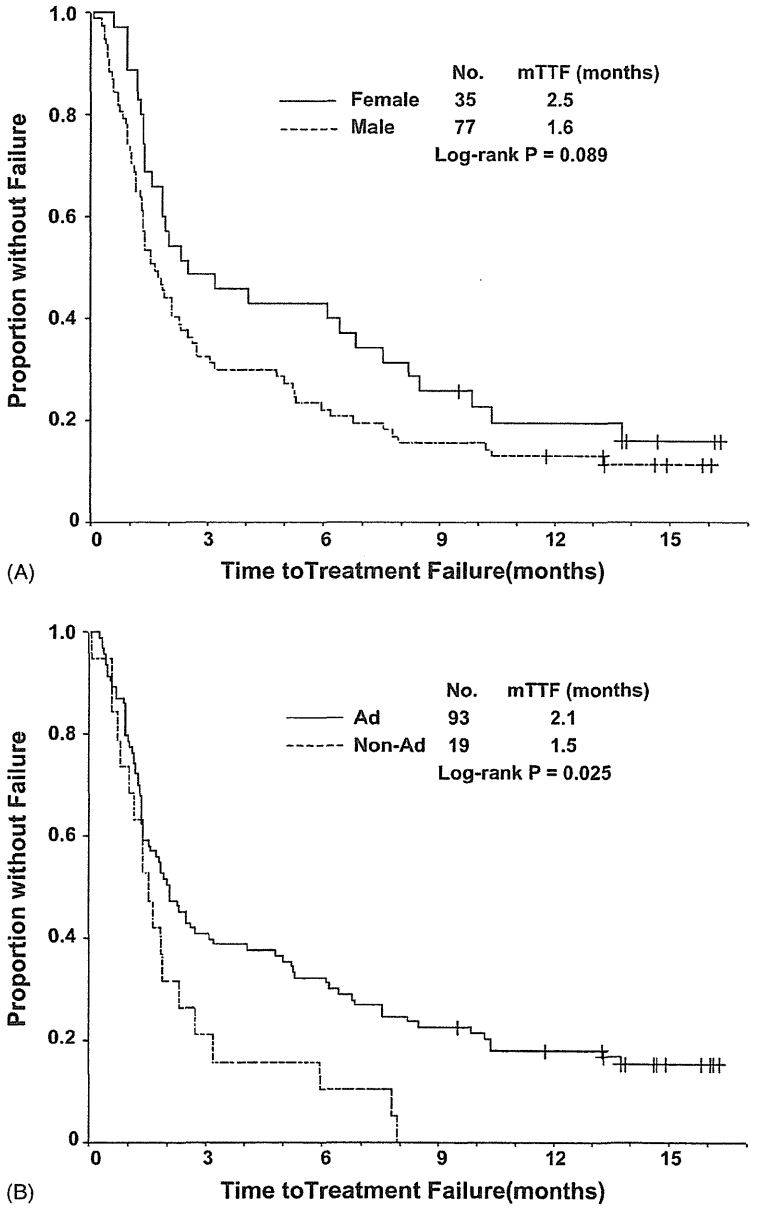

Fig. 1 Kaplan–Meier plot of time to treatment failure according to subgroups: (A) female versus male; (B) adenocarcinoma versus non-adenocarcinoma; (C) never-smokers versus moderate/heavy smokers. mTTF: median time to treatment failure, Ad: adenocarcinoma.

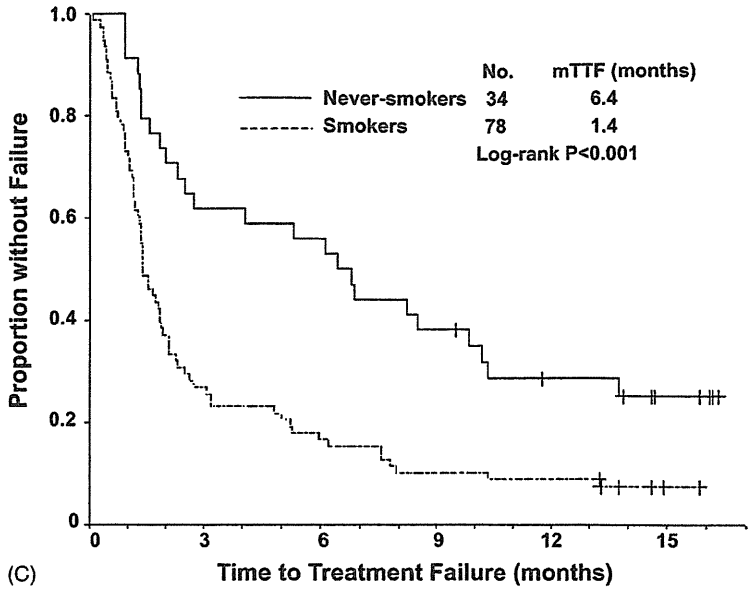

Fig. 1 (*Continued*).

The median follow-up time for survivors was 14.7 months, and ranged from 11.0 to 16.8 months. Sixty-nine patients (62%) died: 65 of disease progression and 4 of toxicity. Gefitinib treatment was terminated in 97 patients (87%) because of disease progression (68 patients), no tumor shrinkage (7 patients), toxicity (19 patients), or at the patients' request (3 patients). The median TTF and the median survival time (MST) for all patients were 1.9 and 10.7 months, respectively. The 1-year survival rate was 45%. The Kaplan–Meier plots of TTF and OS in each subgroup are shown in Figs. 1 and 2. The women had a longer TTF and OS than the men, but the difference was not significant. Patients with adenocarcinoma had a significantly longer TTF than those with non-adenocarcinoma, and "adenocarcinoma" was a marginally significant predictor of longer survival. "No history of smoking" was a highly significant predictor of longer TTF ($P < 0.001$) and longer survival ($P = 0.007$); the MST was 15.3 months in never-smokers and 8.8 months in moderate/heavy smokers.

We observed an association between efficacy and toxicity. As shown in Table 6, those who experienced skin rash or elevation in GOT/GPT levels tended to

Table 6 Association between efficacy and toxicity

	No. of patients	Response rate (%)	*P*-values*	Median survival (months)	1-year survival (%)	*P*-values†
Skin rash						
Grade 0	21	12	0.043	3.0	24	0.011
Grade 1	59	33		10.6	44	
Grade 2	29	46		15.3	66	
Diarrhea						
Grade 0	48	33	0.903	9.3	35	0.037
Grade 1–2	61	32		13.6	54	
GOT/GPT						
Grade 0	57	21	0.004	7.8	31	0.006
Grade 1	31	48		15.1	55	
Grade 2–3	18	50		Not reached	83	

* *P*-values for chi-square test between grade 0 and 1–3.
† *P*-values for log-rank test.

exhibit a response, and skin rash, diarrhea and elevation in GOT/GPT levels were significant prognostic factors of survival.

4. Discussion

Gefitinib is a promising agent for the treatment of advanced NSCLC, but risk assessment is of critical importance to using it properly. Gefitinib was thought to be a relatively safe agent at first, and physicians in Japan tended to prescribe it without careful consideration of risks. In the first 4 months after its approval, 17,000 patients began taking gefitinib, the most rapid adoption of any antitumor agent in Japan. The Ministry of Health, Labour and Welfare has estimated that the incidence of ILD was 2.2%. However, since a follow-up survey of all of the cases has not been conducted and only limited data from sporadic reports by physicians were available, many ILD cases may not have been reported, and the actual incidence may have been higher than 2.2%. Although the sample size in the present study was small, the incidence of ILD was

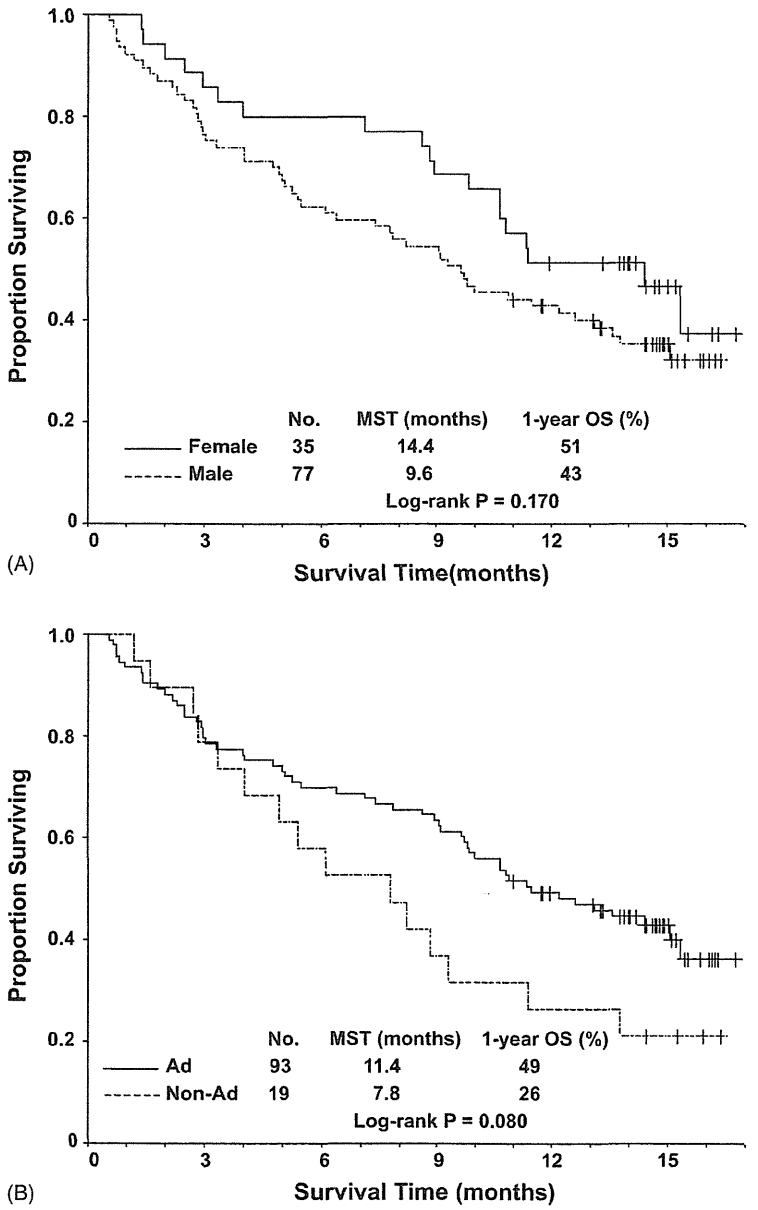

Fig. 2 Kaplan–Meier plot of overall survival according to subgroups: (A) female versus male; (B) adenocarcinoma versus non-adenocarcinoma; (C) never-smokers versus moderate/heavy smokers. MST: median survival time, OS: overall survival, Ad: adenocarcinoma.

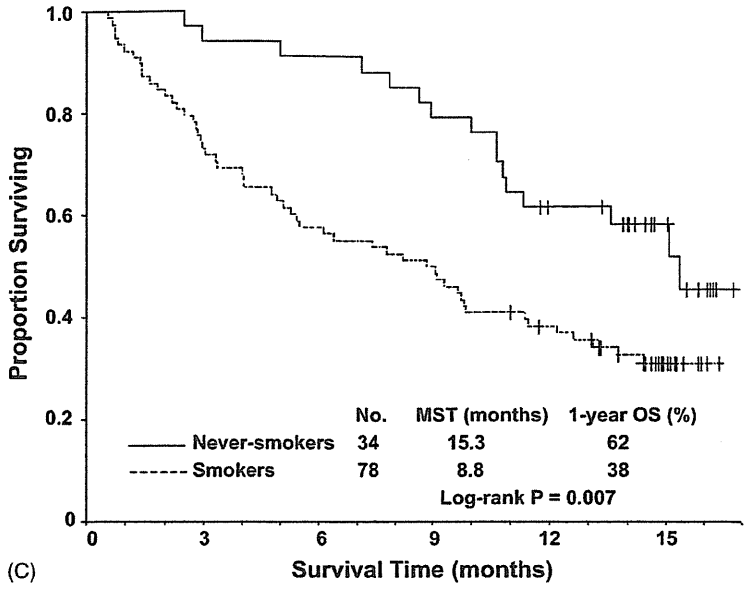

Fig. 2 (*Continued*).

as high as 5.4%. The risk of ILD appears to be around 2–5% if gefitinib is given to patients without careful risk assessment. We think that the incidence can be reduced by patient selection after a thorough risk assessment and that the proper use of gefitinib may enable great benefit, far exceeding its potential risks.

Our analysis of the risk factors for the development of ILD revealed pre-existing PF as a strong risk factor. Of the 112 patients in this study, 12 had PF at the start of gefitinib administration. Four (33%) of these patients subsequently developed ILD, 3 (25%) died as a result, and no response was seen in any of these 12 patients. A panel of experts convened by AstraZeneca Japan retrospectively analyzed 104 patients with NSCLC who developed ILD during gefitinib therapy in Japan and reported that 30 (29%) of them were diagnosed as pre-existing PF by chest X-rays or computed tomography scans taken before gefitinib administration [8]. The panel also noted that the patients with PF had a significantly higher mortality rate after the onset of ILD: it was 77% (23/30) among the patients with PF and 34% (25/74) among the patients without PF ($P < 0.001$) [8]. We conclude that gefitinib treatment may be harmful to patients with PF and recommend that gefitinib not be used if PF is apparent on the chest X-rays.

In our study, all patients were Japanese and a 33% response rate was observed. In the IDEAL 1 trial, 102 Japanese and 106 non-Japanese patients received gefitinib, and the response rate was 27.5% in the Japanese and 10.4% in the non-Japanese [5]. Whether this difference was attributable to ethnicity or an imbalance in other characteristics is unknown, but a high response rate in Japanese patients has been consistently observed in clinical practice.

Both the IDEAL 1 and 2 trials suggested "female gender" and "adenocarcinoma" as predictive factors for tumor response to gefitinib [5,6], and a retrospective analysis of gefitinib monotherapy for advanced NSCLC showed that "adenocarcinoma" (especially with bronchioloalveolar features) and "no history of smoking" were significantly correlated with response to gefitinib [9]. We observed the same tendency with a response rate of 53% in women, 38% in patients with adenocarcinoma, and 63% in never-smokers. "No history of smoking" was a significant predictive factor for response in multivariate analysis, and it was also a significant predictor of longer TTF and longer survival. Since both female gender and adenocarcinoma were significantly associated with no history of smoking, which of these characteristics are true predictive factors remains uncertain. It was also suggested that heavier smokers and male smokers specifically had a lower response rate among the patients with smoking history. Since heavier smokers tended to have a higher risk of ILD, we should carefully assess their risk-benefit ratio of gefitinib therapy before selecting therapeutic strategies.

There are some biological explanations for these clinical characteristics associated with response to gefitinib [10]. Although gefitinib inhibits the intracellular tyrosine kinase domain of EGFR, no correlation between expression of EGFR and response

has been demonstrated [11]. When EGFR and human epidermal growth factor receptor 2 (HER2) are coexpressed, HER2 is the preferred dimerization partner of EGFR, and EGFR-HER2 heterodimers have more signaling potency than EGFR homodimers [12]. Preclinical studies have indicated that tumor cell lines overexpressing HER2 or coexpressing EGFR and HER2 are sensitive to gefitinib [13–16]. Since EGFR/HER2-coexpression is more common in adenocarcinoma of the lung than in squamous cell carcinoma [13,17], the high response rate in adenocarcinoma may be attributable to it. In women, estrogens and estrogen receptors are involved in the development of NSCLC [18], and estrogens binding to its receptors upregulates EGFR and EGFR ligands [19]. The presence of estrogens and its receptors may impact EGFR signaling and the response of NSCLC to gefitinib in women. NSCLC in never-smokers may also have a different biology. Since several studies have indicated fewer mutations of the p53 and K-ras genes in never-smokers than in smokers [20,21], the relation between such tobacco-related mutations and gefitinib response should be investigated. Subgroups of patients who obtain a clinical benefit from gefitinib administration are needed to be identified more precisely, and molecular markers predictive of tumor response should be sought by using DNA microarrays and a proteomics-based approach.

Our analysis suggests that patients who suffer from skin toxicity, diarrhea, or liver toxicity have a greater clinical benefit from gefitinib treatment. A correlation between skin toxicity and survival has also been shown in a study of gefitinib for head and neck cancer [22] and in studies of erlotinib, another EGFR tyrosine kinase inhibitor [23]. Because these findings may be attributable to the responders having taken gefitinib for longer periods and the toxicities in these patients being evaluated more carefully, further studies are needed to confirm them. If the early onset of toxicities has predictive value for survival, it can be used for clinical decision making regarding continuation of gefitinib treatment.

5. Conclusion

When gefitinib is used to treat advanced NSCLC, it confers a higher risk of ILD on patients with PF and a greater clinical benefit on never-smokers, women, patients with adenocarcinoma, and patients with no history of thoracic radiotherapy. Gefitinib therapy is an important treatment option for patients with advanced NSCLC, but the proper use of it based on individual risk-benefit assessments is crucial.

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SYNERGISTIC INTERACTION BETWEEN THE EGFR TYROSINE KINASE INHIBITOR GEFITINIB ("IRESSA") AND THE DNA TOPOISOMERASE I INHIBITOR CPT-11 (IRINOTECAN) IN HUMAN COLORECTAL CANCER CELLS

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Epidermal growth factor receptor [EGFR (HER1, erbB1)] is a receptor with associated tyrosine kinase activity, and is expressed in colorectal cancers and many other solid tumors. We examined the effect of the selective EGFR tyrosine kinase inhibitor (EGFR-TKI) gefitinib ("Iressa") in combination with the DNA topoisomerase I inhibitor CPT-11 (irinotecan) on human colorectal cancer cells. EGFR mRNA and protein expression were detected by RT-PCR and immunoblotting in all 7 colorectal cancer cell lines studied. Gefitinib inhibited the cell growth of the cancer cell lines *in vitro* with an IC_{50} range of 1.2–160 μM by 3,(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Lovo cells exhibited the highest level of protein and autophosphorylation of EGFR and were the most sensitive to gefitinib. The combination of gefitinib and CPT-11 induced supra-additive inhibitory effects in COLO320DM, WiDR and Lovo cells, assessed by an *in vitro* MTT assay. Administration of gefitinib and CPT-11 had a supra-additive inhibitory effect on WiDR cells and tumor shrinkage was observed in Lovo cell xenografts established in nude mice, whereas no additive effect of combination therapy was observed in COLO320DM cells. To elucidate the mechanisms of synergistic effects, the effect of CPT-11-exposure on phosphorylation of EGFR was examined by immunoprecipitation. CPT-11 increased phosphorylation of EGFR in Lovo and WiDR cells in time- and dose-dependent manners. This EGFR activation was completely inhibited by 5 μM gefitinib and gefitinib-induced apoptosis was enhanced by combination with CPT-11, measured by PARP activation although no PARP activation was induced by 5 μM CPT-11 alone. These results suggested that these modification of EGFR by CPT-11, in Lovo cells, is a possible mechanism for the synergistic effect of CPT-11 and gefitinib. These findings imply that the EGFR-TKI gefitinib and CPT-11 will be effective against colorectal tumor cells that express high levels of EGFR, and support clinical evaluation of gefitinib in combination with CPT-11, in the treatment of colorectal cancers.

Key words: *combination; gefitinib; "Iressa"; colorectal cancer; irinotecan*

Colorectal cancer is a major public health concern. Although chemotherapy appears to be of very limited value in advanced colorectal cancer, there have been many efforts to apply combination chemotherapy in patients with primary disease.[1–3]

The combination of fluorouracil and leucovorin used to be recognized as standard therapy for colorectal cancer, but the topoisomerase I inhibitor, irinotecan (CPT-11), has recently been demonstrated to be active against colorectal cancer that was resistant to prior therapy.[4,5] Moreover, the CPT-11/5-FU/LV combination has been approved as standard chemotherapy by the US FDA for metastatic colorectal cancer.[6] However, patients treated with CPT-11 plus bolus 5-FU/leucovorin have been found to have a 3-fold higher rate of treatment-induced or treatment-exacerbated death than patients treated with other arms of the respective studies.[7] We have therefore been seeking a new combination regimen containing CPT-11 and target-based drugs.

The development of target-based drugs, including receptor tyrosine kinase inhibitors (TKI), is one of the promising strategies for cancer chemotherapy.[8,9] Colorectal cancers express receptors of the type 1 tyrosine kinase family, including epidermal growth factor receptor (EGFR) and c-erbB-2,[10–12] and the EGFR has emerged as a central molecular target for modulation in cancer therapeutics. The correlation between high expression of EGFR and clinically aggressive malignant disease has made EGFR a promising target of therapy for many epithelial tumors, which represent approximately 2/3 of all human cancers. In solid cancers, including colorectal cancers, high EGFR expression correlates with poor prognosis.[11] Gefitinib ("Iressa") is an orally active, selective EGFR-TKI that blocks signal transduction pathways involved in the proliferation and survival of cancer cells and in other host-dependent processes promoting cancer growth.[13,14] In EGFR tyrosine kinase assays, gefitinib has an IC_{50} of 0.033 μM. Inhibition of c-erbB-2 and KDR occurs at doses 100-fold higher than for EGFR inhibition.[15] We have previously demonstrated that gefitinib exerts high growth-inhibitory activity against EGFR-positive tumors in a xenograft model,[16] and gefitinib is therefore expected to be a potent therapeutic agent against EGFR-positive colorectal cancers. In recent years, it has been shown that the combined treatment of established human colorectal cancer xenograft with anti-EGFR drug (cetuximab or gefitinib) and with topoisomerase I inhibitor, topotecan, increase the antitumor activity of these drugs.[17,18] The aim of the present study was to investigate the combination effect of gefitinib and CPT-11 and to elucidate the biochemical mechanism of synergistic interaction in colorectal cancers.

MATERIAL AND METHODS

Drugs and chemicals

Gefitinib (N-(3-chloro-4-fluorophenyl)-7-methoxy-6-[3-(morpholin-4-yl)propoxy] quinazolin-4-amine) was provided by AstraZeneca (Cheshire, UK). Gefitinib was dissolved in dimethyl sulfoxide (DMSO) for the *in vitro* study and suspended in 5% glucose, pH 6, for the *in vivo* study. CPT-11 was obtained from Yakult Honsha (Tokyo, Japan). CPT-11 was dissolved in 45 mg/ml solvitol (pH 3-4) for both the *in vivo* and *in vitro* studies.

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Animals

Female BALB/c nude mice, 6-weeks-old, were purchased from Japan Charles River Co., Ltd. (Atsugi, Japan). All mice were maintained in our laboratory under specific-pathogen-free conditions.

Cells and culture

Human colorectal cancer cell lines WiDR, LS-174T, COLO320DM, COLO320HSR, Lovo, SW480 and HCT116 were obtained from ATCC (Lockville, MD). Lovo cells, SW480 and HCT116 cells were maintained in HAM's F12 medium (GIBCO BRL, Grand Island, NY), Leibovitz's L-15 medium and McCoy's 5A medium (GIBCO BRL), respectively, all supplemented with 10% heat-inactivated fetal bovine serum (FBS). Other cell lines were maintained in RPMI1640 (Nikken Bio Med. Lab., Kyoto, Japan) supplemented with 10% FBS.

Growth-inhibition assay

We used the tetrazolium dye [3,(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, MTT] assay to evaluate the cytotoxicity of various drug concentrations. A 200 ml volume of an exponentially growing cell suspension (5×10^3–1.5×10^4 cells/ml) was seeded into a 96-well microtiter plate and 20 μl of each drug at various concentrations was added. After incubation for 72 hr at 37°C, 20 μl of MTT solution (5 mg/ml in phosphate buffered saline, PBS) was added to each well and the plates were incubated for a further 4 hr at 37°C. After centrifuging the plates at 200*g* for 5 min, the medium was aspirated from each well, and 180 μl of DMSO was added to each well to dissolve the formazan. Optical density was measured at 562 and 630 nm with a Delta Soft ELISA analysis program interfaced with a Bio-Tek Microplate Reader (EL-340, Bio-Metallics, Princeton, NJ). Each experiment was performed in 6 replicate wells for each drug concentration and carried out independently 3 or 4 times. The IC_{50} value was defined as the concentration needed for a 50% reduction in the absorbance calculated based on the survival curves. Percent survival was calculated as follows: (mean absorbance of 6 replicate wells containing drugs − mean absorbance of 6 replicate background wells)/(mean absorbance of 6 replicate drug-free wells − mean absorbance of 6 replicate background wells) × 100.

RT-PCR

Specific primers designed for EGFR CDS were used for detection of EGFR mRNA as described elsewhere.[16] First-strand cDNA was synthesized from the cells' RNA with an RNA PCR Kit (TaKaRa Biomedicals, Ohtsu, Japan). After reverse transcription of 1 μg of total RNA with Oligo(dT)-M4 adaptor primer, the whole mixture was used for PCR with 2 oligonucleotide primers (5′-AATGTGAGCAGAGGCAGGGA-3′,5′GGCTTGGTTTG-GAGCTTCTC-3′). PCR was performed with initial denaturation at 94°C for 2 min, 25 cycles of amplification (denaturation at 94°C for 30 sec, annealing at 55°C for 60 sec and extension at 72°C for 105 sec).

Immunoprecipitation and immunoblotting

The cultured cells were washed twice with ice-cold PBS, lysed in EBC buffer (50 mM Tris-HCI, pH 8.0, 120 mM NaCI, 0.5% Nonidet P-40, 100 mM NaF, 200 mM Na orthovanadate and 10 mg/ml each of leupeptin, aprotinin and phenylmethylsulfonyl fluoride). The lysate was cleared by centrifugation at 20,000*g* for 5 min, and the protein concentration of the supernatant was measured by BCA protein assay (Pierce, Rockford, IL). For Immunoblotting, 20 μg samples of protein were electrophoretically separated on a 7.5% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was probed with rabbit polyclonal antibody against EGFR (1005; Santa Cruz Biotech, Santa Cruz, CA), HER2/neu (c-18; Santa Cruz), phospho-EGFR specific for Tyr 845, Tyr 1045, and Tyr 1068 (numbers 2231, 2235 and 2234; Cell Signaling, Beverly, MA) and cleaved PARP (number 9544; Cell Signaling) as the first antibody, followed by horseradish peroxidase-conjugated secondary antibody. The bands were visualized by electrochemiluminescence (ECL, Amersham, Piscataway, NJ). For immunoprecipitation, 5×10^6 cells were washed, lysed in EBC buffer, and centrifuged. The resultant supernatants (1,500 μg) were incubated with the anti-EGFR antibody (1005) at 4°C overnight. The immunocomplex were absorbed onto protein A/G-Sepharose beads, washed 5 times with lysate buffer, denatured and subjected to electrophoresis on a 7.5% polyacrylamide gel followed by immunostaining probed with antiphosphotyrosine antibody (PY-20, BD Bioscience Clontech, Tokyo, Japan).

Combined effect of gefitinib and CPT-11 in vitro

The combined effect of gefitinib and CPT-11 on colorectal cancer cell growth was evaluated by the combination index (CI) analysis method.[6] For any given drug combination, CI represents the degree of synergy, additivity or antagonism. CI was expressed in terms of fraction-affected (F_a) values, which represents the percentage of cells killed or inhibited by the drug. Using the mutually exclusive ($\alpha=0$) or mutually nonexclusive ($\alpha=1$) isobologram equation, the F_a/CI plots for each cell line was constructed by computer analysis of the data generated from the median effect analysis. CI values were interpreted as follows: <1.0 = synergism; 1.0 = additive and >1.0 = antagonism.

Using the median-effect method, developed by Chou and Talalay, the dose-response curve was plotted for each drug and for multiple doses of a fixed-ratio combination by using the equation:

$$f_a/f_u = (D/D_m)^m ,$$

where, D is the dose-administered, Dm is the dose required for 50% inhibition of growth, f_a is the fraction affected by dose D, f_u is the unaffected fraction and m is a coefffect curve. The dose-response curve was plotted by logarithmic conversion of the equation to determine the m and D_m values, and the dose D_x required for x percent effect $(f_a)_x$ was then calculated as

$$D_x = D_m[\, f_a]_x/(\, f_u)\, x]^{1/m} .$$

Thus, Cl can be defined by the isobologram equation

$$CI = (D)_1/(D_x)^1 + (D)_2/(D_x)2 + \alpha(D)_1(D)_2/(D_x)_1(D_x)_2 ,$$

where $(D_x)_1$ is the dose of Drug-1 required to produce x percent effect alone, and $(D)_1$ is the dose of Drug 1 required to produce the same x percent effect in combination with Drug 2; similarly, $(Dx)_2$ is the dose of Drug 2 required to produce x percent effect alone and $(D)_2$ is the dose of Drug 2 required to produce the same x percent effect in combination with Drug 1. Theoretically, Cl is the ratio of the combined dose to the sum of the single-drug doses at an isoeffective level. Consequently, Cl values <1 indicate synergism, values >1 indicate antagonism and a value of 1 indicates additive effects. The Cl values obtained from both the classical nonconservative ($\alpha=0$) and conservative ($\alpha=1$) isobologram equations are presented in this report.

Growth-inhibition assay in vivo

Experiments were performed in accordance with the United Kingdom Coordinating Committee on Cancer Research Guidelines for the welfare of animals in experimental neoplasia (second edition).

In vivo experiments were scheduled to evaluate the combined therapeutic effect on preexisting tumors of oral or intraperitoneal administration of gefitinib and intravenous injection of CPT-11. The dose of each drug was set based on the results of a preliminary experiment involving administration of each drug alone. Ten days before administration, 1×10^7 WiDR and COLO320DM or 2×10^6 Lovo cells were injected subcutaneously into the back of mice. Five or 6 mice per group were injected with tumor cells. Tumor bearing mice were either given gefitinib, 40 mg/kg/day *p.o.* on days 1–10, or CPT-11, 40 mg/kg/day *i.v.* on days 1, 5 and 9, or

both, or placebo (5%(w/v) glucose solution). Alternatively, gefitinib, 30 or 60 mg/kg, *i.p.* days 1–14, and *i.v.* CPT-11, 16.7 or 33.3 mg/kg, *i.v.* on days 1, 5 and 9, were administered to the mice. Tumor diameters were measured with calipers on days 1, 4, 7, 10, 14, 18 and 22 to evaluate the effects of treatment, and tumor volume was determined by using the following equation: tumor volume = $ab^2/2$ (mm^3) (where a is the largest diameter of the tumor and b is the shortest diameter). Day "x" denotes the day on which the effect of the drugs was estimated, and day "0" denotes the first day of treatment. All mice were sacrificed on day 22 after measuring their tumors.

Statistical analysis

Differences between the test groups were analyzed by 1-factor ANOVA followed by Fisher's protected least significant difference (PLSD). A value of $p<0.05$ was considered statistically significant.

RESULTS

EGFR and HER2 expression and EGFR autophsophorylation in colorectal cancer cells

We examined EGFR mRNA expression by RT-PCR analysis using 2 specific primers. Approximately 570 bp-long PCR products were amplified in all cell lines that exhibited expression of EGFR mRNA (Fig. 1*a*). Comparison of the protein expression levels of EGFR in colorectal cancer cells by immunoblotting (Fig. 1*b*) revealed high expression in Lovo and WiDR cells. EGFR protein was also detected in LS-174T, COLO320DM, COLO320HSR, HCT116 and SW480 cells, although the expression levels in COLO320DM and COLO320HSR are subtle. The highest expression level of phosphrylated EGFR measured by phospho-specific EGFR antibody (Tyr845, Tyr1045 and Tyr1068) was observed in Lovo cells (Fig. 1*b*). Because the function of EGFR is closely related to that of other HER families including HER2/neu, we also examined the protein level of HER2/neu. High expression of HER2/neu were observed in LS-174T, HCT-116 and SW480 (Fig. 1*b*).

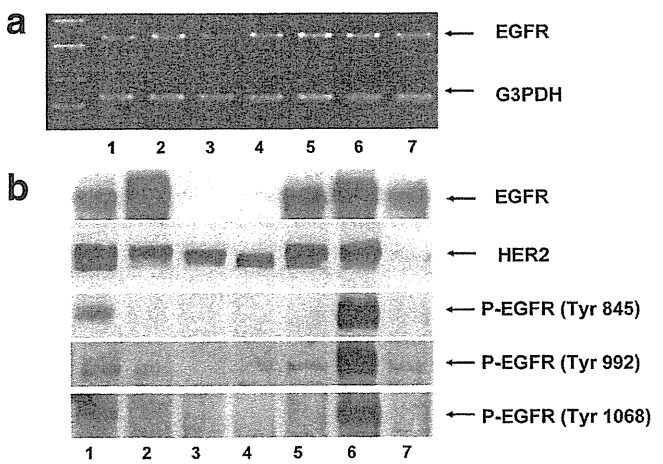


FIGURE 1 – EGFR expression in colorectal cancer cells. (*a*) Expression of EGFR mRNA in each cell line was detected by RT-PCR using specific primers designed for EGFR CDS. Expression of G3PDH mRNA was detected. Twenty-five cycles of PCR amplification were performed for each PCR product. Lanes 1–7 represent LS-174T, WiDR, COLO320DM, COLO320HSR, HCT116, Lovo and SW480 cells, respectively. (*b*) A 20 μg sample of total cell lysates was separated by 7.5% SDS-PAGE, transferred to PVDF membrane, and incubated with a specific anti-human EGFR, HER2/neu and phospho-EGFR (Tyr845, Tyr992 and Tyr1068).

Cellular sensitivity of colorectal cancer cells to gefitinib and CPT-11

The growth inhibitory effect of gefitinib and CPT-11 on colorectal cancer cells was examined by MTT assay. The IC_{50} values of gefitinib for the cell lines ranged from 1.2 μM (Lovo cells) to 160 μM (HCT116 cells) (Table I). No significant relationship was observed between EGFR expression levels and IC_{50} values among these cell lines. However, Lovo cells, which exhibited the highest EGFR expression and its phosphorylation, were the most sensitive to gefitinib. On the other hand, the IC_{50} values of CPT-11 for the cell lines ranged from 5.2 μM (Lovo) to 35 μM (SW480). The range of sensitivity to gefitinib was wider than to CPT-11.

In vitro *combined effect of gefitinib and CPT-11 on colorectal cancer cell lines*

Based on the results of the evaluation of *in vitro* growth-inhibition, 4 cell lines (WiDR, COLO320DM, Lovo, and SW480 cells) were selected for the *in vitro* combination study. Cells were treated with gefitinib or CPT-11 alone or in concomitant combination at fixed molar ratio for 72 hr. The ratios of gefitinib and CPT-11 were set based on the IC_{50} values of each cell line. Growth rate values are averages of data from at least 3 independent experiments. The effects of combinations of gefitinib and CPT-11 on cell growth are shown in Figure 2. CI values of <1, >1 and 1 indicate a supra-additive effect (synergism), antagonistic effect and additive effect, respectively. A low CI index was observed in WiDR, COLO320DM and Lovo cells over a wide range of inhibition levels. Synergistic effects were also observed in the relatively high F_a values in SW480 cells. These results suggest that gefitinib and CPT-11 had a synergistic effect on most of the colorectal cancer cell lines *in vitro.*

In vivo *combination effects of gefitinib and CPT-11*

In order to determine whether the combination of these 2 drugs is also synergistic against colorectal cancer *in vivo*, the growth-inhibitory effect of the combination was evaluated against the colorectal cancer cells in tumor xenografts. The growth inhibitory effect of gefitinib, 30 mg/kg, *i.p.* days 1–10, and CPT-11, 40 mg/kg, *i.v.* days 1, 5 and 9, on WiDR cells was evaluated (Fig. 3*a,b*). Administration of gefitinib or CPT-11 alone suppressed the tumor volume of WiDR cells with a T/C value of 73.9% and 69.2%, respectively, at day 22, (Fig. 3*c*), whereas gefitinib+CPT-11 suppressed WiDR tumors with T/C value of 51.8% at day 22, but this was not statistically significant (Fig. 3*d*, $p=0.164$ by 1-factor ANOVA). A 10% body weight loss was observed until day 15 in mice given the combination, but body weight recovered by day 22 (Fig. 3*e*). No growth inhibitory effect of single or combined therapy of CPT-11 and gefitinib in COLO320DM cells were observed (data not shown). In mice transplanted with Lovo cells, with a high EGFR expression level, marked tumor growth inhibition was achieved with gefitinib+CPT-11 (Fig. 3*f*). The T/C of the combination schedule at day 11 was 22.8% and significantly lower than in the control ($p<0.0012$ by Fisher's PSLD, Fig. 3*g*). A 10% maximum body weight loss until day 15 was also observed in mice treated with the combination (Fig. 3*j*).

Alternatively, the combined effect of oral administration of gefitinib and intravenous administration of CPT-11 was evaluated in mice transplanted with Lovo cells. Gefitinib, 30 or 60 mg/kg *p.o.* days 1–14, and CPT-11, 16.7 or 33.3 mg/kg *i.v.* days 1, 5 and 9, were administered (schedule 2, Fig. 4*a*), and greater growth inhibition was observed in mice treated with this combination, compared to the controls (Fig. 4*b*) A more marked growth-inhibitory effect was observed at a higher dose of CPT-11 (16.7 *vs.* 33.3 mg/kg), but there was no difference between 30 mg/kg and 60 mg/kg of gefitinib in the combination. The combination of gefitinib (30 and 60 mg/kg) and CPT-11 (33.3 mg/kg/*i.v.*) resulted in tumor reduction during treatment that was significant at day 15 (Fig. 4*c*). The T/C values imme-

TABLE I – *IN VITRO* GROWTH-INHIBITORY ACTIVITY OF GEFITINIB AND CPT-11 IN HUMAN COLORECTAL CANCER CELLS (MTT ASSAY)[1]

Cell line	gefitinib		CPT-11	
	IC_{50} (μM)	Concentration range (μM)	IC_{50} (μM)	Concentration range (μM)
WiDR	10 ± 1.1	0.83–53	33 ± 7.5	1.6–160
LS-174T	100.4 ± 10.1	N.D.	13	N.D.
COLO320DM	11 ± 3.8	0.63–100	11 ± 0.6	1.6–160
COLO320HSR	22	N.D.	5.5	N.D.
HCT116	177.0 ± 12.2	N.D.	11	N.D.
SW480	23 ± 0.6	1.6–10	35 ± 5.5	1.6–50
Lovo	1.2 ± 0.59	0.31–25	5.2 ± 0.82	0.16–10

[1]The IC_{50} value (μM) of each drug was measured by MTT assay, as described in the Materials and Methods. Each value is a mean ± SD of 3 or 4 independent experiments–N.D., not determined.

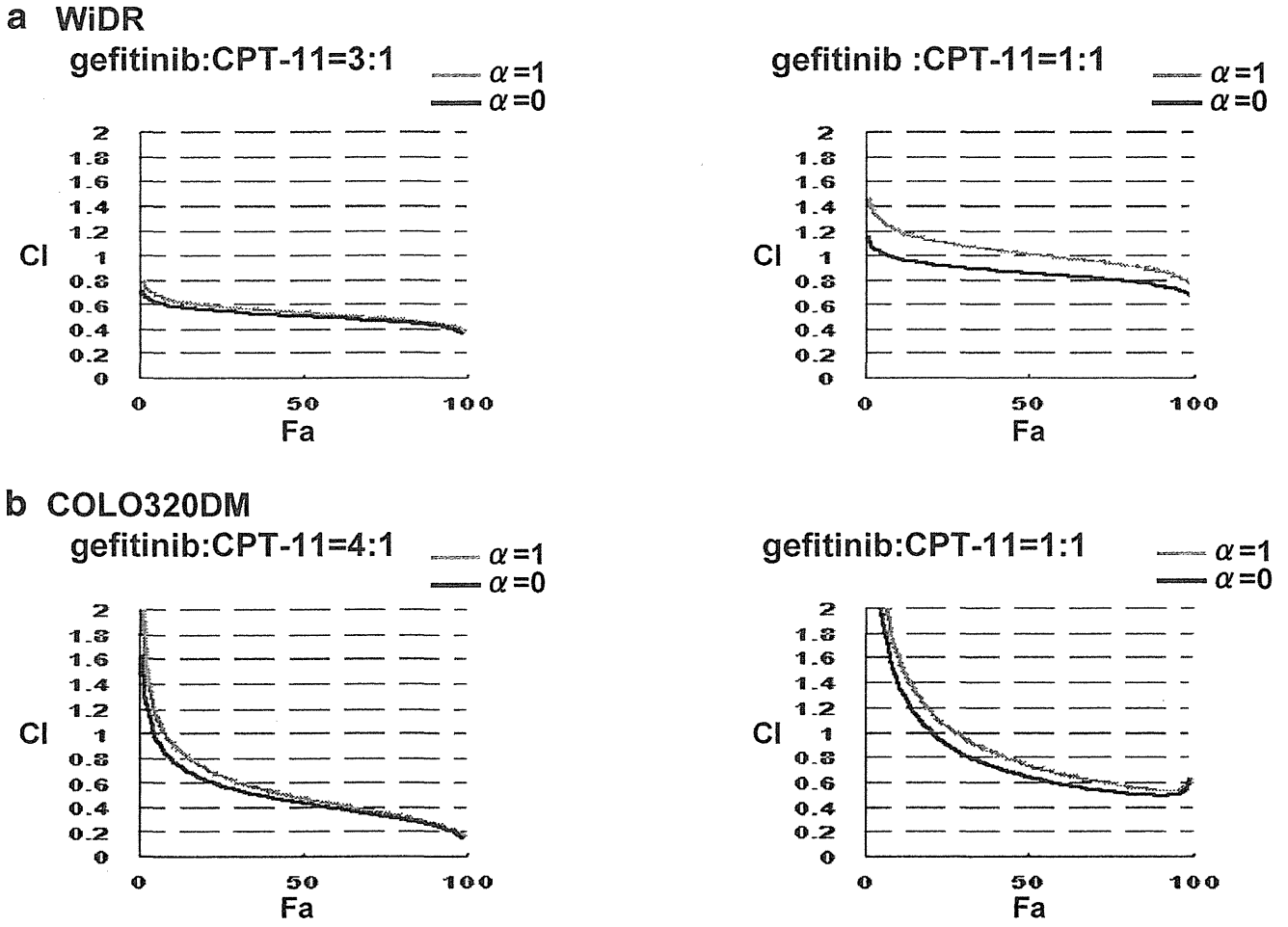


FIGURE 2 – Combination index (CI) plots of interactions between gefitinib and CPT-11. Cells were treated with gefitinib and CPT-11 alone and in combination at fixed molar ratios (molar ratios of gefitinib to CPT-11 of 3:1 and 1:1 [(*a*) WiDR], 4:1 and 1:1 [(*b*) COLO320DM], 1:2 and 1:5 [(*c*) Lovo], 1:1 [(d) SW480]. Using the mutually exclusive (CI) or mutually nonexclusive (CI′) isobologram equation, the affected fraction (F_a)-CI plot for each cell was constructed by computer analysis of the data generated from the median effect analysis. CI values <1 occurred over a wide range of inhibition levels, indicating synergy.

diately after the completion of treatment (at day 15) and at day 22 are summarized in Fig.4*d*. More severe body weight loss was observed, ~20% at day 15, in mice treated with 60 mg/kg of gefitinib alone or with CPT-11, suggesting that CPT-11 does not enhance the body weight loss induced by gefitinib. Body weight recovered by day 22 (Fig. 4*e*). No deaths of were observed during the treatment or observation period.

Induction of EGFR phosphorylation and enhanced gefitinib-induced PARP activation by CPT-11

To elucidate the synergistic effects of CPT-11 and gefitinib, we examined the effect of exposure of CPT-11 on EGFR phosphorylation in Lovo and WiDr cells. Phosphorylated EGFR was detected with anti-phosphotyrosine antibody (PY-20)

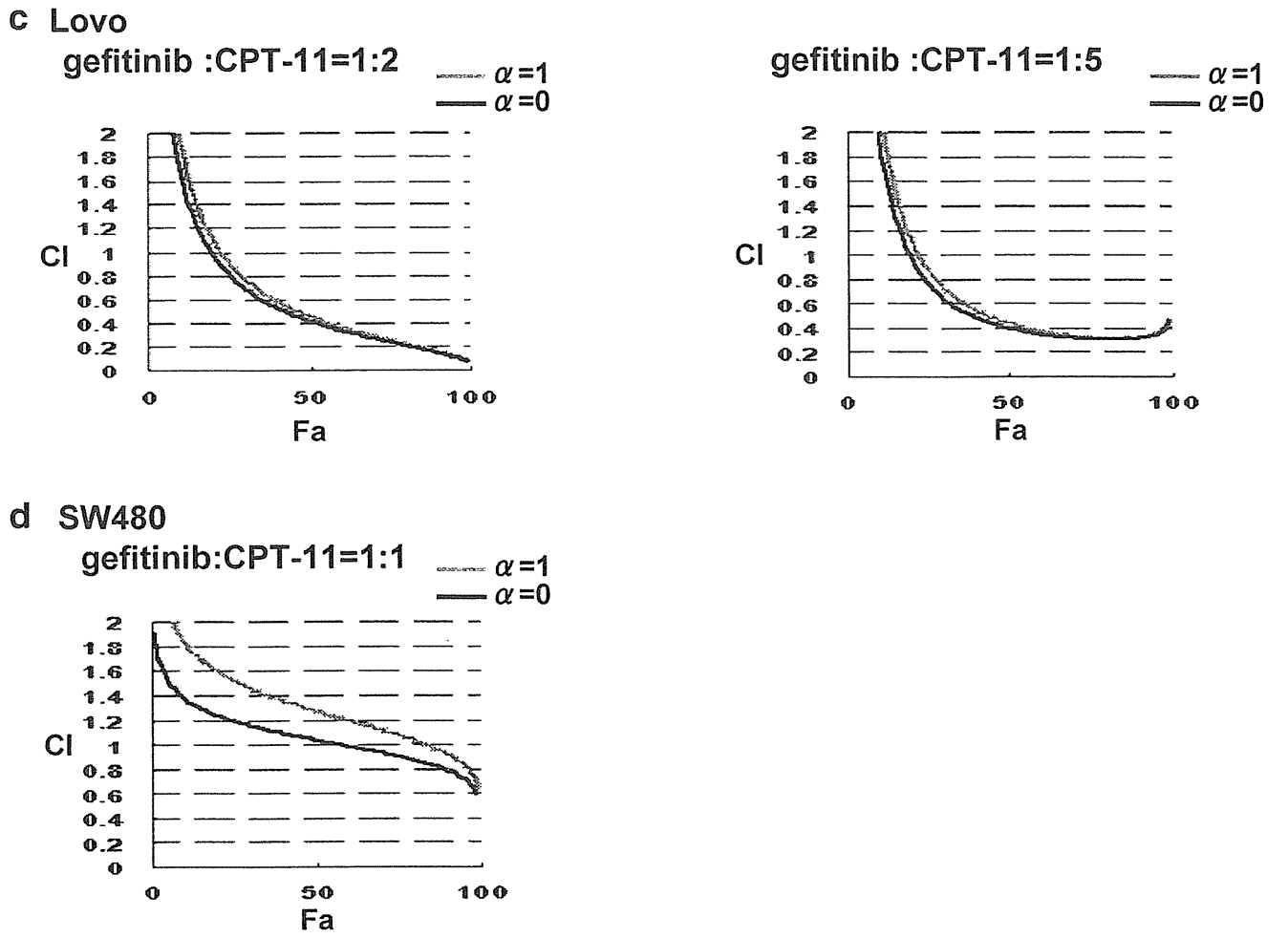


FIGURE 2 – CONTINUED.

against immunoprecipitated EGFR and increased phosphorylation of EGFR was observed after exposure to CPT-11 in Lovo cells in dose- and time- dependent manner (3–24 hr) (Fig. 5*a*). The dose-dependent activation of EGFR by CPT-11 was also obtained in WiDR cells (Fig. 5*b*). CPT-11-induced phosphorylation of EGFR was observed without ligand-stimulation. The EGFR activation was completely inhibited by 24 hd exposure of 5 μM gefitinib. gefitinib-induced apoptosis measured by PARP activation was enhanced by combination with CPT-11, although no PARP activation was induced by CPT-11 alone (Fig. 5*c*). These results suggest that the modification of EGFR by CPT-11 increases the cellular sensitivity to gefitinib, resulting the synergistic effect of CPT-11 and gefitinib. We also observed the effect of gefitinib on the expression and the activity of topoisomerase I by immunoblotting and decatenatnion assay. No modification of topoisomerase I by gefitinib was observed (data not shown).

DISCUSSION

Evidence has suggested that the new EGFR-targeting drug gefitinib is active against gastrointestinal malignancies as well as non-small cell lung cancer. Combination of gefitinib with cytotoxic drugs has been evaluated in the U.S. and Europe,[19,20] but combination with CPT-11 has not been evaluated. CPT-11 is a potent DNA-targeting drug in patients with colorectal cancer that is refractory to treatment with fluorouracil and leucovorin,[4,5] although a higher rate of treatment-induced toxicity was suspected in a retrospective analysis.[7] In preclinical study, Ciadiello *et al.*[17,18] reported that supra-additive combination effect of EGFR-targeting drug (cetuximab or gefitinib) and topoisomerase I inhibitor, topotecan was observed in human colorectal cancer GEO xenograft. We have therefore studied the synergistic potential for a new combination regimen containing CPT-11 and gefitinib. The synergistic potential of CPT-11 combined with gefitinib demonstrated in our study suggests that the gefitinib/CPT-11 combination is a promising regimen for colorectal cancer patients. Schedule 2, administration of oral gefitinib and intravenous CPT-11 designed in a xenograft model, was based on possible clinical administration of the drugs, and thus a treatment schedule consisting of intermittent *i.v.* CPT-11 and continuous gefitinib *p.o.* may be applicable to colorectal cancer in humans.

In xenograft models, body weight loss was observed when administered in combination as well as when each drug was administered alone. However, body weight loss rapidly recovered immediately after the completion of administration, and no deaths were observed. Diarrhea is the dose-limiting toxicity of CPT-11 in humans,[7] and it is also observed in patients treated with gefitinib.[21,22] However, no diarrhea or related phenomena were observed in the mouse model treated with combinations of these

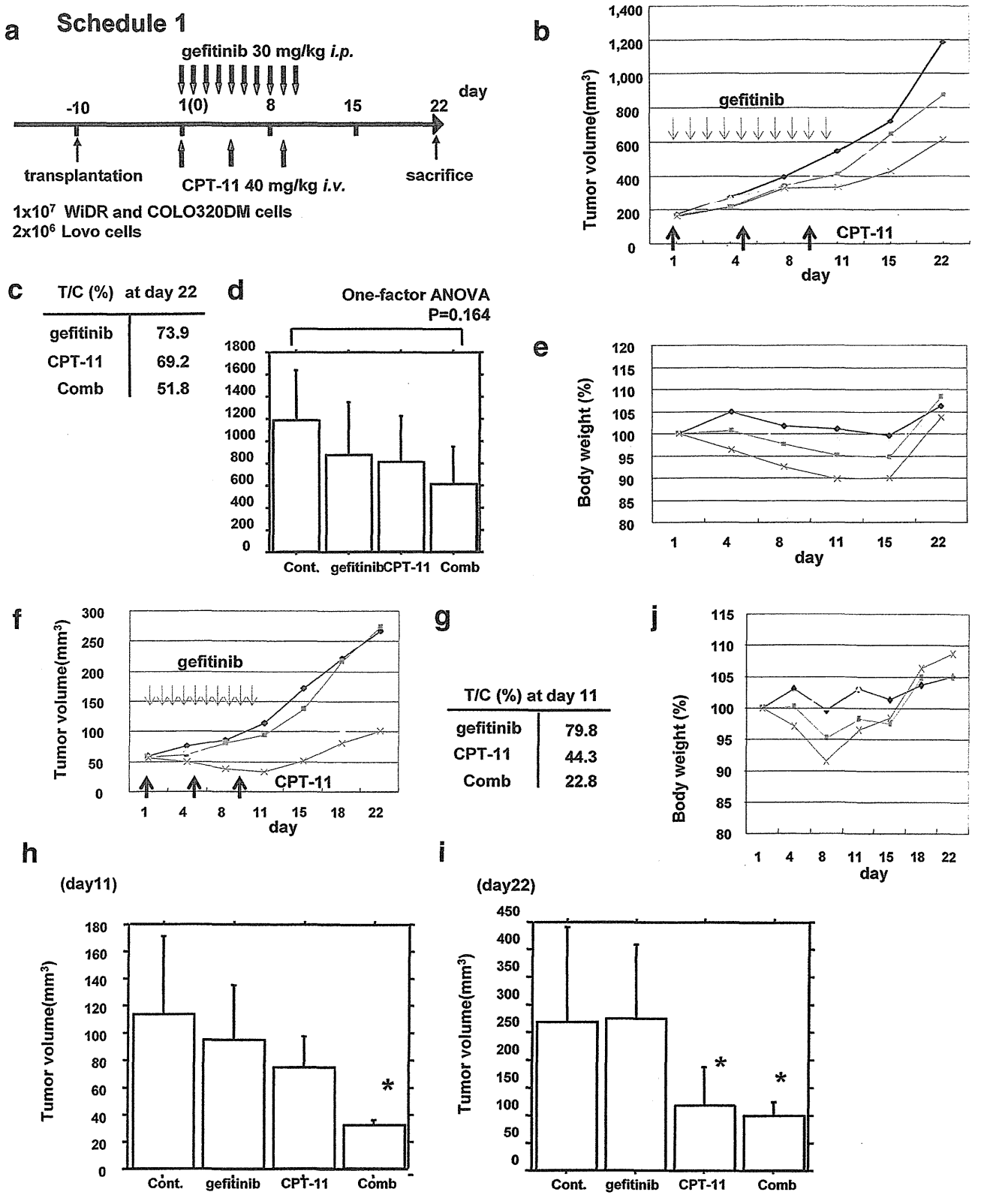
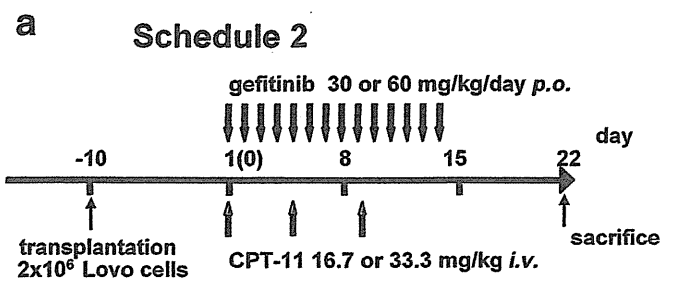
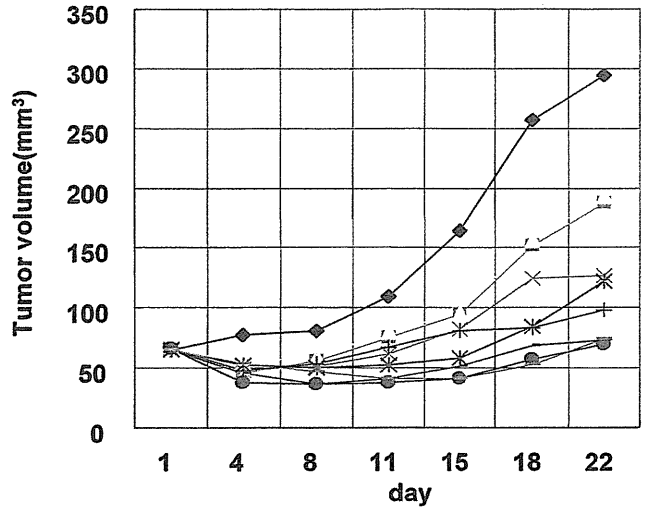
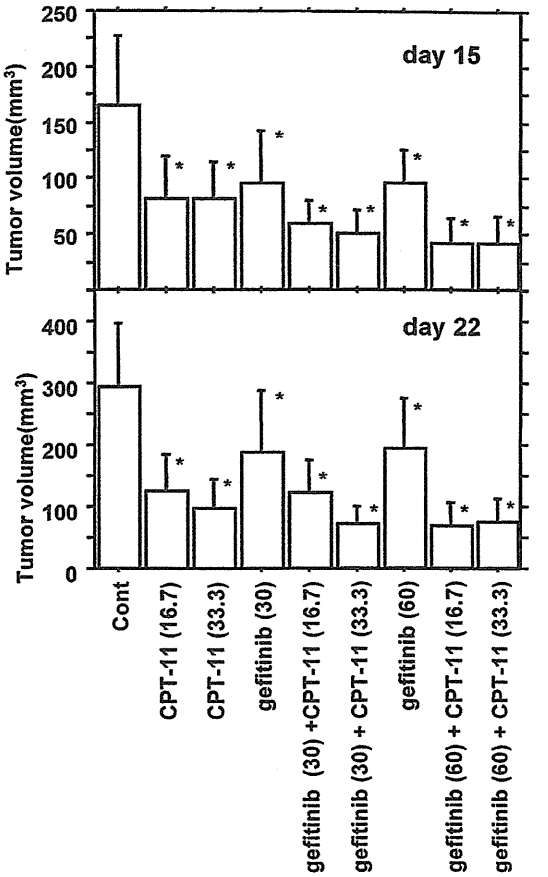


FIGURE 3 – *In vivo* combined effect of gefitinib and CPT-11 on WiDR and Lovo tumor xenografts. (*a*) Treatment schedule. (*b*) (WiDR) and F (Lovo), Tumor growth curves. Female nude mice bearing WiDR or Lovo xenografts were randomly allocated to treatment with 5% (w/v) glucose solution (diamond), gefitinib (square), CPT-11 (triangle), or the combination (×). Tumor volume was calculated as described in Material and Methods. Each data point represents the mean tumor volume of 5 mice. E (WiDR) and J (Lovo) Percent change in body weight in the gefitinib (hatched square) and combination (×) group. C (WiDR) and G (Lovo) Ratio of tumor volume in the control (C) to tumor volume in the treatment group (T) at day 22 and day 15. D (WiDR), H and I (Lovo) Histogram of mean tumor volume at day 11 and day 22 bars, *S.D.* Statistical analysis was performed by 1-factor ANOVA, followed by Fisher's PLSD between 2 groups, as described in the Material and Methods section. *Significant difference ($p<0.05$; Fisher's PLSD) compared to the control.







d

	T/C (%) at day 15
gefitinib 30	57.6
gefitinib 60	58.4
CPT-11 16.7	49.6
gefitinib 30+CPT-11 16.7	35.4
gefitinib 60+CPT-11 16.6	25.1
CPT-11 33.3	49.3
gefitinib 30+CPT-11 33.3	30.9
gefitinib 60+CPT-11 33.3	25.3

	T/C (%) at day 22
gefitinib 30	63.7
gefitinib 60	65.7
CPT-11 16.7	43.0
gefitinib 30+CPT-11 16.7	41.6
gefitinib 60+CPT-11 16.6	23.6
CPT-11 33.3	33.2
gefitinib 30+CPT-11 33.3	24.9
gefitinib 60+CPT-11 33.3	25.3

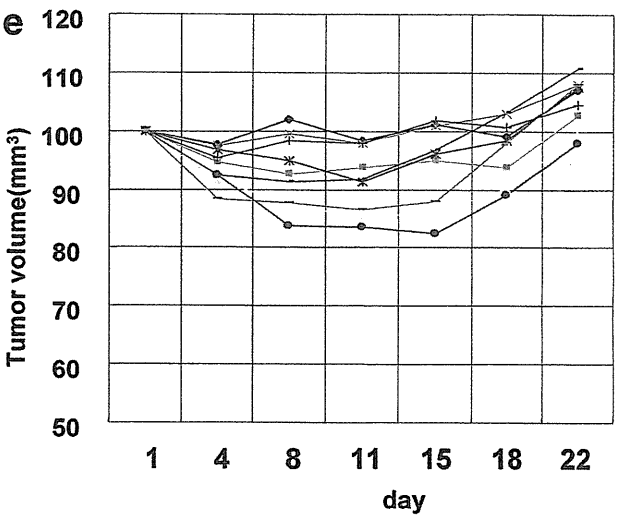


FIGURE 4 – The dose-dependent effect of combination therapy on Lovo cells *in vivo*. (*a*) Treatment schedule. (*b*) Significant growth-inhibition was observed in mice treated with the combination. Mice were allocated to 9 groups (6 mice/group) [closed diamond, 5%(W/V) glucose solution; ×, CPT-11 16.7 mg/kg; + CPT-11 33.3 mg/kg; square, gefitinib 30 mg/kg; star, gefitinib 30 mg/kg + CPT-11 16.7 mg/kg; blue line, gefitinib 30 mg/kg + CPT-11 33.3 mg/kg; open triangle, gefitinib 60 mg/kg; circle, gefitinib 60 mg/kg + CPT-11 16.7 mg/kg; light blue line, filled square, gefitinib 60 mg/kg + CPT-11 33.3 mg/kg]. (*c*) Mean tumor volumes and results of the statistical analysis at days 15 and 22, bars, *S.D.* *Significant difference ($p<0.05$) compared to the control. (*d*) T/C(%) at day 15 and 22. (*e*) Treatment-related body weight loss occurred in mice treated with gefitinib 60 mg/kg (triangle, circle, and light blue line).