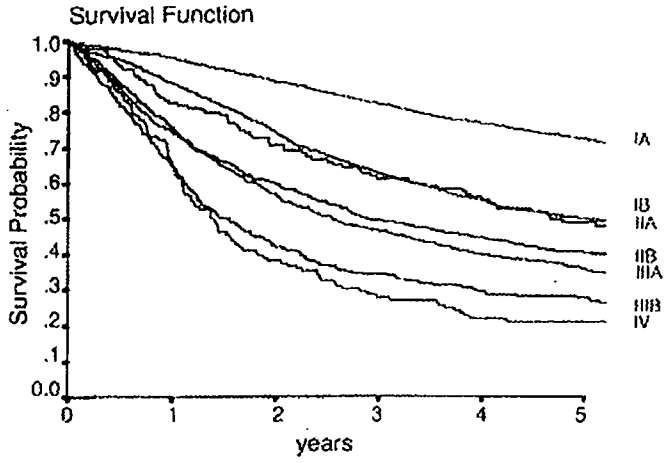

Fig. 1. Survival curves according to clinical (c)-stage. The 5-year survival rates according to c-stage were as follows: 72.1% for IA (n = 2423), 49.9% for IB (n = 1542), 48.7% for IIA (n = 150), 40.6% for IIB (n = 746), 35.8% for IIIA (n = 1270), 28.0% for IIIB (n = 366), and 20.8% for IV (n = 147). There was a significant difference in survival between stages IA and IB (P = 0.0000), between stages IIA and IIB (P = 0.0458), between stages IIB and IIIA (P = 0.0439), and between stages IIIA and IIIB (P = 0.0000). There was no difference in survival between stages IB and IIA (P = 0.4969) or between stages IIIB and IV (P = 0.1577).

Autofluorescence bronchoscopes (AFBs)

Central-type early-stage lung cancer can be cured by noninvasive endoscopic treatment, such as PDT, which has advantages for patients with poor pulmonary reserve; however, the detection of carcinoma in situ (CIS) is a challenge for bronchoscopists. Such lesions show only subtle changes in the bronchial mucosa,[5] and Woolner[6] reported that 60% of CIS lesions showed no macroscopically abnormal findings. This is particularly true with slightly edematous or superficial mucosal changes that can easily be missed, even by experienced bronchoscopists, because they are only a few millimeters thick. Autofluorescence diagnosis is a powerful method to detect macroscopically subtle lesions of the bronchus. Autofluorescence bronchoscopes (AFBs) have been used in leading facilities throughout the world, and the sensitivity for detection of intraepithelial lesions was reported to be 1.5 to 6 times higher than that of conventional white-light bronchoscopy.

Endobronchial ultrasonography (EBUS)

In order to decide indications for PDT, knowledge of the depth of the bronchial tumor is important. Previously, we assessed depth of tumor invasion by the shape of the tumor and loss of bronchus folds. Endobronchial ultrasonography (EBUS) can image the bronchial wall structure in order to assess the depth of bronchial tumor invasion.

Malignant tissues are imaged as hypoechoic areas, and tumor invasion of the cartilage layer is clearly detected. The bronchial wall structure can be imaged as six distinct layers. The cartilage layer is easily identified and can be used to evaluate bronchial wall invasion.

Optical coherence tomography (OCT)

Optical coherence tomography (OCT) is a new modality to detect early-stage lung cancer. OCT can obtain high-resolution, cross-sectional microscopic images of tissue, potentially enabling an optical biopsy to substitute for conventional excisional biopsy. We sought to investigate the capability of OCT to image the microstructure of normal and abnormal bronchial tissue. To assess the depth of bronchial tumor invasion, OCT imaging of the bronchial wall structure was clearer than EBUS, but OCT could detect only the surface of the bronchus.

The OCT system we used was produced by Light Lab Imaging (Boston, MA, USA) and Pentax (Tokyo, Japan). We inserted the OCT catheter via the working channel of the bronchoscope to evaluate the bronchial lumen. The catheter delivers a radial OCT beam and scans circumferentially to generate a transluminal image. In central-type lung cancers, the tumors showed unevenly distributed high backscattering areas and resultant loss of the normal layer structure. We believe that OCT will be able to detect nuclear structure and be used for diagnose is similarly to biopsy in the future.[7]

Possibility of limited resection by video-assisted thoracoscopic surgery (VATS)

The standard therapeutic procedure for peripheral-type early-stage lung cancer is believed to be lobectomy with mediastinal lymph node dissection. However the question has been raised whether lobectomy is really needed for tiny tumors, particularly those less than 1 cm in greatest dimension. There are several reports on limited resection of small lung cancers.[8,9] Some of these results showed satisfactory 5-year survival rates. Clinical trials to clarify the possibility of limited resection are needed for particularly small lung cancers showing ground-glass opacity (GGO), or ground-glass attenuation (GGA). Most of such lesions showed no lymph node metastases, and a 5-year survival of 100% was obtained in patients with such cases who underwent resection. Wedge resection of small lung cancers by VATS without lymph node dissection is one type of minimally invasive surgery. If some types of lung cancer could be shown to be resected by VATS without any increase in local recurrence, this method could become a future standard treatment for peripheral small lung cancers.

Rate of lymph node metastasis of peripheral small nodular cancers

In the past 5 years, 983 patients with lung cancer underwent surgery at our institution. Among them, a total of 159 pa-

tients were studied (Table 1). The tumor size was classified into three categories: 1 cm or less, 1 to 1.5 cm, and 1.5 to 2 cm (47, 49, and 63 patients, respectively). There were 147 pathological N0 patients; lymph node metastasis was recognized in 12 patients (7.5%); this was N1 in 3, and N2 in 9. Table 2 shows the rate of lymph node involvement according to tumor size. In patients with tumors of 1 cm or less, 98% showed no lymph node involvement; however, even in these tiny tumors, 2% showed N2 disease. In tumors between 1 and 1.5 cm, 94% showed no metastasis, but 6% were either N1 or N2. In tumors between 1.5 and 2 cm, lymph node involvement was recognized in 13%.

In this study, the percentages of GGO in tumors were extensively analyzed. We divided tumors into two categories according to how much of the lesion consisted of GGO findings. According to these criteria, 44 tumors showed more than 50% GGO and 115 showed less than 50% GGO. Tumors with a GGO ratio of more than 50% showed no lymph node metastases. On the contrary, all node-positive tumors showed a GGO ratio of less than 50% (Table 3). The relationship between percent GGO area on High Resolution Computed Tumography (HRCT) and the Noguchi classification[10] is shown in Table 4.

Twenty-five of the 44 tumors (57%) showing a GGO component of more than 50% on HRCT were Noguchi type A and B. Seventeen of the 71 tumors (24%) of type C showed more than 50% GGO, and the remaining 54 type C tumors (76%) showed less than 50% GGO. Fifty-three of the 55 (96%) type D, E, and F tumors showed less than 50% GGO. A good correlation between the CT findings and the Noguchi classification was recognized.

The relationship between representative clinicopathological factors and the percent GGO area is shown in Table 5. According to the χ^2 test, the percent GGO area was related to tumor size ($P = 0.0135$) and pathological stage ($P = 0.04$). In particular, a significant relationship with percent GGO was obtained for pathological features including the Noguchi classification ($P = 0.0001$), vascular invasion, and lymphatic invasion.

The overall 5-year survival rate of the patients studied was 88.0%, but it was 96.7% in those with tumors less than 1 cm in diameter, 81.6% in those with tumors between 1 and 1.5 cm, and 84.4% in those with tumors between 1.5 and 2 cm.

The 5-year survival rate was also analyzed according to percent GGO in the lesion. In patients with more than 50% GGO, a 100% 5-year survival rate was obtained, but those with less than 50% GGO had an 83.9% 5-year survival rate.

According to the Noguchi classification, a 5-year survival rate of 100% was obtained in types A and B, with 5-year survivals of 97.4% in type C, 67.1% in types D, E, and F, respectively, which was significantly lower than the results for types A and B and C.

Future surgical procedures for peripheral early-stage lung cancer

Tumors with 100% GGO findings on CT images could indicate suitability for limited surgical resection by VATS. Lesions showing between 50% and 100% GGO may also be indicated for limited resection in tumors less than 2 cm in

Table 1. Patient characteristics

Characteristics	
Age (years)	
Average	63
Minimum	40
Maximum	84
Sex	
Male	67
Female	92
Smoking habit	
Non-smoker	89
Smoker	70
Operative procedure	
Lobectomy	112
Segmentectomy	20
Wedge resection	27

Table 2. Tumor size and nodal status

Tumor size	N0	N1	N2
1.0 cm or less ($n = 47$)	46	0	1
1.0–1.5 cm ($n = 49$)	46	1	2
1.5–2.0 cm ($n = 63$)	55	2	6

Table 4. GGO area and Noguchi classification

GGO%	A, B	C	D, E, F	
More than 50%	25	17	2	44
Less than or equal to 50%	8	54	53	115

Table 5. Relationship between prognostic factors and percent GGO on HRCT

Prognostic factor	χ^2	P value
Sex	0.162	0.687
Tumor size	8.616	0.0135
Pathological stage: I or II–IV	4.168	0.0412
Noguchi classification: A, B, C or D, E, F	14.442	0.0001
Vascular invasion	6.76	0.0093
Lymphatic invasion	5.326	0.0206

Table 3. GGO area and TN status

GGO%	$T \leqq 1$ cm	$1 < T \leqq 5$ cm	$1.5 < T \leqq 2$ cm	
More than 50%	18	16	10	44
Less than or equal to 50%	29 (1)	33 (3)	53 (8)	115 (12)

Numbers in parentheses are numbers of node-positive tumors

diameter, and also, perhaps, in lesions showing between 10% and 50% GGO findings with a tumor size less than 1 cm in diameter. Evaluation of limited resection for small peripheral nodules was reported previously by several researchers.[8,9,11] However, different opinions concerning the modalities used have been reported.[12,13] There are still controversies concerning limited resection of peripheral small lung cancers. A randomized clinical trial by the Lung Cancer Study Group (LCSG) demonstrated the disadvantages of limited resection for T1N0 tumors in relation to lobectomy.[13] Therefore, clinical evidence of the usefulness of limited resection for peripheral early-stage lung cancers should be established. The features of peripheral lung cancers suitable for limited resection without lymph node dissection should be clarified. This will make it possible to determine the optimal CT findings for limited resection.

In our experience, even if the primary lesion was less than 1 cm in size, nodal involvement was confirmed histologically in some patients. Prognostic factors may depend not solely on tumor size but also on the percent GGO area. It is necessary to clarify the findings of CT images of noninvasive cancer by a clinical multicenter study.

Low-dose CT screening for lung cancer

Helical (spiral) CT imaging in the early 1990s provided a promising test for the detection of smaller nodules in the lungs, compared with traditional chest radiography, as images of the chest could be obtained in less than 20 s at a low dose of radiation. It is generally accepted that low-dose CT screening leads to early diagnosis of lung cancer in a high percentage of cases. Based on this evidence, annual CT screening provides for detecting the disease at earlier and presumably more commonly curable stages. The Early Lung Cancer Action Project (ELCAP) showed the great superiority of CT imaging over chest radiographic imaging in identifying cancerous "nodules" in the lungs.[14,15]

Adjuvant chemotherapy for early-stage lung cancer

Recently, some reports have shown significant survival results with adjuvant chemotherapy. The Japan Lung Cancer Research Group (JLCRG) conducted a randomized phase III trial of adjuvant chemotherapy with and without uracil plus tegafur (UFT) after complete surgical resection for stage I adenocarcinoma patients. Subgroup analysis of 263 stage IB patients showed a highly significant result for the UFT arm (5-year survival, 84.9% versus 73.5%; $P = 0.005$).[16]

Conclusions

Good results have been obtained in early-stage lung cancer treatments. Photodynamic therapy (PDT) is suitable for central-type early-stage lung cancer. VATS is a good indication for peripheral-type early lung cancer. Recently, less invasive therapies, such as stereotactic radiation therapy,[17] charged-particle therapy,[18] and microwave coagulation therapy[19] have shown promising results. PDT could be a good modality for peripheral lung cancer, too.[20] The important thing is to find the early-stage lung cancers.

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LUNG CANCER

Effect of Histologic Type on Recurrence Pattern in Radiation Therapy for Medically Inoperable Patients with Stage I Non-Small-Cell Lung Cancer

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Abstract Japanese randomized trials showed that there was a significant impact on survival from stage I adenocarcinoma (AD) of the lung by adjuvant chemotherapy with uracil-tegaful after complete resection but there was no effect for patients with squamous cell carcinoma (SQ). The purpose of this study was to examine the correlation of tumor histology and clinical outcome of radiation therapy (RT) for stage I non-small-cell lung cancer (NSCLC) and to consider the necessity of adjuvant chemotherapy after RT for these patients. The subjects were 83 patients, 54 with SQ and 29 with AD; they had received definitive RT with the total dose ranging from 60 to 80 Gy with conventional fractionation at a daily dose of 2 Gy. The differences between SQ and AD with respect to survival and recurrence pattern were investigated. The 5-year overall survival and cause-specific survival rates were 26.5% and 49.1%, respectively. No difference in survival was observed between SQ and AD patients, and the recurrence rates were almost identical (44% for SQ and 45% for AD). However, the 5-year primary control rate of SQ was significantly poorer than that of AD (SQ: 61.5%; AD: 87.6%; $p = 0.03$). Conversely, the 5-year metastasis-free survival rate of SQ was significantly better than that of AD (SQ: 88.2%; AD: 53.0%; $p = 0.005$). The different failure pattern, according to tumor histology, indicates that taking into consideration the difference in their clinical behaviors would also be important for planning RT and surgery for early lung cancer.

Keywords Non-small-cell lung cancer · Histologic type · Radiation therapy · Survival · Recurrence

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Introduction

The incidence and mortality rate of non-small-cell lung cancer (NSCLC) have been increasing in Japan. Surgery and radiation therapy (RT) have been recognized as curative therapies for NSCLC. In particular, RT is a typical treatment modality for patients with unresectable or inoperable tumors [6, 8, 10, 35]. Because of the increase in the number of elderly patients with NSCLC, RT will play a more important role in the management of these patients.

The majority of histologic types of NSCLC are squamous cell carcinoma (SQ) and adenocarcinoma (AD), but the influence of histologic type on the treatment outcomes in RT has not been investigated in detail because it was thought that the difference in survival between SQ and AD was not significant in NSCLC. Recently, however, a difference in the clinical behavior of the different histologic types of NSCLC has been found [11, 22, 25]. Our previous study of

two-year survivors of NSCLC suggested that patients with AD had a higher possibility of future recurrence than patients with SQ [19]. Hence, the patterns of failure may be different according to the histologic type of tumor of NSCLC in RT.

It is possible that more aggressive treatments such as combined chemoradiotherapy (CRT) can improve the prognosis [2, 27, 28], but these intensive treatments seem intolerable, especially for impaired patients because they tend to be elderly and be in poor general condition. Therefore, from the strategic point of view, to plan for or modify a treatment for NSCLC, more stratified treatment modalities should be developed according to a patient's condition, including the tumor histologic type. With this in mind, we retrospectively analyzed the treatment outcomes, including the recurrence pattern and survival, of stage I NSCLC treated with RT. We also investigated whether the tumor histologic type of NSCLC may serve as a guide for selection of the treatment modalities.

Patients and Methods

Patients

Between January 1980 and June 2002, 87 patients with stage I NSCLC were treated with a curative intent with definitive RT alone at Gunma University. The histologic examination found that SQ and AD were observed in 54 and in 29 patients, respectively. The remaining four patients were excluded from this analysis because of other histologic types of NSCLC. Patient characteristics are summarized in Table 1. Of the 83 patients, 68 were males and 15 were females. The median age was 74 years (range = 43–91 years) at the start of treatment and 18 patients (22%) were over 80 years old. The sites of primary tumors were the upper lobes in 47 patients, the middle lobes in 7, and the lower lobes in 29. Twenty-four patients had T1 disease and the remaining 59 tumors were judged as T2. The reasons for medical inoperability were poor pulmonary function in 29 patients, cardiovascular disease in 17, other medical disease in 12, and advanced age in 19; the remaining 6 patients refused surgery. There were no significant differences in characteristics between the SQ and AD patients, although there was a tendency for the tumors in SQ patients to be larger than those in AD patients ($p = 0.07$).

Table 1 Characteristics of patients according to tumor histologic type

Characteristics	No. of patients (%)			
	SQ ($n = 54$)	AD ($n = 29$)	Total ($n = 83$)	p value
Gender				
Male	47 (87)	21 (72)	68 (82)	0.18
Female	7 (13)	8 (28)	15 (18)	
Age (yr)				
–70	20 (37)	6 (21)	26 (31)	0.13
71+	34 (63)	23 (79)	57 (69)	
Performance status				
0–1	49 (91)	27 (93)	76 (92)	0.96
2–3	5 (9)	2 (7)	7 (8)	
Site				
Upper	31 (57)	16 (56)	47 (57)	0.46
Middle	3 (6)	4 (14)	7 (8)	
Lower	20 (37)	9 (30)	29 (35)	
T-stage				
T1	15 (28)	9 (31)	24 (29)	0.76
T2	39 (72)	20 (69)	59 (71)	
Tumor size (cm)				
–3.9	30 (56)	22 (76)	52 (63)	0.07
4.0+	24 (44)	7 (24)	31 (37)	
Total dose (Gy)				
–69	28 (52)	19 (66)	47 (57)	0.23
70+	26 (48)	10 (34)	36 (43)	
Nodal irradiation				
Yes	29	7	36	<0.01
No	25	22	47	

SQ = squamous cell carcinoma; AD = adenocarcinoma

Treatment

Our treatment policy for patients with NSCLC has been previously described [10, 19] and was similar during this period. In brief, all patients were treated with RT using 10-MV X-rays with a once-daily conventional fractionation, 5 fractions a week. The total dose ranged from 60 to 80 Gy (mean dose = 66.8 ± 5.8 Gy) with a fraction dose of 2 Gy. Thirty-six patients, whose tumors were located around the ipsilateral hilus, received prophylactic irradiation to regional lymph nodes at the ipsilateral side with a total dose of 38–46 Gy (median dose = 40 Gy), whereas the remaining 47 patients, who had a peripheral tumor in the lung, were irradiated with a localized field to the tumors. The doses were calculated at the central axis without correcting for lung heterogeneity. No patient received surgery or additional chemotherapy as part of the initial treatment.

Followup and Statistics

The last followup was performed in August 2005. The median followup period for all patients was 66 months (range = 8–222 months) or until death. The followup examination included a physical examination, chest radiography, and serum tumor marker study. The first examination was performed two weeks after treatment.

followed by examinations at 4-week intervals within a year after RT and then at 1–3-month intervals thereafter. When a recurrence was suspected as a result of these assessments, other modalities, including computed tomography, gallium-67 scintigraphy, positron emission tomography (PET) with 2-[F-18]fluoro-2-deoxy-d-glucose (FDG), bone scintigraphy, sputum cytology, bronchoscopy, and biopsy, were used. Local failure was defined by pathologic determination of recurrent carcinoma or progressive radiographic abnormalities thought to be consistent with tumor progression rather than radiation-related changes. Intrathoracic recurrence included recurrent tumors developing in the lung, including pulmonary metastasis, lymph nodes, or pleura. The survival time was calculated from the date of the start of RT to death or the last followup according to the Kaplan-Meier method [12]. The survival curves were statistically compared by the log rank test, and the differences were considered significant if the $p < 0.05$.

Results

Toxicity

All treatment courses were completed successfully and no patient needed the split-time during RT. Two patients with radiation pneumonitis required administration of steroids but the symptoms were immediately resolved by medication only. One patient, who received a total dose of 80 Gy to the hilum, developed pulmonary insufficiency due to severe stenosis of the proximal bronchus, which was confirmed by bronchoscopic examination 6 months after RT. No patient had other serious complications related to RT.

Survival, Local Control, and Recurrence Pattern

At the time of the last followup, 58 patients had died from the progression of the lung cancer ($n = 33$) or a concurrent disease ($n = 25$). The median survival time (MST) for all patients was 32 months. The 5-year rates of overall survival (OS) and cause-specific survival (CSS) were 26.5% and 49.1%, respectively (Fig. 1). In addition, the 5-year CSS rates according to clinical stage were 54.8% in T1N0M0 (stage IA) and 46.8% in T2N0M0 (stage IB).

Recurrences were observed in 37 (45%) of the 83 patients in this study. The initial sites of recurrence are summarized in Table 2. Recurrences arose from primary lung tumors in 18 patients (22 %), regional lymph nodes in 5 (6 %), malignant pleural effusion in 4 (5 %),

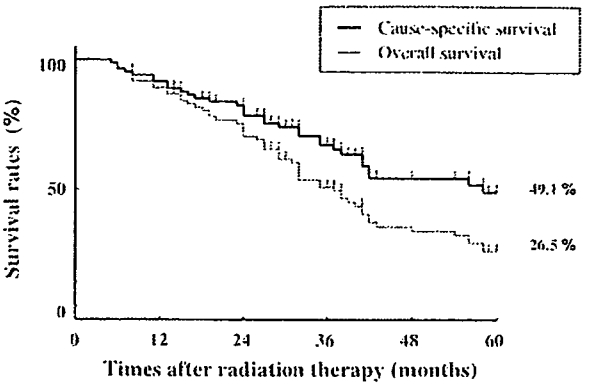

Fig. 1 The curves of overall survival (OS) and cause-specific survival (CSS) for stage I NSCLC. The 5-year OS (gray line) and CSS (bold line) rates were 26.5% and 49.1 %, respectively

and distant organs in 16 (19 %). There were locoregional and intrathoracic recurrences in 23 (28 %) and 31 patients (37 %), respectively. In this series, the 5-year rates of primary tumor control (PTC) and locoregional control (LRC) were 71.6% and 65.0%, respectively.

In addition, 15 (25%) of 59 patients with a local recurrence had T2 tumors, whereas there were only 3 (13%) local recurrences in 24 patients with T1 tumors. The 5-year PTC rate of patients with T1 disease was 84.1%, whereas the corresponding rate of T2 disease was 64.1%. However, the difference according to T stage did not reach a statistical significance ($p = 0.11$).

Difference in Clinical Outcome Between Squamous Cell Carcinoma and Adenocarcinoma

Table 3 gives the initial recurrent patterns of SQ and AD. Recurrences developed in 24 patients (44%) with SQ and 13 (45%) with AD, i.e., the total recurrence rates of the two groups were almost same. However, primary tumor recurrence, locoregional recurrence, and distant metastasis for SQ as initial recurrences were found in 16 (29%), 20 (37%), and 5 (5%) patients, respectively, whereas those for AD occurred in 2 (7%), 3 (10%), and 11 (38%) patients, respectively. There were significant differences in the initial recurrent types between SQ and AD (primary tumor recurrence: $p = 0.03$; locoregional recurrence: $p = 0.02$; distant metastasis: $p = 0.004$).

The MST of patients with SQ was 32 months and was equal to that of AD, and the 5-year rates of OS and CSS were 31.2% and 51.6% in SQ and 18.0% and 44.5% in AD, respectively. Furthermore, recurrence-free survival rates at 5 years of SQ and AD were 49.4% and 48.7%, respectively, which was no significant difference (Table 4). However, the 5-year rates of PTC

Table 2 Initial recurrent sites of stage I non-small-cell lung cancer after RT

Patterns	No. of patients (%)
Local only	15 (18)
Distant only	11 (13)
Regional only	3 (3)
Pleural effusion only	2 (2)
Local + distant	2 (2)
Regional + distant	2 (2)
Local + effusion	1 (1)
Distant+effusion	1 (1)

Table 3 Initial recurrent patterns according to tumor histologic type

	No. of patients (%)			
Patterns	SQ (n = 54)	AD (n = 29)	Total (n = 83)	p value
All recurrences	24 (44)	13 (45)	37 (45)	0.97
Local	16 (30)	2 (7)	18 (22)	0.03
Regional	4 (7)	1 (3)	5 (6)	0.81
Distant	5 (9)	11 (38)	16 (19)	< 0.01
Locoregional	20 (37)	3 (10)	23 (28)	0.02
Pleural effusion	1 (2)	3 (10)	4 (5)	0.24
Intrathoracic	21 (39)	10 (34)	31 (37)	0.69

SQ = squamous cell carcinoma; AD = adenocarcinoma

and LRC for patients with SQ were 61.5% and 54.8%, respectively, whereas the corresponding rates for AD were 87.6% and 84.0%, respectively. The survival curves of PTC and LRC for AD were significantly superior to those for SQ [p = 0.03 for PTC (Fig. 2A); p = 0.02 for LRC (Fig. 2B)]. On the other hand, the distant metastasis-free survival (DMFS) of AD was significantly inferior to that of SQ, and 5-year DMFS rates of AD and SQ were 53.0% and 88.2%, respectively (p = 0.005; Fig. 2C).

In 60 patients who achieved locoregional tumor control, the 5-year CSS rates of SQ (n = 34) and AD (n = 26) were 86.8% and 52.3%, respectively. The difference between SQ and AD was statistically significant (Fig. 3, p = 0.02).

Discussion

When making an appropriate choice of treatment modality for NSCLC, it is important to take the natural history of each case of NSCLC into consideration. Vrodoljak et al. [34] and McGarry et al. [15] demonstrated that the MSTs of nontreated, medically inoperable, early NSCLC were 14–17 months. On the other

Table 4 Survival rates according to tumor histologic type

	5-year rates (%)			
Types	SQ	AD	All	p value
OS	31.2	18.0	26.5	0.26
CSS	51.6	44.5	49.1	0.61
RFS	49.4	48.7	49.3	0.97
PTC	61.5	87.6	69.6	0.03
LRC	54.8	84.0	63.7	0.02
DMFS	88.2	53.0	74.5	0.005

SQ = squamous cell carcinoma; AD = adenocarcinoma; OS = overall survival; CSS = cause-specific survival; RFS = relapse-free survival; PTC = primary tumor control; LRC = locoregional control; DMFS = distant metastasis-free survival

hand, the MSTs of patients with stage I NSCLC after conventional RT ranged from 18 to 27 months, and their 5-year CSS rates were estimated at 30%–40% [6, 10, 20, 29]. The current study on conventional RT for medically inoperable stage I NSCLC was, by population size, one of the largest at a single institution. In this study the MST was 32 months and the 5-year rates of OS and CSS were 26.5% and 49.1%, respectively. Taking into account that 59 of 83 patients (71%) had T2 diseases, these results were comparable to or even a little better than those of the previously referenced reports. Furthermore, Endo et al. [3] concluded that the 5-year survival rates following surgical treatment were 65% and 45% in patients with clinical stage IA and IB, respectively. Therefore, the comparable 5-year CSS rates of 54.8% for stage IA and 46.9% for stage IB in the inoperable patients of the present study indicate that RT is an effective treatment method for medically inoperable patients with stage I NSCLC.

NSCLC contains various histologic types with SQ and AD being seen most common. Despite their histologic difference, they have usually been treated with the same treatment protocol as NSCLC and their outcomes are also investigated en masse, because it has been assumed that there is no difference between survival rates of patients with SQ and AD who were treated with not only surgery but also with RT. Our results also demonstrated that the survival rate of AD 5 years after treatment was similar to that of SQ. However, several differences in the clinical behaviors of the two histologic types have been recognized [4, 11, 14, 22, 25]. It was reported that AD had a relatively higher possibility of developing distant metastases without any sign of progression in the primary tumor [22], and Thomas et al. [32] also reported that patients with non-SQ NSCLC had a higher possibility of distant metastases after radical surgery. Our study demonstrated that the rate of distant metastasis development in AD was significantly higher than that of SQ in early

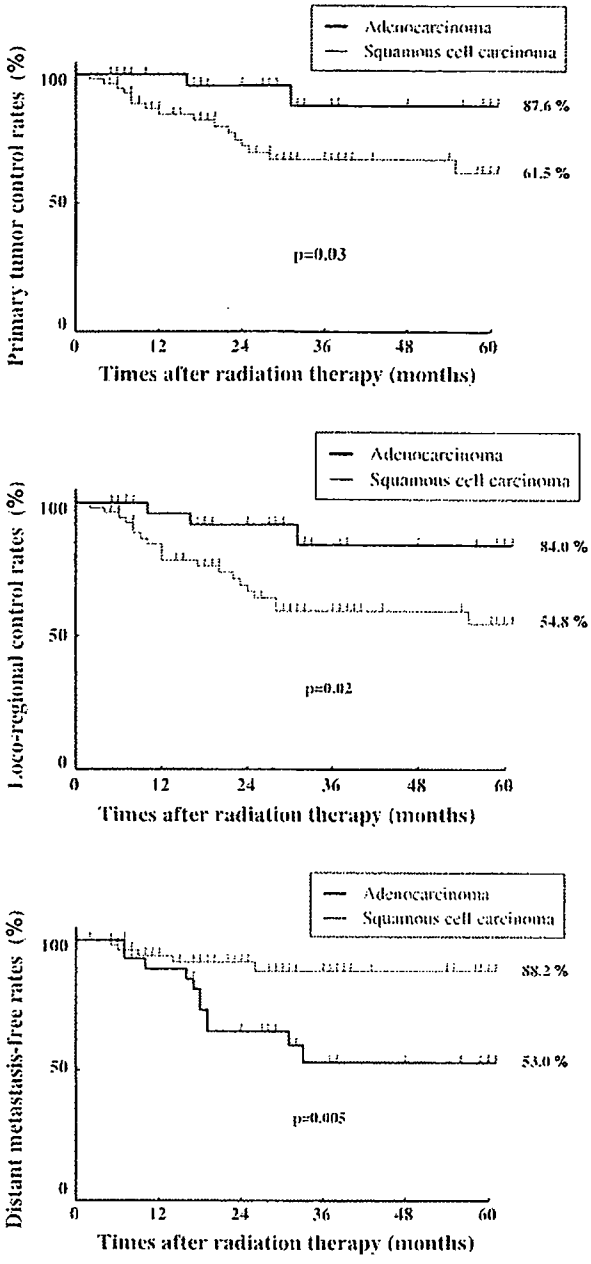

Fig. 2 The curves of primary tumor control (PTC), locoregional control (LRC), and distant metastasis-free survival (DMFS) according to the histologic type: Both curves of PTC and LRC for AD (bold line) were significantly superior to those for SQ (gray line) (**A, B**). On the other hand, the 5-year rate of DMFS for SQ was significantly better than that for AD (**C**)

NSCLC. This supported the frequent development of distant metastases after treatment in AD rather than in SQ in NSCLC patients. Another characteristic of the AD type is that patients with well-differentiated AD could survive for a relatively long time, even with recurrent tumors [5, 7]. Although the data were not shown, two of three patients with well-differentiated

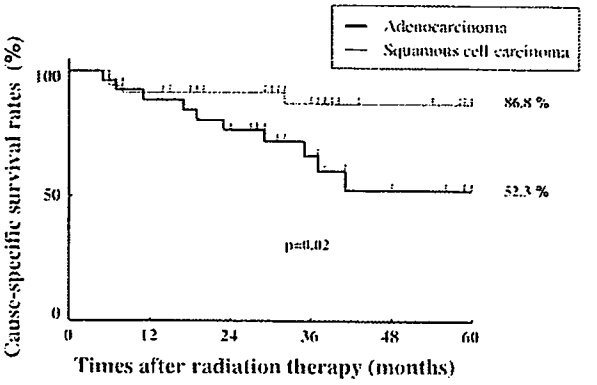

Fig. 3 The curves of cause-specific survival (CSS) of patients who achieved locoregional tumor control according to the histologic type. The 5-year CSS rate of SQ (gray line) was 86.8% and the corresponding rate of AD was 52.3%. The difference of the CSS rates between the two groups was statistically significant ($p = 0.02$)

AD had survived for more than two years after developing a distant metastasis, but no patients in the groups with another histologic NSCLC type survived for two years if their tumors recurred at distant organs.

On the other hand, it seems particularly important to achieve local tumor control in the management of SQ compared with AD [9, 23]. In fact, this study showed that the 5-year CSS rate for patients with SQ who obtained locoregional control was 86.8%, superior to the 52.3% of patients with AD. These results suggested that only local treatments, including radiotherapy, were considered appropriate for SQ but that additional systemic therapy would be necessary for AD.

Many reports have suggested that the combination of chemotherapy with RT for advanced NSCLC has some advantages with respect to the clinical outcome [2, 24, 27, 28, 31]. However, it is very difficult to investigate the influence of the histologic type on the prognosis because of the difficulty of accumulating a large enough number of long-term advanced NSCLC survivors. Therefore, we analyzed the impact of combination therapy on patients with early NSCLC. Sause et al. [11, 27] reported that the usefulness of the combination of chemotherapy and RT was found in a randomized study of patients with non-SQ, whereas no benefit was shown for those with SQ. Furthermore, Japanese trials suggested that adjuvant chemotherapy with uracil-tegaful significantly improved the survival of patients with pathologic stage I AD of the lung who had undergone complete resection, whereas this approach showed little benefit for SQ patients [13, 18]. Considering our data, such a phenomenon might be reproduced with RT, and the histologic type is

therefore a very important factor in selecting what treatment to use.

Dose escalation studies for stage I NSCLC using some modern stereotactic approaches have demonstrated not only better local control rates but also better survival rates with an increasing RT dose [16, 17, 21, 33]. Ohnishi et al. [21] reported that the local recurrence rate of patients with T2 tumors was 41.4% in a Japanese multi-institutional study of stereotactic high-dose irradiation for stage I NSCLC, if the biological effective dose (BED) was less than 100 Gy. They also demonstrated that there was no difference in local recurrence rates between T1 and T2 tumors if the BED was 100 Gy or more and the overall local recurrence rate was less than 10%. The BED in the present study ranged from 72 to 96 Gy, lower than in the Ohnishi et al. study, and the local recurrence rate of T2 disease was as low as 25.4%. Therefore, patients with T2 tumors in the present study should have received higher doses to control the primary tumor.

However, some problems still exist with our approach, similar to those of conventional RT, in that the recurrences often develop not only at the primary site but also in lymph nodes out of the irradiation field. Even for patients with a T1N0 tumor, the incidence of unsuspected nodal metastasis is expected be 15%–20% in peripheral NSCLC [1]. It was also reported that the incidence of lymph node metastasis after radiotherapy for stage I NSCLC was about 10% [21, 30, 33]. There were 5 patients (6%) with nodal recurrences in our study, but there was no significant efficacy of elective nodal irradiation on the regional control. In reality, 3 of 36 patients who received the prophylactic RT to regional lymph nodes had nodal recurrences. However, based on our data we cannot conclude that elective nodal irradiation is necessary for early NSCLC, because only 36 patients whose tumors were located around the ipsilateral hilus received the elective irradiation and these cases seemed to have a greater possibility of future lymph node recurrence than the remaining 47 patients who had a peripheral tumor in the lung.

In conclusion, RT is a successful treatment modality for inoperable stage I NSCLC. To take the histologic type into consideration and to choose a suitable option in RT as well as surgery are very important when attempting to improve treatment results for these patients.

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Effect of the introduction of minimum lesion size on interobserver reproducibility using RECIST guidelines in non-small cell lung cancer patients

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We evaluated interobserver reproducibility for the response evaluation criteria in solid tumors (RECIST) guidelines and the influence of minimum lesion size (MLS) on reproducibility. The 110 consecutive patients with non-small cell lung cancer were treated with platinum-based chemotherapy. Five observers measured target lesions according to both the World Health Organization (WHO) criteria and RECIST. The percentage changes for unidimensional measurements (UD; RECIST type) and bidimensional measurements (BD; WHO type) were calculated for each patient. Interobserver reproducibility among five observers, that is 10 pairs, was expressed as the Spearman's correlation coefficient for the percentage changes, the proportion of agreement and the kappa statistics for response categories. The same analysis was carried out using MLS. BD was more reproducible than UD (Spearman rank correlation coefficient, 0.84 vs 0.81; proportion of agreement, 84.4% vs 82.5%; kappa value, 0.69 vs 0.61). When MLS was applied to UD, eligible cases decreased by 6.4% and the number of target lesions by 44.6%, whereas interobserver reproducibility for UD improved (Spearman rank correlation coefficient, 0.81–0.84; proportion of agreement, 82.5–84.2%; kappa value, 0.61–0.65). The introduction of MLS to UD could also improve intercriteria reproducibility between WHO and RECIST. It is important to apply the MLS when using RECIST for the comparable interobserver reproducibility attained with WHO. (*Cancer Sci* 2006; 97: 214–218)

Tumor response to chemotherapy was previously evaluated using the WHO criteria, which stipulate bidimensional measurement (BD; WHO type) of lesions.[1] With these standardized criteria for evaluating tumor response, valid and reproducible results could be obtained by all investigators. However, a number of modifications to the WHO criteria have been developed by different institutions, which made it difficult to compare response rates for screening new anticancer agents across different investigators. This has led to the introduction of a new system, the RECIST guidelines,[2] which have been widely accepted as the new standard.

In order to standardize the methodology for evaluating tumor response, RECIST simplified the response evaluation through the use of unidimensional measurements (UD: RECIST type) instead of the BD used by the WHO criteria. Furthermore, the MLS allowable for measurement at baseline study was defined as being no less than double the slice thickness on CT or MRI.

The validity and intercriteria reproducibility between the new RECIST guidelines and the previous WHO criteria have been investigated.[2–7] However, to the best of our knowledge, no analysis of the influence of MLS on interobserver reproducibility, specified for measurability in tumor response evaluation according to the RECIST guidelines, has been published in the literature.

The purpose of the present study was therefore to evaluate interobserver reproducibility in tumor response evaluation using RECIST, intercriteria reproducibility between WHO and RECIST, and whether this reproducibility is affected by the application of MLS.

Materials and Methods

Patient population

This is a retrospective study of the radiological findings for patients who underwent chemotherapy for advanced NSCLC. The subjects were patients treated during clinical trials at the Medical Oncology Division of the National Cancer Center Hospital in Tokyo, Japan, between January 1999 and January 2001. All clinical trials were conducted in accordance with the Helsinki Declaration and the protocols were approved by the institutional review board. Written informed consent was obtained from each patient for the treatment protocols, which included the secondary use of treatment-associated documents. Patients were staged according to the Union Internationale Contra le Cancer TNM classification of malignant tumors.[8] The 110 eligible patients included those histologically or cytologically diagnosed with NSCLC. Patients were required to undergo CT scans periodically for evaluating tumor response prior to and once after

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Abbreviations: BD, bidimensional measurements; CR: complete response; CT, computed tomography; MLS, minimum lesion size; MRI, magnetic resonance imaging; NSCLC, non-small cell lung cancer; PD, progressive disease; PR, partial response; RECIST, response evaluation criteria in solid tumors; SD, stable disease; TNM, tumor node metastases; UD, unidimensional measurements; WHO, World Health Organization.

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treatment, to have at least one bidimensionally measurable lesion, and to be treated with chemotherapy in clinical trials.

Patients treated in clinical practice were considered to be unsuitable and excluded from this study as tumor response evaluation in the clinical practice of oncology is not always carried out according to predefined criteria, but rather is made by subjective medical judgment based on clinical and laboratory data. In addition, tumor response evaluation is not always carried out by CT examination, and the intervals between tumor evaluations can be irregular.

Image analysis

Almost all images were acquired with a TCT-900S Superhelix (Toshiba Medical, Tokyo, Japan), with the remainder scanned on an X-Vigor helical CT scanner (Toshiba Medical). Helical CT was carried out with fixed scanning parameters, including a table speed of 15 mm/s, a pitch ratio of 1:1.5 per rotation time 1 s, and the same contrast agent for both baseline and follow-up evaluations. Image reconstruction was carried out at intervals of 10 mm.

On chest CT obtained during baseline examination before the initiation of chemotherapy, target lesions up to a maximum of five lesions per patient with longest and perpendicular diameters that could be measured accurately were selected by one diagnostic radiologist. In addition, one follow-up chest CT examination, indicating tumors with the greatest response to chemotherapy, was selected retrospectively. Target lesions included primary lung lesion, pulmonary metastases and lymph nodes.

For the target lesions, the two parameters consisting of the longest diameter and the diameter perpendicular to it were measured with electronic calipers on digitized images. Five observers of different backgrounds, blinded to patient profiles, reviewed all patients independently and no attempt was made to arrive at a consensus. These observers included one diagnostic radiologist, one thoracic physician, two medical oncologists and one thoracic surgeon.

Tumor response evaluation

The sum of the longest diameters for all target lesions was calculated for pretreatment and post-treatment UD. Similarly, the sum of the products of the longest diameters and their perpendicular diameters for all target lesions was calculated for pretreatment and post-treatment BD. If there were two or more lesions, the sum of all target lesions was calculated. The baseline sum was used as the reference from which objective tumor response could be calculated. The percentage changes were calculated as post-treatment value divided by pretreatment value for both UD and BD.

Percentage changes were then classified using the current RECIST guidelines and the previous WHO criteria tumor response classification system. Tumor response was categorized into CR, PR, SD and PD based on both RECIST guidelines and WHO criteria. The RECIST PR was defined as a 50% decrease in the percentage changes for UD, and the WHO PR was defined as a 30% decrease in the percentage changes for BD. The RECIST PD was defined as a 20% increase in the sum of the longest diameters, and the WHO PD was defined as a 25% increase in the sum of the products of the two diameters of all lesions or in the product of the diameters of one lesion. For the present study, no minimum interval was required for the confirmation of either CR or PR.

Analysis of intercriteria reproducibility

To examine intercriteria reproducibility, the mean and ranges of differences in the response rate between UD and BD were calculated. We then estimated those between UD-MLS and BD. Interobserver differences among the five observers yielded 20 pair comparisons. Intraobserver differences of the same observer yielded five pair comparisons.

Analysis of interobserver reproducibility

First, to examine the interobserver reproducibility of the percentage changes according to the two different dimensional measurements, we estimated the Spearman's correlation coefficient of the percentage changes among the five observers, calculated for each pair observed (five observers yielded 10 pair comparisons).

Second, to examine the interobserver reproducibility for two tumor response criteria, we estimated the proportion of agreement to the categories of CR, PR, SD and PD for both UD and BD among the five observers (10 pair comparisons). We then calculated the kappa statistics, a measure of agreement in which agreement is taken into consideration by chance, to assess interobserver reproducibility for tumor response categories.[9]

Third, we examined the influence of MLS on the number of eligible cases and target lesions. The same analyses on interobserver reproducibility were conducted applying the MLS. MLS was introduced into the RECIST guidelines, which specify a minimum lesion size of less than double the slice thickness on images. The slice thickness was 10 mm in the present study, so the MLS was set at no less than 20 mm at baseline evaluation before treatment. Cases that only had tumors smaller than the MLS were excluded from the present study. We defined the RECIST guidelines as the evaluation by UD for measurable cases and the WHO criteria as the evaluation by BD for all cases.

SAS version 8.02 (SAS Institute, Cary, NC, USA) was used for all analyses.

Results

Patient population

The characteristics of the 110 patients were as follows: male/female = 80/30, median age = 59 years (range 36–72 years), stage IIIB/IV = 33/77. Chemotherapy regimens are listed in Table 1. A total of 220 CT images were reviewed, comprising 110 CT images each from the baseline study (pretreatment) and from the follow-up (post-treatment) study.

Tumor response evaluation between UD and BD

The tumor response evaluation was categorized into CR, PR, SD and PD without MLS. The response rate results are shown in Table 2. None of the patients were rated CR. The use of UD resulted in response categories by observers A, B, C, D and E of 35, 28, 26, 34 and 36 PR, 73, 79, 81, 73 and 71 SD, and 2, 3, 3, 3 and 3 PD, respectively. The response rate ranged from 23.6 to 32.7%. For BD, the corresponding response categories were 37, 30, 33, 36 and 36 PR, 67, 73,

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Table 1. Characteristics of the 110 patients enrolled in the present study

Characteristic	*n*
No. patients	110
Age (years)	
Median	59
Range	36–72
Sex	
Male	80
Female	30
Disease stage at study entry	
IIIB	33
IV	77
Tumor histology	
Adenocarcinoma	78
Squamous	22
Large-cell	1
Unclassified non-small cell	9
Regimen	
Cisplatin and gemcitabine	21
Cisplatin and paclitaxel	18
Nedaplatin and paclitaxel	15
Cisplatin and vinorelbine	14
Carboplatin and paclitaxel	14
Cisplatin and vindesine	13
Cisplatin, docetaxel and ifosfamide	7
Cisplatin and docetaxel	8

Table 2. Response rate (%) using four different measurements among five observers

Measurement	Observer					Mean
	A	B	C	D	E	
UD	31.8	25.5	23.6	30.9	32.7	28.9
BD†	33.6	27.3	30.0	32.7	32.7	31.3
UD-MLS‡	33.0	27.2	32.0	30.1	32.0	30.9
BD-MLS	35.0	32.0	33.0	33.0	34.0	33.4

†WHO criteria; ‡RECIST guidelines. BD, bidimensional measurement; MLS, minimum lesion size; UD, unidimensional measurement.

68, 68 and 68 SD, and 6, 7, 9, 6 and 6 PD, respectively. The response rate ranged from 27.3 to 33.6%.

Tumor response evaluation between UD-MLS and BD-MLS

When the MLS criteria were applied, the number of eligible cases decreased by 6.4% from 110 to 103, and the number of target lesions decreased by 44.6% from 402 to 223.

The response rate results are shown in Table 2. None of the patients were rated CR. When UD was used with MLS, the respective response evaluations made by observers A, B, C, D and E were 34, 28, 33, 31 and 33 PR, 68, 73, 67, 72 and 68 SD, and 1, 2, 3, 0 and 2 PD. The response rates of UD applying MLS ranged from 27.2 to 33.0%, showing a reduction in interobserver difference compared with those of UD not applying MLS. With BD using the MLS, the corresponding response categories were 36, 33, 34, 34 and 35 PR, 63, 66, 65, 63 and 64 SD, and 4, 4, 4, 6 and 4 PD. The response rate ranged from 32.0 to 35.0%.

Intercriteria reproducibility

The intercriteria reproducibility in the response rates is shown in Table 3. Between UD and BD, the intraobserver difference in the response rates ranged from 0 to 6.4% with a mean of 2.36%, and the interobserver difference ranged from 0 to 10.0% with a mean of 4.25%. Between UD-MLS and BD, the intraobserver difference in the response rates ranged from 0.1 to 2.6% with a mean of 1.26%, and the interobserver difference ranged from 0.1 to 6.4% with a mean of 2.76%.

Correlations between UD and BD

The mean and ranges of interobserver reproducibility among five observers using the two dimensional measurements are shown in Table 4. The mean value of the Spearman rank correlation coefficient for the percentage changes when using UD (0.81) was lower than that using BD (0.85), and the same tendency was observed for the mean value of proportion of agreement for the tumor response categories (82.5%, 908/1100 vs 84.4%, 928/1100) and the mean kappa statistics for the tumor response categories (0.61 vs 0.69). The lowest kappa statistics among the 10 pair comparisons were 0.49 with UD and 0.61 with BD. The kappa statistics obtained with BD were higher than those with UD in nine out of 10 pair comparisons (Fig. 1).

Correlations between UD and UD-MLS

The mean values and ranges of interobserver reproducibility when applying the MLS are shown in Table 4. The mean value of Spearman's correlation coefficient for UD-MLS (0.84) was higher than that for UD (0.81), and the same tendency was observed for the mean value of proportion of agreement for the tumor response categories (84.2%, 867/1030 vs 82.5%, 908/1100) and the mean kappa statistics for the tumor response categories (0.65 vs 0.61). The lowest kappa statistics among the 10 pair-based comparisons was 0.57 with MLS and 0.49 without. When MLS was used together with UD, the kappa statistics increased in eight out of 10 pair comparisons (Fig. 2).

Table 3. Intercriteria reproducibility: difference in the response rate (%) among five observers

Category	UD and BD†		UD-MLS‡ and BD†	
	Mean	Range	Mean	Range
Overall (25 comparisons)	3.87	0–10	2.45	0.1–6.4
Interobserver (20 comparisons)	4.25	0–10	2.76	0.1–6.4
Intraobserver (5 comparisons)	2.36	0–6.4	1.26	0.1–2.6

†WHO criteria; ‡RECIST guidelines. BD, bidimensional measurement; MLS, minimum lesion size; UD, unidimensional measurement.

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Table 4. Interobserver reproducibility (10 pair comparisons) using four different measurements among five observers

Category	No. patients	Spearman's correlation coefficient		Proportion of agreement (%)		Kappa statistic	
		Mean	Range	Mean	Range	Mean	Range
UD	110	0.81	0.76–0.86	82.5	77.3–89.1	0.61	0.49–0.75
BD[1]	110	0.85	0.79–0.89	84.4	80.0–89.1	0.69	0.61–0.78
UD-MLS[2]	103	0.84	0.75–0.89	84.2	80.6–88.3	0.65	0.57–0.73
BD-MLS	103	0.86	0.80–0.89	84.0	78.6–89.3	0.68	0.58–0.78

[1]WHO criteria; [2]RECIST guidelines. BD, bidimensional measurement; MLS, minimum lesion size; UD, unidimensional measurement.

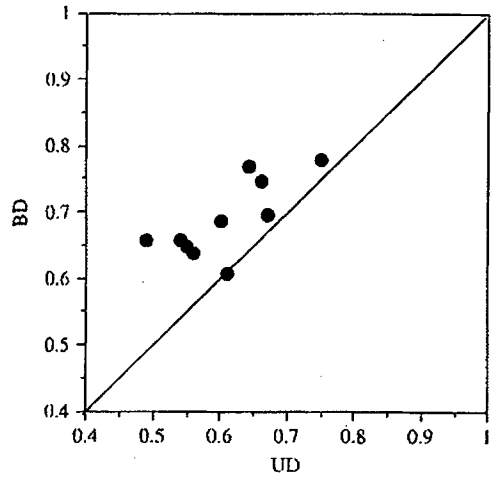

Fig. 1. Scattergram showing the kappa statistics for the use of unidimensional (UD) and bidimensional (BD) measurements. The kappa values for BD were higher than those for UD in nine of 10 pair comparisons.

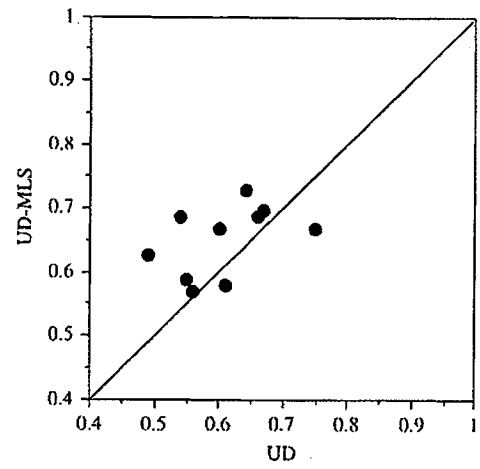

Fig. 2. Scattergram showing the kappa statistics for the use of unidimensional measurement (UD) and UD with minimum lesion size (MLS). When MLS was applied to UD, the kappa values increased in eight of 10 pair comparisons.

Discussion

Standardized tumor response evaluation systems are considered reliable in clinical trials when they are valid and reproducible among different observers. Although the intercriteria reproducibility between the new RECIST guidelines and the previous WHO criteria had been investigated,[2–7] little information was available concerning interobserver reproducibility of tumor response evaluation. In addition, statistical analysis results regarding the effect of MLS on interobserver reproducibility had not been provided in previous reports. This is the first study to investigate interobserver reproducibility of the RECIST guidelines evaluating the MLS.

The importance of interobserver reproducibility for any classification scheme has been discussed previously for other grading systems.[10–12] Clinical investigators must take into account interobserver reproducibility in tumor response evaluation, which can greatly affect the results in clinical trials. Our findings demonstrated that interobserver variability exists for bidimensional measurements, as in studies published previously.[13,14] For example, Hopper *et al.* showed considerable interobserver variability in CT tumor measurements between radiologists interpreting thoracic and abdominal/pelvic CT scans.[13] In another report, the impact of an evaluation committee on patients' overall response status in a large multicenter trial in oncology was evaluated.[14] Major disagreements occurred in 40% of cases and minor disagreements occurred in 10.5% of the cases reviewed. The number of responders was reduced by 23.2% after review by the evaluation committee.

The range of response rates among five observers was clearly narrowed by the MLS (Table 2). The response rates assessed by UD varied from 23.6 to 32.7%. When assessed by BD, the response rates ranged from 27.3 to 33.6%. Response rates assessed with UD-MLS ranged from 27.2 to 33.0%, which was almost identical when BD was used.

The results of the present study also suggested that BD was more reproducible than UD. When MLS was applied to UD, the mean values and ranges of Spearman's correlation coefficient, proportion of agreement and the kappa statistics improved (Table 4). In order to ensure comparable interobserver reproducibility (as was originally achieved with the WHO criteria) it is essential that the MLS be used in combination with UD when using RECIST.

Because of the need to retain some ability to compare results of future therapies with those available currently, no major discrepancy should exist between the old (WHO) and

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new (RECIST) criteria, although measurement criteria would be different. The mean values and ranges of intercriteria reproducibility in the response rates between UD-MLS and BD were lower and narrower than those between UD and BD (Table 3). The introduction of MLS to UD improved the intercriteria reproducibility between WHO and RECIST.

As for intercriteria reproducibility, the mean values and ranges for intraobserver reproducibility were better than those for interobserver reproducibility (Table 3). Erasmus *et al.* have suggested that consistency can be improved if the same reader carries out serial measurements for any one patient.[15]

When MLS is included in the eligibility criteria, the number of patients with measurable lesions is less than that obtained with the previous WHO criteria because patients with only small lesions are excluded from measurement. In the present study, when MLS criteria were used the number of eligible cases decreased by 6.4% from 110 to 103 and the number of target lesions by 44.6% from 402 to 223. This reduction could affect the number of patients enrolled in clinical trials.

The present study had several limitations. First, the study cohort comprised NSCLC patients only and the application of the measurement modalities was limited to chest CT. Second, intraobserver variability between evaluations with different intervals was not investigated. Third, our reference was a 10 mm slice thickness and therefore the minimum lesion size was defined as 20 mm. However, RECIST guidelines allow for a minimum lesion size of 10 mm as a slice thickness of 5 mm measured by helical CT is used. Recently, multidetector CT, which creates a thinner slice thickness, has been developed and is being used in daily clinical practice. Therefore, the addition of the outcomes of patients ineligible for our study as a result of using a thinner slice thickness might change our results and should be evaluated in a further study.

In conclusion, the results of the present study suggest that UD yields poorer interobserver reproducibility of tumor response evaluation than BD; however, if MLS is applied to UD, interobserver reproducibility can improve and become the same as that obtained with BD. The introduction of MLS to UD could also improve intercriteria reproducibility between WHO and RECIST. It is therefore essential that investigators include MLS when using RECIST guidelines to ensure interobserver reproducibility comparable with the WHO criteria.

Acknowledgments

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Effects of different combinations of gefitinib and irinotecan in lung cancer cell lines expressing wild or deletional EGFR

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Summary EGFR mutations are a major determinant of lung tumor response to gefitinib, an EGFR-specific tyrosine kinase inhibitor. Obtaining a response from lung tumors expressing wild-type EGFR is a major obstacle. The combination of gefitinib and cytotoxic drugs is one strategy against lung cancers expressing wild-type EGFR. The DNA topoisomerase inhibitor irinotecan sulfate (CPT-11) is active against lung cancer. We examined the sensitivity of lung cancers expressing wild- or mutant-type EGFR to the combination of gefitinib and CPT-11. The in vitro effect of gefitinib and SN-38 (the active metabolite of CPT-11) was examined in seven lung cancer cell lines using the dye formation assay with a combination index. When administered concurrently, gefitinib and SN-38 had a synergistic effect in five of the seven cell lines expressing wild-type EGFR, whereas the combination was antagonistic in PC-9 cells and a PC-9 subline resistant to gefitinib and expressing deletional mutant EGFR (PC-9/ZD). When administered sequentially, treatment with SN-38 followed by gefitinib had remarkable synergistic effects in the PC-9 and PC-9/ZD cells. In an in vivo tumor-bearing model, this combination had a schedule-dependent synergistic effect in the PC-9 and PC-9/ZD cells. An immunohistochemical analysis of the tumors in mice treated with CPT-11 and gefitinib demonstrated that the number of Ki-67 positive tumor cells induced by CPT-11 treatment was decreased when CPT-11 was administered in combination with gefitinib. In conclusion, the sequential combination of CPT-11 and gefitinib is considered to be active against lung cancer.
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1. Introduction

Lung cancer is one of the leading causes of cancer-related death, despite the use of conventional chemotherapy regi-

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mens. The epidermal growth factor receptor (EGFR) is frequently expressed in non-small cell lung cancer (NSCLC) and is correlated with a poor prognosis. Gefitinib ('Iressa') is an orally active, selective EGFR-tyrosine kinase inhibitor that blocks signal transduction pathways. Its clinical efficacy has been show in refractory NCSLC patients, but the survival benefit of this agent remains unclear. EGFR mutations have been identified in NSCLC, and lung cancers carrying the EGFR mutation have been reported to be hyperresponsive to gefitinib [1,2]. Mutant EGFR is a major determinant of lung tumor response to gefitinib, but the hyperresponsiveness of tumors expressing mutant EGFR has been observed in a small population. Now, obtaining a clinical benefit in lung tumors expressing wild-ype EGFR is a major obstacle. The combination of gefitinib and cytotoxic drugs is one strategy against lung cancers expressing wild-type EGFR. The DNA topoisomerase I inhibitor irinotecan (CPT-11) is a key drug in the treatment of patients with lung cancer and has been shown to prolong survival. SN-38 is the active metabolite of CPT-11 in vitro. The objective of this study was to determine the potential therapeutic utility of gefitinib when combined with CPT-11 therapy to lung cancer cell according to the treatment schedule and EGFR status.

Acquired resistance to gefitinib is also of clinical interest. Recently, Kobayashi et al. [3] reported that an EGFR mutation was related to the development of acquired resistance to gefitinib. We have established subclone PC-9/ZD cells that are resistant to gefitinib [4]. Our results suggested that another mechanism of resistance was active in PC-9/ZD cells. The effect of the combination of gefitinib and SN-38 in these PC-9/ZD cells was also examined.

2. Materials and methods

2.1. 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. CPT-11 and SN-38 were obtained from Yakult Honsha (Tokyo, Japan) and were dissolved in dimethyl sulfoxide (DMSO) for both of the in vitro studies.

2.2. Cells and cultures

Human NSCLC cell lines PC-9, PC-7, and PC-14 derived from untreated patients with pulmonary adenocarcinoma were provided by Professor Y. Hayata, Tokyo Medical College. A small cell lung cancer cell line, H69, was established at the National Cancer Institute (Bethesda, MD, USA). The gefitinib-resistant subline, PC-9/ZD, was established from intrinsic hypersensitive cell PC-9 [5] in our laboratory [4]. A small cell lung cancer cell line, SBC-3, and an adenocarcinoma cell line, A549, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). All cell lines were maintained in RPMI1640 (Nikken Bio Med. Lab., Kyoto, Japan) supplemented with 10% heat-inactivated fetal calf serum, 100 μg/ml streptomycin, and 100 units/ml penicillin in an incubator at 37 °C and 100% humidity in 5% CO_2 and air, as described previously [6].

2.3. RT-PCR

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

2.4. Western blot analysis

The cultured cells were washed twice with ice-cold phosphate buffered saline (PBS), lysate in EBC buffer (50 mM Tris–HCl, pH 8.0; 120 mM NaCl; 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 using a BCA protein assay (Pierce, Rockford, IL, USA). 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, USA). The membrane was then probed with rabbit polyclonal antibodies against EGFR, HER2/neu, Her3 and Her4 (Santa Cruz Biotech, Santa Cruz, CA, USA) and phospho-EGFR specific for Tyr 845, Tyr 1045, and Tyr 1068 (numbers 2231, 2235 and 2234; Cell Signaling, Beverly, MA, USA).

2.5. 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. After incubation for 72 h at 37 °C, 20 μl of MTT solution (5 mg/ml in PBS) was added to each well; the plates were then incubated for a further 4 h at 37 °C. After centrifuging the plates at 200 × *g* for 5 min, the medium was aspirated from each well and 180 μl of dimethylsulfoxide was added to each well to dissolve the formazan. Optical density was measured at 562 and 630 nm using a Delta Soft ELISA analysis program interfaced with a Bio-Tek Microplate Reader (EL-340; Bio-Metallics, Princeton, NJ, USA). Each experiment was performed in six replicate wells for each drug concentration and was independently performed three or four times. The IC_{50} value was defined as the concentration needed for a 50% reduction in the absorbance, as calculated based on the survival curves. Percent survival was calculated as follows:

$$\frac{\text{Mean absorbance of six replicate wells containing drugs} - \text{mean absorbance of six replicate background wells}}{\text{mean absorbance of six replicate drug-free wells} - \text{mean absorbance of six replicate background wells}} \times 100.$$

2.6. Combined effect of gefitinib and SN-38 in vitro

After 24 h of incubation, gefitinib and SN-38 were added to each cell line according to one of the two combination schedules. For the concurrent schedule, gefitinib and SN-38 were added concurrently and were then incubated under the same conditions for 72 h. For the sequential schedule, gefitinib or SN-38 were added sequentially and were then incubated under the same conditions for 72 h. The combined effect of gefitinib and SN-38 on lung cancer cell growth was evaluated using a combination index (CI) [7]. The CI was produced using CalcuSym software (Biosoft, NY, USA). For any given drug combination, the 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 mutually exclusive ($\alpha = 0$) or mutually non-exclusive ($\alpha = 1$) isobologram equations, the F_a/CI plots for each cell line were constructed by computer analysis of the data generated from the median effect analysis. The CI values were interpreted as follows: <1.0 = synergism; 1.0 = additive; >1.0 = antagonism.

2.7. In vivo growth-inhibition assay

Experiments were performed in accordance with the United Kingdom Coordinating Committee on Cancer Research Guidelines for the welfare of animals with experimental neoplasia (second edition). Fig. 2A shows the treatment schedule. For the in vivo experiments, the combined therapeutic effect of orally or intraperitoneally administered gefitinib and intravenously injected CPT-11 was evaluated according to a predetermined schedule. The dose of each drug was set based on the results of a preliminary experiment involving the administration of each drug alone. Ten days before administration, PC-9 and PC-9/ZD cells were injected subcutaneously into the backs of the mice. Six mice per group were injected with tumor cells. Tumor-bearing mice were given either gefitinib (40 mg/kg/day, p.o.) on days 2–6, CPT-11 (50 mg/kg/day, i.v.) on day 1, both, or a placebo (5%(w/v) glucose solution). Alternatively, tumor-bearing mice were given gefitinib on days 2–6 and CPT-1 on days 2. The diameters of the tumors were measured using calipers on days 1, 5, 8, 12, 15 and 20 to evaluate the effects of treatment, and tumor volume was determined 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 20 denotes the day on which the effects of the drugs were estimated, and day "0" denotes the first day of treatment. All mice were sacrificed on day 20 after their tumors had been measured.

2.8. Immunohistochemistry

The tumors were harvested from the mice at the time of sacrifice. For hematoxylin-eosin (HE) and anti-CD31 and Ki-67 staining, the resected tumors were fixed in zinc-buffered formalin (Shandon Lipshaw, Pittsburgh, PA) overnight at 4 °C. After paraffin embedding and sectioning at 6 μm, formalin-fixed sections were stained with Mayer's H&E (Richard Allen, Kalamazoo, MI, USA). For anti-Ki-67 and anti-CD31 immunohistochemistry, the slides were heated in a water bath at 95–99 °C in Target Retrieval Solution (DAKO, Carpinteria, CA, USA) for 20 min, followed by a 20-min cool-down period at room temperature. After heat retrieval, the sections were rinsed well in PBS and stained with rabbit antihuman Ki-67 antigen (DAKO N-series, ready to use) or rat antimouse CD-31 antibody (BD PharMingen, Tokyo, Japan) according to the manufacturer's instructions and then were lightly counterstained with Mayer's hematoxylin. The sections were finally stained with an in situ Death Detection POD Kit (Roche Diagnostic GmbH, Mannheim, Germany), according to the manufacturer's instructions.

TUNEL staining was performed using the Apoptosis Detection System, Fluorescein (Promega, Madison, WI, USA). Briefly, 6-μm cryostat sections were fixed in 4% paraformaldehyde for 10 min at room temperature and rinsed in PBS with 0.1% Triton X-100. The sections were then incubated in Equilibration Buffer for 5 min at room temperature followed by incubation in TUNEL Mix, prepared according to the manufacturer's instructions, for 1 h at 37 °C. After successive washes in PBS, the sections were coverslipped using an antifade reagent.

Microvessel density was determined by calculating the proportion of CD31-positive cells. The Proliferation Index was determined by Ki-67 immunostaining and calculating the population of Ki-67-positive cells in five fields at 200×. The Apoptosis Index, determined by TUNEL staining, was calculated from the population of TUNEL-positive cells in five fields at 200×. The apoptosis:proliferation ratio equals the apoptosis index/proliferation index × 100. At least 1000 tumor cell nuclei from the most evenly and distinctly labeled areas were examined in each examination.

At least 1000 cancer cells were counted and scored per slide. Both the percentage of specifically stained cells and the intensity of immunostaining were recorded. Blood vessels were detected with an anti-von Willebrand factor (vWF) antibody (Chemicon). Microvessel density was determined by calculating the proportion of vWF-positive cells.

3. Results

3.1. Expression of Her-receptors and cellular sensitivity to gefitinib or SN-38 in lung cancer cell lines

The expression levels of EGFR in seven lung cancer cell lines were examined using RT-PCR with a primer set for exon 20 in EGFR. PC-14, SBC-3, H69, PC-7, and A549 cells showed a 570-bp-long PCR amplified product exhibiting wild-type EGFR mRNA (data not shown). On the other hand, a smaller PCR product was also detected in the PC-9 and PC-9/ZD cells, and this band was confirmed to be an in-frame 15-base deletion of exon 20 (E746_A750del).

We examined the protein levels of EGFR, Her2, Her3, and Her4 in the lung cell lines using immunoblotting. The quantitative data obtained by densitometorical analysis is summarized in Table 1. The protein levels of EGFR, Her2, and Her3 in the PC-9 cells were one- to four-fold higher than those in the other cell lines (PC-7, H69, PC-14, A549, and SBC-3).

Table 1 Comparison of Her family protein levels and gefitinib- and SN-38-induced growth inhibition

Cell lines	Relative expression[a]				Growth inhibition[b], IC_{50} ± S.D.	
	EGFR	Her2	Her3	Her4	Gefitinib (μM)	SN-38 (nM)
PC-9	2.8[c]	3.2	3.7	ND	0.047 ± 0.061	8.09 ± 1.9
PC-9/ZD	1.6[c]	2.6	3.8	ND	7.7 ± 0.5	38.9 ± 7.0
PC-14	1.5	2.8	1.1	ND	17.1 ± 0.8	42.1 ± 2.6
SBC-3	2.4	2.6	1.0	ND	19.9 ± 5.4	1.07 ± 0.1
A549	2.3	2.3	1.4	ND	30.2 ± 2.2	293 ± 64.5
H69	1.3	1.3	2.0	ND	56.5 ± 3.2	27.2 ± 4.1
PC-7	1.0	1.0	1.2	ND	68.8 ± 14.8	20.5 ± 8.2

The IC_{50} value (μM) of each drug was measured by MTT assay, as described in Section 2. Each value is the mean ± S.D. of three or four independent experiments.

[a] Protein expression levels were analyzed by Western blotting.

[b] Drug concentration responsible for 50% growth inhibition in MTT assay at 72 h, calculated data for at least three dependent experiments.

[c] 15-base deletion EGFR, ND: not determined.

3.2. Cellular sensitivity of lung cancer cells to gefitinib and SN-38

The growth inhibitory effect of gefitinib and SN-38 on lung cancer cells was examined using an MTT assay. The IC_{50} values of gefitinib for the cell lines ranged from 46 nM (PC-9 cells) to 68 μM (PC-7 cells). The PC-9/ZD cells were ~200-fold resistant to gefitinib, compared with the parental PC-9 cells. Cellular sensitivity to gefitinib and the expression levels of EGFR and Her2 were negatively correlated with the IC_{50} values of gefitinib (Table 1). The IC_{50} values of SN-38 for these cell lines ranged from 1 nM (SBC-3) to 300 nM (A549). The range of sensitivity to gefitinib was wider than that to SN-38. No correlation in cellular sensitivity to gefitinib and SN-38 was seen.

3.3. In vitro combined effect of gefitinib and SN-38 on lung cancer cell lines

To evaluate the potential combined effect of gefitinib and SN-38, the combination index was determined using an MTT assay. The combined effects of gefitinib and SN-38 under the concurrent schedule are shown in Fig. 1. CI values of <1, >1, and 1 indicate a supra-additive effect (synergism), an antagonistic effect, and an additive effect, respectively. An additive to supra-additive growth-inhibitory effect was observed for all doses of gefitinib and SN-38 tested in cell lines expressing wild-type EGFR. On the other hand, a high CI index was observed in PC-9 cells and PC-9/ZD cells expressing mutant EGFR over a wide range of inhibition levels. These results suggest that gefitinib and SN-38 are synergistic in lung cancer cells expressing wild-type EGFR but not in cell lines expressing mutant EGFR in vitro.

3.4. Schedule-dependent synergy of gefitinib and SN-38 in lung cancer cells

Next, we examined the schedule dependency of the combined effects of gefitinib and SN-38 in the cell lines. The five cell lines expressing wild-type EGFR showed synergistic (PC-14, H69, and A549 cells) or additive effects (SBC-3 and PC-7 cells) for all three schedules: concurrent administration, SN-38 followed by gefitinib administration, and gefitinib followed by SN-38 administration (Fig. 1A). In the PC-9 cells, concurrent administration and gefitinib followed by SN-38 administration were antagonistic, but SN-38 followed by gefitinib administration was synergistic (Fig. 1B). In the PC-9/ZD cells, concurrent administration was antagonistic, but sequential administration was synergistic. These schedule-dependent combined effects were observed in the cells expressing mutant EGFR.

3.5. Combined effects of gefitinib and SN-38 in vivo

To estimate the schedule-dependent effects in vivo, nude mice bearing tumors were treated with gefitinib and CPT-11 according to sequential or concurrent schedules (Fig. 2A). Mice bearing PC-14 tumors were treated with gefitinib and CPT-11 according to sequential or concurrent schedules. CPT-11 (50 mg/kg) alone potentially reduced the tumor size, and the combination of gefitinib and CPT-11 was synergistic. In particular, the administration of CPT-11 followed by gefitinib cured the mice bearing PC-14 cells (Fig. 2B).

Mice bearing PC-9 or PC-9/ZD tumors were treated with gefitinib and CPT-11 according to sequential or concurrent schedules. Gefitinib (40 mg/kg) alone potentially reduced the PC-9 tumors, and CPT-11 (50 mg/kg) followed by gefitinib administration reduced the tumor size of PC-9 xenografts more dramatically (gefitinib alone: $P = 0.012$, sequential combination: $P = 0.005$) (Fig. 2B). On the other hand, the concurrent schedule produced an antagonistic effect. Body weight loss was not observed in any of the mice treated according to the above schedules (Fig. 2C). CPT-11 followed by gefitinib administration is a potentially beneficial schedule against PC-9 and PC-9/ZD cells expressing mutational EGFR. The results of these in vivo experiments were consistent with those of the in vitro studies.

To elucidate the synergistic mechanisms of CPT-11 and gefitinib in vivo, tumor samples of the PC-9 and PC-9/ZD

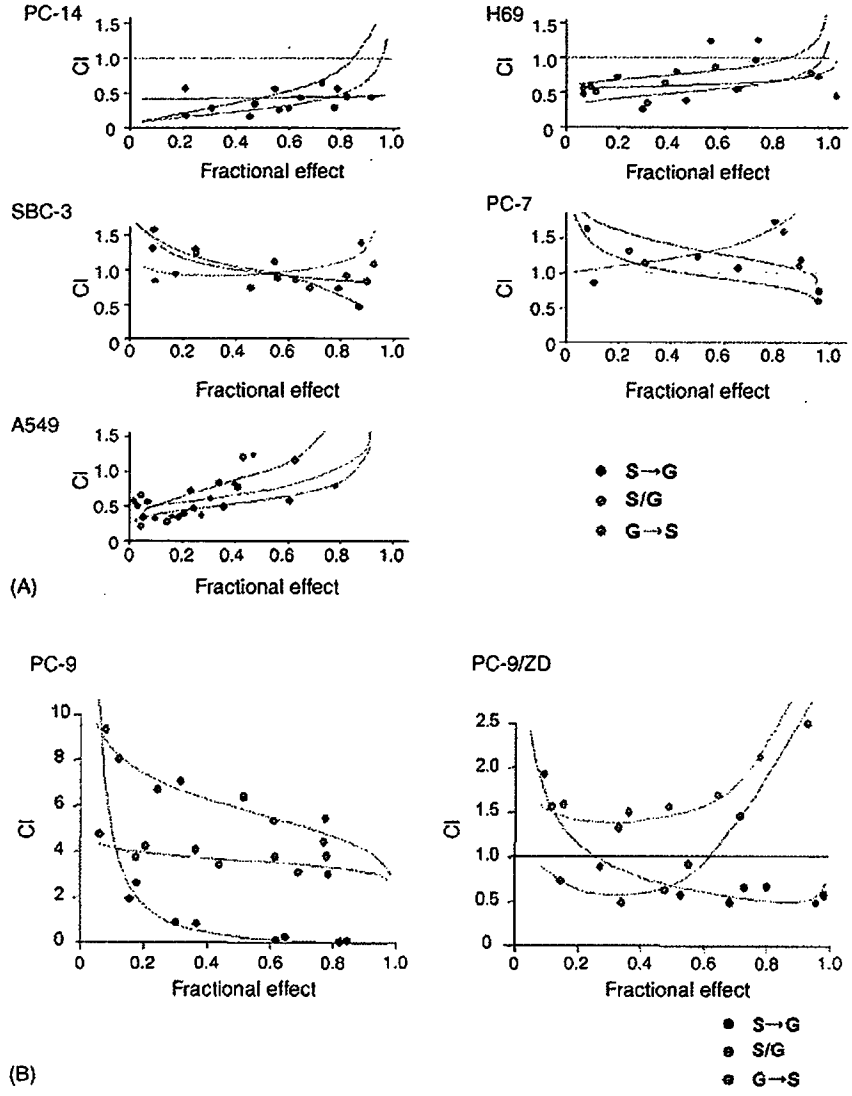

Fig. 1 Combination index (CI) plots of interactions between gefitinib and SN-38 in lung cancer cell lines. Each cell line was treated with gefitinib and SN-38, either alone or in combination at a fixed molar ratio. (A) (PC-14) gefitinib: SN-38 = 425:1; (SBC-3) 20000:1; (A549) 100:1; (H69) 2000:1; (PC-7) 3500:1. (B) (PC-9) gefitinib: SN-38 = 6:1; (PC-9ZD) 175:1. Treatment schedule: (1) SN-38 was applied first and gefitinib was applied 12 h later, followed by incubation in medium for 72 h (blue). (2) SN-8 and gefitinib were applied concurrently, followed by incubation in medium for 72 h (red). (3) Gefitinib was applied first and SN-38 was applied 12 h later, followed by incubation in medium for 72 h (green). S → G: sequential combination (SN-38 followed by gefitinib); C/G: concurrent combination; G → S: sequential combination (gefitinib followed by SN-38).

cells were stained with anti-Ki-67, anti-CD31 and the TUNEL assay (Fig. 3A and B). A reduction in tumor cell proliferation (Ki-67 staining), a reduction in tumor vasculature (CD31 staining), and an increase in tumor apoptosis (TUNEL staining) were observed in tumors treated with gefitinib alone or gefitinib and CPT-11. The administration of CPT-11 alone increased the number of Ki-67 positive tumor cells. In the PC-9 tumors, sequential treatment resulted in a 2.7-fold increase in tumor cell apoptosis and a 1.9-fold decrease in vessel staining, compared with the results obtained in tumors treated concurrently. The ratio of apoptosis:proliferation increased 1.7-fold in sequentially treated tumors compared with tumors treated with both drugs concurrently. Quantitative analysis of tumor cell proliferation and apoptosis showed a significant difference between the effects of the concurrent and sequential schedules ($P < 0.001$), but not between concurrent and gefitinib-alone ($P > 0.01$ for all comparisons, Fig. 3C). No significant difference in CD31-positive cells was observed between the control and gefitinib-alone treatments, suggesting that gefitinib exerts no remarkable anti-angiogenetic effects ($P > 0.01$, Fig. 3C). Similar findings were observed in PC-9/ZD tumors. These findings suggest that the antitumor activity of sequential treatment using gefitinib and CPT-11 is mediated by an increase in tumor cell apoptosis, compared with concurrent treatment.