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Radiotherapy quality assurance review in a multi-center randomized trial of limited-disease small cell lung cancer: the Japan Clinical Oncology Group (JCOG) trial 0202

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Abstract

Background: The purpose of this study was to analyze the radiotherapy (RT) quality assurance (QA) assessment in Japan Clinical Oncology Group (JCOG) 0202, which was the first trial that required on-going RT QA review in the JCOG.

Methods: JCOG 0202 was a multi-center phase III trial comparing two types of consolidation chemotherapy after concurrent chemoradiotherapy for limited-disease small cell lung cancer. RT requirements included a total dose of 45 Gy/30 fx (bis in die, BID/twice a day) without heterogeneity correction; elective nodal irradiation (ENI) of 30 Gy; at least 1 cm margin around the clinical target volume (CTV); and interfraction interval of 6 hours or longer. Dose constraints were defined in regards to the spinal cord and the lung. The QA assessment was classed as per protocol (PP), deviation acceptable (DA), violation unacceptable (VU), and incomplete/not evaluable (I/NE).

Results: A total of 283 cases were accrued, of which 204 were fully evaluable, excluding 79 I/NE cases. There were 18 VU in gross tumor volume (GTV) coverage (8% of 238 evaluated); 4 VU and 23 DA in elective nodal irradiation (ENI) (2% and 9% of 243 evaluated, respectively). Some VU were observed in organs at risk (1 VU in the lung and 5 VU in the spinal cord). Overall RT compliance (PP + DA) was 92% (187 of 204 fully evaluable). Comparison between the former and latter halves of the accrued cases revealed that the number of VU and DA had decreased.

Conclusion: The results of the RT QA assessment in JCOG 0202 seemed to be acceptable, providing reliable results.

Introduction

Quality assurance (QA) and quality control are an integral part of multi-center clinical trials involving radiotherapy (RT). Several reports have shown that failure to adhere to the treatment protocol deteriorated the outcome in clinical trials [1-5]. To provide reliable results in clinical trials, it is important to keep each treatment as uniform as possible. In addition, a QA program is indispensable for patient safety, preventing increased or unexpected toxicity, and ensuring a certain effect.

In 1999, Japan Clinical Oncology Group (JCOG) trial 9812 was started to evaluate whether RT with carboplatin would result in longer survival than RT alone in elderly patients with unresectable stage III non-small cell lung cancer; however, due to excessive serious adverse events, the trial was terminated early when 46 patients were registered. By retrospective RT QA review, a protocol violation was revealed in 60% of the cases [6].

JCOG 0202 was a multi-center phase III trial comparing two types of consolidation chemotherapy after concurrent chemoradiotherapy for limited-disease small cell lung cancer (Figure 1).

The primary endpoint of JCOG 0202 was overall survival and the secondary endpoints included disease-free survival and the toxicity profile of each treatment. This trial was the first in JCOG to require on-going RT QA to improve the quality of clinical trials. This is a retrospective evaluation of the protocol compliance of JCOG 0202.

Methods

Study design and RT requirements

After enrolling in this trial, patients received cisplatin 80 mg/m^2 on day 1 and etoposide 100 mg/m^2 on days 1–3, with concurrent RT. Patients were randomized after chemoradiotherapy and received either 3 cycles of the same chemotherapy of cisplatin and etoposide every 3 weeks, or cisplatin 60 mg/m^2 on day 1 and irinotecan 60 mg/m^2 on days 1, 8 and 15 every 4 weeks.

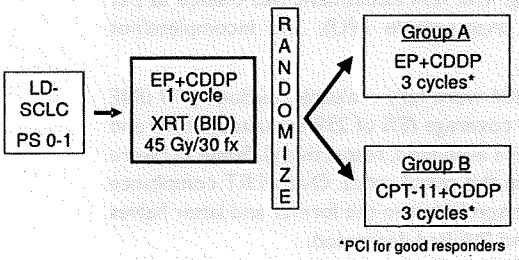

Figure 1
Schema of JCOG 0202. Abbreviations. LD-SCLC, limited-disease small cell lung cancer; PS, performance status; EP, etoposide; CDDP, cisplatin; XRT, thoracic radiotherapy; BID; bis in die/twice a day; CPT-11, irinotecan; PCI, prophylactic cranial irradiation.

RT requirements included a total dose of 45 Gy in 30 fractions (bis in die, BID/twice a day) with an interfraction interval of over 6 hours. For treatment planning, both conventional 2-dimensional (2-D) X-ray simulation and 3-dimensional (3-D) CT simulation were allowed. PET scanning was not required in RT planning. Gross tumor volume (GTV) was defined as the primary tumor demonstrated by CT scan as well as metastatic lymph nodes measuring 1 cm or greater in short axis. In this trial, the clinical target volume (CTV) for the primary tumor and metastatic lymph nodes was created without adding any margins to GTV. CTV also included a regional (elecitve) nodal area which consisted of ipsilateral hilum and bilateral mediastinal (pretracheal, paratracheal, tracheobroncheal, and subcarinal) lymph nodes. Contralateral hilar lymph nodes were not included in the CTV. The planning target volume (PTV) was created by adding margins at the discretion of radiation oncologists (typically 0.5–1 cm for lateral margin and 1–2 cm for cranio-caudal margin, depending on respiratory motion and patient fixation). A dose of 30 Gy was prescribed at the center of the PTV, including elective nodal irradiation (ENI), followed by a boost dose of 15 Gy to the primary tumor and metastatic lymph nodes. Tissue heterogeneity correction was not used for monitor unit calculation, because if heterogeneity correction was required and different calculation algorithms were allowed, inter-institutional variation of the delivered dose would have been significant, and the convolution-superposition algorithm was not available in some participating institutions at the beginning of this trial.

Dose constraints were defined in regard to the dose to the spinal cord and the lung. The dose to the spinal cord was kept at ≤ 36 Gy. A posterior spinal shield was not allowed. The percentage of normal lung volume minus PTV receiving 20 Gy or greater (V_{20}) was kept ≤ 35%. In 2-D planning, the field size was limited to ≤ half of the ipsilateral lung (for upper lobe tumors, ≤ 2/3).

Quality assurance review

For initial QA review, copies of pre-treatment diagnostic chest X-ray and CT, simulation and portal films, worksheets for monitor unit calculation of the prescribed dose, and RT charts with the record of the irradiated time were collected. Information on the initial RT plan was required to be sent to the QA review center within 7 days after the start of RT. Information on the total course of RT, including the boost treatment plan, was required to be sent within 30 days after completion of RT. These were reviewed periodically at least twice a month by the RT

principal investigator (S.I.), and also by an independent radiation oncologist (N.S.) after patient accrual. RT QA for prophylactic cranial irradiation was not performed. After the review of the initial RT plan, the RT principal investigator sent each institution a letter reporting whether they had complied with the treatment protocol as well as an inquiry about QA documentation when necessary (Figure 2). Progress remarks and problems were reported at periodical meetings for investigators.

To assess protocol compliance for RT, the following parameters were reviewed: the dose and field border placement for PTV (adequacy of margins for GTV and ENI), doses to organs at risk, such as the spinal cord and the normal lung, overall treatment time, interfraction interval, and dose calculation without heterogeneity correction. The QA assessment was given as per protocol (PP), deviation acceptable (DA), violation unacceptable (VU), and incomplete/not evaluable (I/NE). The criteria were set for each parameter as follows. For the dose and field coverage of GTV, VU was defined as a dose less than 40.5 Gy, more than 49.5 Gy, or the distance between the field edge of the blocks or multileaf collimators and the rim of GTV less than 1 cm or more than 3.5 cm. For the dose and field coverage of ENI, a dose less than 27 Gy, more than 36 Gy or inclusion of the contralateral hilum was judged as VU. If hererogeneity correction was used for dose calculation and the recalculated uncorrected dose deviated more than 10%, it was judged as VU. Other criteria for the QA assessment are listed in Table 1. These criteria were arbitrary rather than based on the literature. We set these criteria based on the patterns of practice in Japan at the start of this trial. After parameter compliance was assessed, overall RT compliance was determined as PPoverall, no DA or VU in any parameter; VUoverall, at least one VU in any parameter; or DAoverall, neither PP nor VU. The proportion of 2-D X-ray simulation vs. 3-D CT simulation was analyzed, and a comparison was also made between compliance in the first half vs. the second.

Results

From September 2002 to September 2006, 283 cases were accrued. Of these, 204 (72%) were fully evaluable, excluding 79 cases (Table 2). Partially evaluable cases were included to evaluate each item.

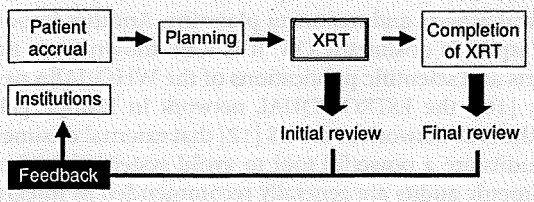

Figure 2
Flow of QA review. After the QA review, feedback was given to the institutions. Treatment planning was modified when possible.

Among 258 patients evaluable for the treatment planning method, conventional 2-D X-ray simulation was performed in 62 (24%) patients, while 196 (76%) had 3-D CT simulation. Of 35 participating institutions, 24 institutions had introduced 3-D CT simulation, 6 used only 2-D X-ray simulation, and 5 used both.

RT compliance for each parameter is listed in Table 3. There were 18 VU in GTV (8% of 238 evaluated), of which, 14 (78%) had insufficient lateral margins, while 3 (17%) and 2 (11%) had insufficient caudal and cranial margins, respectively (one case, both lateral and caudal margins). There was no VU in the GTV dose. With regard to ENI, 4 VU and 23 DA (2% and 9% of 243 evaluated, respectively) were observed. Of these 4 VU, a total dose of 45 Gy instead of 30 Gy was given in 3, and the contralateral hilum was irradiated in one case. Of these 23 DA, 17 had larger field placement than required in the protocol, such as the inclusion of uninvolved supraclavicular fossa, upper mediastinum, or subaortic/paraaortic lymph node area, etc, whereas 3 had insufficient margins. Three had both larger field placement and insufficient margins. No VU was found in overall treatment time, interfraction interval and dose calculation, while some VU were observed in organs at risk (1 VU in the lung and 5 VU in the spinal cord). Overall RT compliance (PP + DA) was 92% (187 of 204 fully evaluable).

In regard to the 35 participating institutions, 17 (49%) had no VU. In 18 institutions with VU, 15 (83%) had only one VU and 3 (17%) had 2 or more VU. Sixteen institutions (89%) had VU in their first 3 cases.

Comparison between the former and latter halves of the accrued cases (141 and 142 cases, respectively) revealed that the number of VU and DA had decreased: for GTV, the number of VU was 13 in the early period (9%; 95% CI, 5%–15%), while 5 in the late period (4%; 95% CI, 1%–8%). In regard to ENI, DA decreased from 20 (14%; 95% CI, 9%–21%) to 3 (2%; 95% CI, 0.4%–6%), respectively.

Discussion

In clinical trials, patients must receive optimal treatment. Since the 1980s, a number of reports have focused on the relationship between RT compliance and treatment outcomes in various types of malignancy [1-5]. These results suggested that failure to adhere to RT protocol guidelines compromises survival. Overall compliance of 92% in the current trial seemed acceptable to provide reliable results. More than half of the participating institutions did not have VU, and even with VU, the majority had only one VU; however, there is room for improving compliance in

Table 1: Criteria for QA scores

	PP	DA	VU
GTV			
distance to field borders	1 – 3.5 cm	NA	< 1 cm or > 3.5 cm
prescribed dose	45 Gy	Neither PP nor VU	< 40.5 Gy or > 49.5 Gy
ENI			
distance to field borders	1 – 3.5 cm	Neither PP nor VU	contralateral hilum included
prescribed dose	27 – 36 Gy	NA	< 27 Gy or > 36 Gy
Overall treatment time	21 – 42 days	NA	> 42 days
Interfraction interval	≥ 5.5 hrs	4 – 5.5 hrs or <4 hrs (once)	< 4 hrs more than once
Organs at risk			
Spinal cord	≤ 36 Gy	Neither PP nor VU	> 39 Gy
Lung	≤ 1/2 ipsilateral hemithorax (≤ 2/3, upper lobe tumor) or V_{20} ≤ 35%	Neither PP nor VU	> 1/2 ipsilateral hemithorax (> 2/3, upper lobe tumor) or V_{20} > 40%
Heterogeneity correction	No	Yes (≤ 10% total dose difference)	Yes (> 10% total dose difference)

Abbreviations: PP, per protocol; DA, deviation acceptable; VU, violation unacceptable; GTV, gross tumor volume; ENI, elective nodal irradiaton; NA, not applicable; hrs, hours; V_{20}, percentage of the total lung minus PTV receiving ≥ 20 Gy.

future trials incorporating RT. GTV and ENI violations and/or deviations were more frequent in the early period. In addition, among institutions with VU, the majority had VU in the first 3 cases. This may be because the institutions received feedback on how to better comply with the treatment protocol by the RT principal investigator, which enabled participants to follow the protocol guidelines in their later cases.

In the current study, more suboptimal treatments were observed in field placement than in the dose for tumors or risk organs. A similar trend was reported in other studies [7,8]. The majority of VU consisted of smaller lateral margins. The reason may have been a discrepancy between the protocol guidelines and their daily practices. The physicians tended to reduce lateral margins rather than craniospinal margins for fear of radiation pneumonitis. The varied ENI coverage also suggested a discrepancy. In this trial, a dry-run procedure was not attempted and therefore the radiation oncologists in each institution might not have been familiar with the protocol guidelines in the initial period of this trial. Wallner et al. [4] speculated the influence of clinical trial experience by reviewing a large number of cases in RTOG studies for lung and head and neck cancer. They reported that adequate primary and lymph node margins and dose prescriptions had progressively improved over the years, suggesting long-lasting learning experiences in clinical trials. As the need for immediate monitoring was described by Schaake-Koning et al. [9] from a quality control study in the EORTC lung cancer trial, some early interventions, such as a dry-run and immediate feedback before the start of treatment, will be more effective to improve compliance in clinical trials involving RT.

Table 2: Number of evaluable cases and overall RT compliance

	number	(%)
Total	283	
Data insufficient/partially evaluable	62	
Off-protocol	12	
Ineligible	5	
Fully evaluable	204	(100)
PPoverall	158	(77)
DAoverall	29	(14)
VUoverall	17	(8)
Compliance (PPoverall+DAoverall)	187	(92)

Abbreviations: PP, per protocol; DA, deviation acceptable; VU, violation unacceptable

There were several limitations of our study. We did not perform 3-D volumetric data analyses due to technical limitations. Other factors, such as inter-observer contouring variations, 2-D vs. 3-D planning, may have had a much greater impact on the outcome of this trial than protocol compliance. The transition from 2-D to 3-D treatment planning is now almost complete in Japan, and more precise QA analyses using digital data, exported from treatment planning systems with the DICOM-RT format, have been introduced in recent JCOG 3-D RT trials.

In addition, all described QA activities focused on the medical aspects and treatment planning. Another important aspect is dosimetric QA. It is well known from the reports and scientific publications of the WHO/IAEA network [10], the ESTRO-EQUAL network in Europe [11] and the NCI network in the US [12] that external dosimetric audits are a powerful tool to avoid systematic errors. Dosimetric audits are generally recommended as integral parts of QA activities for clinical trials. In Japan, dosimetric audits were introduced in 2003, and were therefore not available at the beginning of this trial, and have been implemented in recent JCOG radiotherapy trials [13]. We

Table 3: RT compliance for each parameter

	Evaluable cases	PP	(%)	DA	(%)	VU	(%)
GTV	**238**	**220**	(92)	**NA**		**18**	(8)
ENI	**243**	**216**	(89)	**23**	(9)	**4**	(2)
Overall treatment time	**227**	**227**	(100)	**NA**		**0**	(0)
Interfraction interval	**205**	**195**	(95)	**10**	(5)	**0**	(0)
Organs at risk							
Spinal cord	**236**	**231**	(98)	**0**	(0)	**5**	(2)
Lung	**246**	**245**	(100)	**0**	(0)	**1**	(0.4)
Heterogeneity correction	**244**	**228**	(93)	**16**	(7)	**0**	(0)

Abbreviations: PP, per protocol; DA, deviation acceptable; VU, violation unacceptable; GTV, gross tumor volume; ENI, elective nodal irradiaton; NA, not applicable.

also believe that these activities will have run-on effects in routine practice and lead to higher quality cancer care.

Conclusion

In conclusion, the results of the RT QA assessment of JCOG 0202 seemed to be acceptable, providing scientifically reliable results. The time trend toward improved compliance in this trial showed the importance of introducing an RT QA program. A dry-run procedure and intensive feedback to participating institutions are being implemented to further improve JCOG trials.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NS performed the QA evaluation. SI was in charge of the QA program and performed the QA evaluation. KH participated in the design of the QA program and helped to draft the manuscript. KK, and YN and TT conceived the study and helped to draft the manuscript.

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Lung cancer RT

Relation between elective nodal failure and irradiated volume in non-small-cell lung cancer (NSCLC) treated with radiotherapy using conventional fields and doses

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ABSTRACT

Introduction: The role of elective nodal irradiation of non-small-cell lung cancer (NSCLC) patients treated with radiotherapy remains unclear. We investigated the significance of treating clinically uninvolved lymph nodes by retrospectively analyzing the relationship between loco-regional failure and the irradiated volume.

Methods: Between 1998 and 2003, patients with IA–IIIB NSCLC were treated with radiotherapy. The eligibility criteria for this study were an irradiation dose of 60 Gy or more and a clinical response better than stable disease. Typical radiotherapy consisted of 40 Gy/20 fr to the tumor volumes (clinical target volume of the primary tumor [CTVp], of the metastatic lymph nodes [CTVn], and of the subclinical nodal region [CTVs]), followed by off-cord boost to CTVp+n to a total dose 60–68 Gy/30–34 fr. The relationship between the sites of recurrence and irradiated volumes was analyzed.

Results: A total of 127 patients fulfilled the eligibility criteria. Their median overall and progression-free survival times were 23.5 (range, 4.2–109.7) and 9.0 months (2.2–109.7), respectively. At a median follow-up time of 50.5 months (range, 14.2–83.0) for the surviving patients, the first treatment failure was observed in 95 patients (loco-regional; 41, distant; 42, both; 12). Among the patients with loco-regional failure, in-field recurrence occurred in 38 patients, and four CTVs recurrences associated with CTVp+n failure were observed. No isolated recurrence in CTVs was observed.

Conclusions: In-field loco-regional failure, as well as distant metastasis, was a major type of failure, and there was no isolated elective nodal failure. Radiation volume adequacy did not seem to affect elective nodal failure.

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Radiation therapy is an integral component of the multi-modal treatment of non-small-cell lung cancer (NSCLC). Recent phase III studies have demonstrated that concomitant chemoradiotherapy improves survival, and this has resulted in the general acceptance of concurrent chemoradiotherapy as one of the standard treatments for locally advanced NSCLC [1]. Despite the improved survival, however, most patients die from their disease as a result of local or distant failure.

Local failure remains a major challenge when treating NSCLC with radiotherapy. A number of studies of dose escalation to the gross tumor volume (GTV) have been conducted as a means of improving local control [2–5]. The conventional radiation fields for NSCLC typically encompass the entire mediastinum and ipsilateral hilum (elective nodal region) to deliver a dose of 40 Gy, even without evidence of disease in these areas, followed by a 20 Gy boost to the GTV. However, the conventional treatment has added considerable morbidity and can limit the dose escalation. In phase I–II dose escalation studies, there is a trend toward omitting the practice of elective nodal irradiation (ENI) after their experiences with toxicity, which is not based on direct evidence [2–5]. According to those studies, omitting ENI has not sacrificed treatment outcomes so far. They also analyzed patterns of recurrence in relation to irradiated volume in a dose escalation setting [6].

By contrast, the current literature provides limited information regarding patterns of failure when conventional fields and doses are used [7,8]. Since it is important to know whether loco-regional failure is within or outside the irradiation field, we retrospectively analyzed patterns of failure after radiation therapy for NSCLC, especially in regard to the relationship between local failure and irradiated volume.

Methods and materials

Patients

Between January 1998 and March 2003, 263 patients with newly diagnosed NSCLC were treated with thoracic radiation therapy,

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with or without chemotherapy, at the National Cancer Center Hospital. All tumors were cytologically or histologically confirmed NSCLC. Patients' disease was staged by the tumor-node-metastasis (TNM) staging system (UICC, version 6, 2002). The diagnostic workup included a bone scan, brain scan by computed tomography (CT) or magnetic resonance imaging, CT scan of the chest, and CT or ultrasound imaging of the abdomen. The criteria for inclusion in this study were irradiation with a dose of 60 Gy or more as a part of the initial treatment and a clinical response better than stable disease. After excluding patients with metastatic disease, whose primary tumor was located in the apex of the lung (superior sulcus), and whose post-treatment evaluation was inadequate, the remaining 127 patients served as the subjects of the analysis.

Details of treatment

Radiotherapy

Gross tumor volume (GTV) was defined as the demonstrable extent of the primary tumor and the metastatic lymph nodes, GTVp and GTVn, respectively. GTVn was defined as abnormally enlarged regional lymph nodes measuring over 1.0 cm along their short axis. Clinical target volume (CTV) consisted of the adjacent mediastinum and ipsilateral hilum (CTV of the subclinical nodal region, CTVs) as well as CTVp and CTVn which were assumed to be equal to GTVp and GTVn, respectively. A planning target volume (PTV) margin of 1–1.5 cm was drawn around each CTV.

External-beam radiotherapy with a 6, 10, or 15 MV photon beam was delivered using a linear accelerator. A majority of the patients were treated with anteroposterior opposing fields encompassing CTV to a dose of 40 Gy/20 fractions (2 Gy per fraction, 5 days per week), followed by an off-cord boost to the GTV by oblique opposing fields, to a total dose of 60–68 Gy/30–34 fractions. No attempt was made to encompass the supraclavicular areas in most patients; the supraclavicular areas were treated only electively. Initially, treatment planning was performed by using an X-ray simulator for the anteroposterior fields and a CT-port for the oblique opposing fields, but after the end of 1999, most treatment planning, especially to define the off-cord boost, was performed using a CT-based planning system (FOCUS, Computed Medical Systems).

The dose to the spinal cord was limited to 45–50 Gy. The size of the treatment fields was adjusted so that it did not exceed half of the hemithorax before introducing CT-based planning system, or so that the volume of normal lung tissue receiving a dose over 20 Gy would be less than 40%.

Chemotherapy

Systemic chemotherapy was used in 87 patients (68.5%), and the majority of the patients received platinum-based chemotherapy sequentially or concurrently with the radiation therapy. One of the representative regimens was 2–3 cycles of cisplatin 80 mg/sqm on day 1 and vinorelbine 25 mg/sqm on days 1 and 8 (or vindesine 3 mg/sqm on days 1, 8, and 15) in 21–28 days. The second most common regimen was cisplatin 80 mg/sqm on day 1, vindesine 3 mg/sqm on days 1 and 8, and mitomycin C 8 mg/sqm on day 1, in 21–28 days. The other regimens are summarized in Table 1.

Evaluation

Patients were followed at 4- to 6-week intervals for 6 months after treatment and at 3- to 6-month intervals thereafter. Chest X-ray and laboratory workups were performed at each post-treatment visit. Unless there were changes in the chest X-ray or in symptoms, a CT scan was performed about 2–3 months after the treatment for the assessment of the treatment response, and every 6–12 months thereafter. Follow-up information was obtained from the medical charts and death certificates.

Table 1
Baseline patient characteristics.

Characteristics	Patients	(%)
Median age (yr)	65 (36–83)	
Gender		
Male	106	83
Female	21	17
Performance status (WHO)		
0	12	9
1	109	86
2	6	5
Stage		
I (A/B)	5(1/4)	4
II (A/B)	12(3/9)	9
III (A/B)	110(59/51)	87
Histology		
Adenocarcinoma	64	50
Squamous cell carcinoma	39	31
Large cell carcinoma	4	3
NSCLC (not otherwise specified)	20	16
Chemotherapy (concurrent/sequential)	87(63/24)	69
Chemotherapy regimens		
Cisplatin + vindesine or vinorelbine	48	55
Carboplatin + paclitaxel	12	14
MVP (cisplatin + vindesine + mitomycin)	12	14
Nedaplatin or nedaplatin + paclitaxel	11	13
Others	4	5

When evaluating overall survival, an event was defined as death from any cause. When evaluating progression-free survival, an event was defined as documented tumor progression (loco-regional or distant) or death from any cause. Local or loco-regional failure was judged to have occurred if there was radiographic evidence of progressive disease. Absence of progression of residual disease for more than 6 months following treatment was considered evidence of loco-regional control. A recurrence in supraclavicular nodes was considered regional failure, not an elective nodal failure, because the supraclavicular regions are not routinely included within the radiation fields in our practice. Treatment failure was not always confirmed histologically. Elective nodal failure (ENF) was defined as recurrence in CTVs without evidence of local failure, as the first event or even after distant metastasis.

The adequacy of field borders was assessed in terms of CTVs coverage and PTV margin in patients with loco-regional failure. The failure patterns were analyzed to distinguish in-field recurrence from out-of-field recurrence; "in-field" included CTVs as well as CTVp and CTVn.

The Kaplan–Meier method was used from the start of the treatment to calculate the overall survival and progression-free survival of all the 127 patients.

Results

A total of 127 patients, median age 65 years (range, 36–83), met the criteria for evaluation in this study. The majority of patients had stage IIIA ($n = 59$) or IIIB ($n = 51$) disease. Other baseline characteristics of the patients and details of their treatment are summarized in Table 1.

At a median follow-up time of 50.5 months (range, 14.2–83.0) of the surviving patients, 95 had experienced treatment failure. Median survival time was 23.5 months (range, 4.2–109.7), and median time to progression was 9.0 months (range, 2.2–109.7). The 2-year cumulative survival rate and 2-year progression-free survival rate were 51.4% and 27.6%, respectively. The survival

curves are shown in Fig. 1. Patients with early progressions were excluded because of the criteria for inclusion in this study: a clinical response better than stable disease.

Eighty-seven (69%) patients received chemotherapy concomitantly or sequentially with the radiotherapy. The overall survival time of the patients who received chemotherapy was 21.7 months (range, 7.6–33.9), as opposed to 19.1 months (range, 6.8–32.7) among those who did not receive chemotherapy, and the difference was not statistically significant ($p = 0.10$). There were no statistically significant differences in disease-free survival nor loco-regional control according to whether the patients had received chemotherapy. Concurrent use of chemoradiotherapy did not affect survival among the 87 patients who received chemotherapy (data not shown).

There were 53 patients with a first loco-regional failure, alone ($n = 41$) or with distant metastasis ($n = 12$), and the majority of the failures were in-field ($n = 38$, 72%). Nine (21%) patients had out-of-field recurrences in the form of supraclavicular node metastasis ($n = 5$) or pleural metastasis ($n = 4$), with or without local recurrence. There were no isolated ENFs (Table 2).

Four patients (7%) experienced nodal failure in CTVs simultaneously with local or distant failure. Three of them had received a prophylactic dose of 40 Gy to the CTVs, and the other had inadequate margin of the CTVs field. Other characteristics of these patients are shown in Table 3. There were no "marginal only" failures among in-field failures; all the failures at the field borders were associated with out-of-field failures.

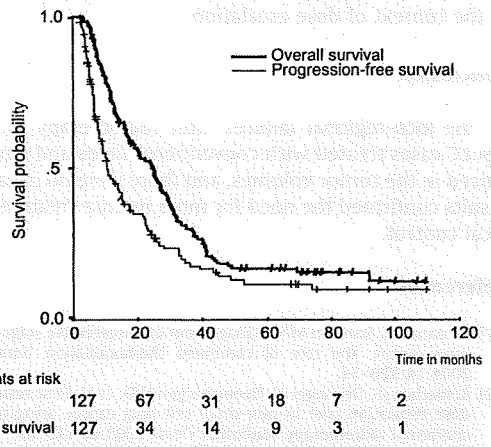

Fig. 1. Overall and progression-free survival curves of all the 127 patients. Patients with early progressions were excluded because of the criteria for inclusion in this study: a clinical response better than stable disease.

Table 2
Details of all the first failures.

Types of event	Patients	%
Loco-regional alone	41	43%
In-field		
CTVpn	30	
CTVpn + CTVs[a]	2	
In-field + out-of-field		
CTVpn + pleural effusion	2	
CTVpn + supraclavicular nodes	2	
Out-of-field		
Supraclavicular nodes	3	
Pleural effusion[b]	2	
Loco-regional + distant	12	13%
In-field + out-of-field		
CTVpn + CTVs	2	
Distant alone	42	44%
All events	95	

[a] One also had concurrent failure in the contralateral hilum.
[b] One also had concurrent supraclavicular recurrence.

Conventional X-ray simulation was performed in 8 (6%) patients, while 70 (55%) had CT-based simulation and remaining 49 (39%) had both (initially with X-ray simulation, followed by CT-based simulation for off-cord boost). A majority ($n = 122$, 96%) of the patients were treated with anteroposterior opposing fields as elective nodal irradiation, followed by oblique opposing fields to the total dose.

ENI was incomplete ($n = 12$) or not performed ($n = 6$) in 18 of the 53 patients with loco-regional failure because of diminished pulmonary function or deteriorated performance status. All the incomplete ENIs were due to insufficient CTVs coverage. In 12 of the 18 patients, the failure was in the tumor volume, in 3 patients it was in the pleura, and in 2 patients it was in the supraclavicular nodes. Only 1 patient had recurrence in both the tumor volume and the uninvolved nodal area.

Discussion

In this series of NSCLC cases treated with conventional fields and doses, the loco-regional failures after radiotherapy mainly occurred in the tumor volumes, and there were no isolated ENFs.

There are several possible reasons for these results. First, micrometastasis in the CTVs may have been controlled by prophylactic delivery of 40 Gy to the region, and depending on the location of the primary tumor, the sites of occult metastasis may often have received additional unintentional radiation doses. Kepka et al. reported an isolated ENF rate of 9% in 185 patients treated with the ENI using 3-dimensional conformal radiotherapy (3D-CRT). Their analysis showed that the ENF occurred more frequently in the regions that received under 40 Gy than in the regions that received higher doses (69% vs. 31%, respectively, $p = 0.04$) [7]. However, despite the same ENF rate of 9% in 1705 patients in the four trials conducted by the Radiation Therapy Oncology Group (RTOG), a retrospective evaluation of in-field progression revealed that neither in-field progression nor survival was affected by the adequacy of ENI [8]. Field adequacy did not have any negative impact on regional control in our series either (Tables 3).

Second, the amount of micrometastasis in unenlarged mediastinal regional nodes may have been small enough to be controlled by chemotherapy, which has been shown to have activity that reduces the incidence of distant micrometastasis in advanced NSCLC. However, the degree of systemic and local efficacy of chemotherapy did not reach statistical significance in our series, probably because of the small number of patients and their heterogeneity (data not shown).

Third, since the failure sites in the majority of patients were distant, they would have died of their disease before the ENF became apparent. As a result, the loco-regional failure rates may have been lower than their true values because we did not investigate regional sites once a patient developed distant metastasis.

The therapeutic significance of treating subclinical nodal regions during and after surgery for NSCLC has been questioned. Some studies have established the presence of considerable microscopic nodal disease in clinically uninvolved lymph nodes [9,10], but the role of mediastinal lymphadenectomy remains controversial and has been limited to the precise staging of the disease [11–13]. A study by Izbicki et al. which compared systemic mediastinal lymphadenectomy with mediastinal lymph node sampling showed that radical systemic mediastinal lymphadenectomy had no effect on the disease-free or overall survival of patients with limited nodal involvement [13,14]. The role of adjuvant radiotherapy after complete resection also remains unclear [15–17]. A systemic

Table 3
Patients with CTVs failure.

	Patient #1	Patient #2	Patient #3	Patient #4
Age (yr)/Sex	45/Female	74/Female	61/Male	78/Male
Reason for inoperability	Unresectable	Unresectable	Decreased pulmonary function	Unresectable, age
Stage	IIIA	IIIA	IIB	IIIB
Primary location	Left lower lobe	Right upper lobe	Right lower lobe	Left upper lobe
Histology	Adenocarcinoma	Adenocarcinoma	Squamous cell carcinoma	Adenocarcinoma
Chemotherapy	Yes	Yes	No	No
Response	Partial response	Partial response	Partial response	Partial response
Site of first failure	Distant and loco-regional	Distant and loco-regional	Loco-regional	Loco-regional
Field border adequacy	Yes	Yes	No	Yes
Dose to CTVs failure	40	40	0	40
Death	No	No	Yes	No

review and meta-analysis [18] showed that postoperative radiotherapy was detrimental to patients with early NSCLC, although there may have been some efficacy in patients with N2 tumors. These arguments also raise questions about the clear benefit of ENI in regard to survival.

In-field loco-regional failure was a major site of failure in the current study: all the recurrences in the CTVs were associated with failure in the gross tumor volume. Thus, more intensive treatment strategies are needed to enhance loco-regional control without sacrificing safety. One possible strategy is to reduce the ENI field in regard to the patients' risk factors while escalating the total dose. Such an attempt has already been made in regard to surgery: Asamura et al. retrospectively reviewed the prevalence of lymph node metastasis with respect to the location of the primary tumor or other characteristics to decide on the optimal lobe-specific extent of systematic lymph node dissection for NSCLC [19,20]. By using such predictors, including the location of the primary tumor, histology, or nodal stage [21–24], it is possible to identify the nodal areas at risk and to optimize the extent of ENI in radiation therapy as well. On the other hand, more precise diagnosis by novel technology, such as positron emission tomography [25], may enable the omission of ENI and avoid unnecessary irradiation to areas at low risk for subclinical disease.

In terms of the technical feasibility of dose escalation, Grills et al. found that intensity-modulated radiation therapy without ENI for NSCLC increased the deliverable mean target dose in node-positive patients by 25–30% over 3D-CRT and by 130–140% over traditional ENI [26].

Because omitting ENI is likely to leave microscopic disease untreated, there is concern that it may result in increased failure in these areas. However, the preliminary results of dose escalation trials have shown that isolated ENF outside the irradiated volume occurred in fewer than 6% of the cases and that omission of ENI did not seem to sacrifice outcome [2–5,27]. There is insufficient evidence to support the use of ENI for any patient with localized NSCLC (Stages I–III), irrespective of whether chemotherapy is administered [28]. There has been only one randomized trial that compared high-dose thoracic radiotherapy without ENI and standard dose radiotherapy with ENI, and it showed a survival benefit of high-dose thoracic radiotherapy without ENI [29]. One possible explanation for this finding is that incidental doses to elective nodal areas may contribute to the eradication of the subclinical disease. The pattern of ENF according to nodal regions was described by Rosenzweig et al., who implemented the use of involved-field radiation therapy with dose escalation in 524 patients [6]. Since the majority of the 42 ENFs that were observed occurred in the areas that received less than 45 Gy, the incidental doses to elective nodal areas may have been substantial despite the attempt not to treat these regions in their study. In addition, Zhao et al. reported that involved-field radiation therapy with a dose escalated to 70 Gy delivered a considerable dose to CTVs, and when the primary tumor was large or centrally located, the percentages of CTVs in the lower paratracheal region, subcarinal region and ipsilateral hilar region receiving over 40 Gy were 33%, 39%, and 98%, respectively [30].

Because of the retrospective nature of our study, no conclusions about the value of ENI for NSCLC can be drawn. However, the finding that in-field loco-regional failure, as well as distant metastasis, was a major type of failure with the standard field and dose of thoracic radiotherapy confirmed the need for more intensive treatment.

Further investigation to verify the true significance of ENI or to identify best candidates for ENI is necessary before it is abandoned in the context of dose escalation.

Conclusion

The loco-regional failures after radiotherapy in this series of NSCLC cases treated with conventional fields and doses mainly occurred in the tumor volumes, and there were no isolated ENFs. The results confirmed the need for more intense treatment to improve local control.

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ORIGINAL ARTICLE

Schedule-dependent synergism and antagonism between pemetrexed and docetaxel in human lung cancer cell lines in vitro

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Abstract
Background Pemetrexed and docetaxel show clinical activities against a variety of solid tumors including lung cancers. To identify the optimal schedule for combination, cytotoxic interactions between pemetrexed and docetaxel were studied at various schedules using three human lung cancer cell lines A-549, Lu-99, and SBC-5 in vitro.
Methods Cells were incubated with pemetrexed and docetaxel simultaneously for 24 or 120 h. Cells were also incubated with pemetrexed for 24 h, followed by a 24 h exposure to docetaxel, and vice versa. Growth inhibition was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and cell cycle analysis. Cytotoxic interactions were evaluated by the isobologram method.
Results Simultaneous exposure to pemetrexed and docetaxel for 24 and 120 h produced antagonistic effects in all three cell lines. Pemetrexed (24 h) followed by docetaxel (24 h) produced additive effects in A-549 cells and synergistic effects in Lu-99 and SBC-5 cells. Docetaxel followed by pemetrexed produced additive effects in A-549 and Lu-99 cells and antagonistic effects in SBC-5 cells. The results of cell cycle analysis were fully consistent with those of isobologram analysis, and provide the molecular basis of the sequence-dependent difference in cytotoxic interactions between the two agents.
Conclusions Sequential administration of pemetrexed followed by docetaxel may provide the greatest anti-tumor effects for this combination in the treatment of lung cancer.

Keywords Pemetrexed · Docetaxel · Isobologram · Lung cancer

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Introduction

Lung cancer is the leading cause of cancer mortality in industrialized countries, with non-small cell lung cancer (NSCLC) accounting for nearly 80% [1]. Although surgery may be curative in early-stage NSCLC, most patients present with inoperable advanced disease. These patients managed with best supportive care alone have a median survival time of only 5 months and a 1-year survival rate of approximately 10% [2]. First-line treatment for patients with advanced NSCLC includes platinum compounds combined with vinorelbine, gemcitabine, or taxanes [3]. This is associated with improved quality of life, but only moderate survival advantages when compared with best supportive

care alone. Therefore, there is an emergent need for effective second-line treatments for NSCLC patients who experience disease progression after first-line chemotherapy. Currently, erlotinib, docetaxel, and pemetrexed are approved as second-line drugs by the US Food and Drug Administration for patients whose tumors have progressed after platinum-based treatments [4, 5].

Small cell lung cancer (SCLC) accounts for approximately 12% of all lung cancers [6]. Compared with NCSLC, SCLC has a rapid doubling time, and earlier development of wide spread metastasis. SCLC is highly sensitive to initial radiotherapy and chemotherapy. The most commonly used regimens include etoposide, cisplatin, doxorubicin, or cyclophosphamide [7]. For limited-stage patients, chemotherapy associated with thoracic radiation was able to produce a cure rate of 10–20%. In extensive disease, the combinations of these agents yields responses of 50–70%, with 20–30% complete remissions, but most patients die from recurrent diseases. The identification of new agents is critical for further progress in the treatment of SCLC, and the evaluation of a variety of agents including docetaxel and pemetrexed has been underway [8–10].

Pemetrexed is a new antifolate that has significant activity against a broad spectrum of solid tumors including lung cancer [11, 12]. Pemetrexed inhibits multiple enzymes involved in folate metabolism including thymidylate synthase, dihydrofolate reductase, and glycinamide ribonucleotide formyltransferase [13]. Pemetrexed arrests cells mainly in S phase and induces apoptosis against tumor cells [14]. Against lung cancers, pemetrexed is non-inferior to docetaxel, with lower hematologic toxicity, and febrile neutropenia and a similar rate of non-hematologic toxicities [12].

The taxanes, paclitaxel and docetaxel, have significant activity in lung cancer. Both inhibit microtubule dynamics and cause G2/M cell cycle arrest. However, there are several differences between them in the pharmacokinetics and pharmacologic actions [15, 16]. Docetaxel demonstrated greater affinity for the tubulin-binding site, wider cell cycle activity, longer intracellular retention time and higher intracellular concentration in tumor cells, more potent antitumor activity in in vitro and in vivo models, and more potent induction of bcl-2 phosphorylation and apoptosis. Paclitaxel has a non-linear pharmacokinetic behavior, while docetaxel demonstrated linear pharmacokinetics and less schedule dependence than paclitaxel.

The combination of pemetrexed and docetaxel may play a major role in the second-line treatment of lung cancers. The wide range of antitumor activity of these agents, their different cytotoxic mechanisms and different toxicity profiles, and the absence of cross-resistance provide the rationale for combining these agents. Since both pemetrexed and docetaxel are cell cycle-specific, disturbances of the cell cycle produced by one drug may influence the cytotoxic effects of the other. Furthermore the drug schedule may play a significant role in the outcome, and therefore, how the drugs are combined requires careful consideration.

We showed that the ordered treatment of pemetrexed followed by paclitaxel may be synergistic, whereas simultaneous administration was potentially antagonistic in a variety of solid tumor cell lines [17]. What is not clear is whether such schedule dependency will be as important for pemetrexed and docetaxel as for pemetrexed and paclitaxel in the treatment of lung cancers. The present study was aimed at characterizing the cytotoxic effects of various pemetrexed and docetaxel combinations and schedules on three human lung cancer cell lines using the isobologram method of Steel and Peckham [18]. Flow cytometry was performed to understand the molecular basis of the schedule-dependent synergism and antagonism of the pemetrexed and docetaxel combination.

Materials and methods

Cell lines

Three human lung cancer lines, A-549 (lung adenocarcinoma), Lu-99 (giant-cell lung cancer), and SBC-5 (small cell lung cancer) were used. A-549 cells were purchased from the American Type Culture Collection (Rockville, MD). Lu-99 and SBC-5 cells were obtained from Health Science Research Resources Bank (Tokyo). These cells were growing as a monolayer in 75-cm^2 plastic tissue culture flasks containing RPMI1640 medium (Sigma Chemical Co., St Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma) and antibiotics (penicillin G and streptomycin) in a humidified atmosphere of 95% air/5% CO_2 at 37°C. Under these conditions, the doubling times of these cells were 20–30 h.

Drugs

Pemetrexed and docetaxel were kindly provided by Eli Lilly and Company (Indianapolis, IN) and Sanofi-Aventis K.K. (Tokyo, Japan), respectively. Drugs were dissolved with RPMI1640 and stored at −80°C. Drugs were diluted with RPMI-1640 plus 10% FBS before use.

Cell growth inhibition using combined anti-cancer agents

Growing cells were collected by trypsinization, separated and resuspended to a final concentration of 5.0×10^3 cells/ml in fresh medium containing 10% FBS and antibiotics. Cell suspensions (100 µl) were dispensed into the individual wells of a 96-well tissue culture plate with a lid (Costar, Corning, NY). Each plate had one 8-well control column

containing medium alone and one 8-well control column containing cells but no drug. Eight plates were prepared for each drug combination.

Simultaneous and continuous exposure to pemetrexed and docetaxel

After a 20–24 h incubation for cell attachment, solutions of docetaxel and pemetrexed (50 μl) at different concentrations were added to individual wells in final volumes of 200 μl per wells. The plates were incubated under the same conditions for 120 h.

Simultaneous 24 h exposure to pemetrexed and docetaxel

After cell attachment, solutions of docetaxel and pemetrexed (50 μl) at different concentrations were added to individual wells in final volumes of 200 μl per wells. The plates were also incubated under the same conditions for 24 h. The cells were then washed twice with culture medium, and then fresh medium (200 μl) and antibiotics were added. The cells were cultured again for four additional days in drug-free medium.

Sequential exposure to pemetrexed (24 h) followed by docetaxel (24 h) or vice versa

After cell attachment, medium containing 10% FBS (50 μl) and solutions of docetaxel or pemetrexed (50 μl) at different concentrations were added to individual wells. The plates were then incubated under the same conditions for 24 h. The cells were washed twice and fresh medium was added, followed by the addition of solutions of docetaxel or pemetrexed (50 μl) at different concentrations. The plates were incubated again under the same conditions for 24 h. The cells were then washed twice, and the cells were cultured for three additional days in drug-free medium.

MTT assay

Viable cell growth was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [19]. For all 4 cell lines examined, we established a linear relation between the MTT assay value and the cell number within the range shown.

Isobologram

The dose–response interactions between pemetrexed and docetaxel were evaluated at the IC_{50} level by the isobologram method of Steel and Peckham (Fig. 1) [18]. The IC_{50} was defined as the concentration of drug that produced 50% cell growth inhibition; i.e. a 50% reduction of absorbance.

The theoretical basis of the isobologram method and the procedure for making the isobologram has been described in detail [18, 20, 21]. Based on the dose–response curves of pemetrexed and docetaxel, three isoeffect curves were constructed (Fig. 1). If the agents act additively by independent mechanisms, combined data points would lie near the Mode I line (hetero-addition). If the agents act additively by similar mechanisms, the combined data points would lie near the Mode II lines (iso-addition) [14, 16, 17].

Since we cannot know in advance whether the combined effects of two agents will be hetero-additive, iso-additive, or an effective intermediate between these extremes, all possibilities should be considered. Thus, when the data points of the drug combination fell within the area surrounded by mode I and/or mode II lines (i.e. within the envelope of additivity), the combination was described as additive.

We used this envelope to evaluate not only the simultaneous exposure combinations of pemetrexed and docetaxel, but also to evaluate the sequential exposure combinations, since the second agent under our experimental conditions could modulate the cytotoxicity of the first agent.

A combination that gives data points to the left of the envelope of additivity (i.e. the combined effect is caused by lower doses of the two agents than is predicted) can confidently be described as supra-additive (synergism). A combination that gives data points to the right of the

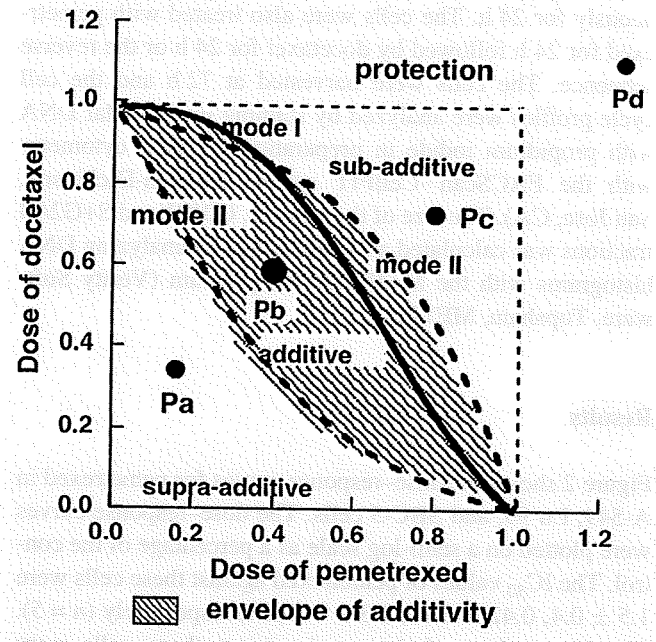

Fig. 1 Schematic representation of an isobologram (Steel and Peckham). The envelope of additivity, surrounded by mode I (*solid line*) and mode II (*dotted lines*) isobologram lines, was constructed from the dose–response curves of pemetrexed alone and docetaxel alone. The concentrations that produced 50% cell growth inhibition were expressed as 1.0 in the ordinate and the abscissa. Combined data points *Pa*, *Pb*, *Pc* and *Pd* show supra-additive, additive, sub-additive, and protective effects, respectively

envelope of additivity, but within the square or on the line of the square can be described as sub-additive (i.e. the combination is superior or equal to a single agent but is less than additive). A combination that gives data points outside the square can be described as protective (i.e. the combination is inferior in cytotoxic action to a single agent). A combination with both sub-additive and/or protective interactions can confidently be described as antagonistic.

Data analysis

Findings were analyzed as described previously [22]. To determine whether the condition of synergism (or antagonism) truly existed, a Wilcoxon signed-rank test was performed to compare the observed data with the predicted minimum (or maximum) data for an additive effect. Probability values (P) ≤ 0.05 were considered significant. Combinations with $P > 0.05$ were regarded as having an additive/synergistic (or additive/antagonistic) effect. All statistical analyses were performed using the Stat View 4.01 software program (Abacus Concepts, Berkeley, CA).

Flow cytometric analysis

SBC-5 cells were treated with 5.0 μM pemetrexed alone, or 1.5 nM docetaxel alone or their combination simultaneously for 24 h. The cells were also treated with pemetrexed for 24 h followed by docetaxel for 24 h or the reverse sequence. The cells were harvested at 72 h and the cell cycle profiles were analyzed by staining intracellular DNA with propidium iodide in preparation for flow cytometry with the FACScan · CellFIT system (Becton-Dickinson, San Jose, CA). The size of the sub-G1, G0/G1 and S+G2/M fractions was calculated as a percentage by analyzing DNA histograms with the ModFitLT 2.0 program (Verity Software, Topsham, ME) [23].

Results

Figure 2 shows the dose–response curves for pemetrexed in A-549, Lu-99, and SBC-5 cells. The dose–response curves were plotted on a semi-log scale as a percentage of the control. The IC_{50} values of pemetrexed against these cells were 1.5 ± 0.4, 0.42 ± 0.10, 1.3 ± 0.2 μM, respectively ($n = 5$). The IC_{50} values of docetaxel against these cells were 1.7 ± 0.2, 1.0 ± 0.1, and 0.82 ± 0.13 nM, respectively ($n = 5$).

The dose–response curves in Fig. 3 show the effect of simultaneous exposure (24 h) (panel a), sequential exposure to pemetrexed followed by docetaxel (panel b), and vice versa (panel c) on the growth of SBC-5 cells. The

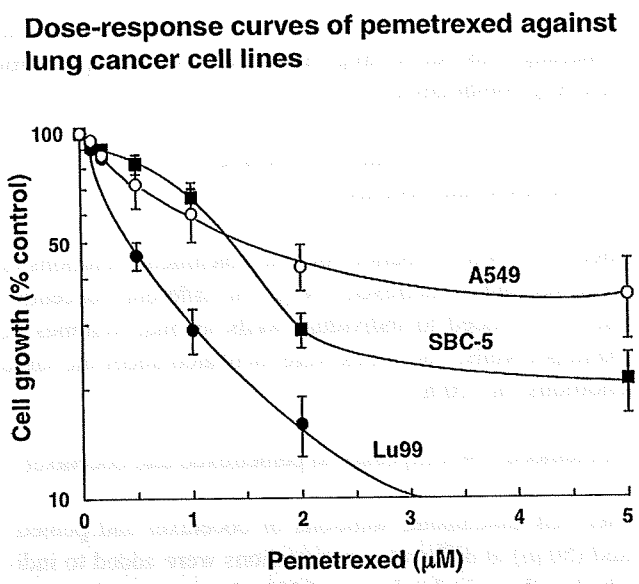

Fig. 2 The dose–response curves of 24 h exposure to pemetrexed against A-549, Lu-99, and SBC-5 cells. Cell growth inhibition was measured using the MTT assay after 5 days and was plotted as a percentage of the control (cells not exposed to drugs). Each point represents the mean ± SEM for at least three independent experiments

pemetrexed concentrations are shown on the abscissa. Dose–response curves in which the docetaxel concentrations are shown on the abscissa are based on the same data (figure not shown). Three isoeffect curves (mode I and mode II lines) were constructed based on the dose–response curves of pemetrexed alone and docetaxel alone. Isobolograms at the IC_{50} level were generated based on these dose–response curves for the combinations.

Simultaneous exposure to docetaxel and pemetrexed for 24 h

Figure 4a shows isobolograms of SBC-5 cells after simultaneous exposure to pemetrexed and docetaxel. The combined data points fell in the areas of subadditivity and protection. The mean values of the observed data (0.71) were larger than those of the predicted maximum values (0.60). The observed data and the predicted maximum data were compared by Wilcoxon signed-rank test. The difference was significant ($P < 0.05$), indicating antagonistic effects (Table 1). Quite similar effects were observed in A-549 and Lu-99 cells (Table 1, isobolograms not shown).

Sequential exposure to pemetrexed for 24 h followed by docetaxel for 24 h

Figure 4b shows isobolograms of SBC-5 cells exposed first to pemetrexed and then to docetaxel. The combined data points fell in the area of supraadditivity. The mean values of the observed data (0.46) were smaller than those

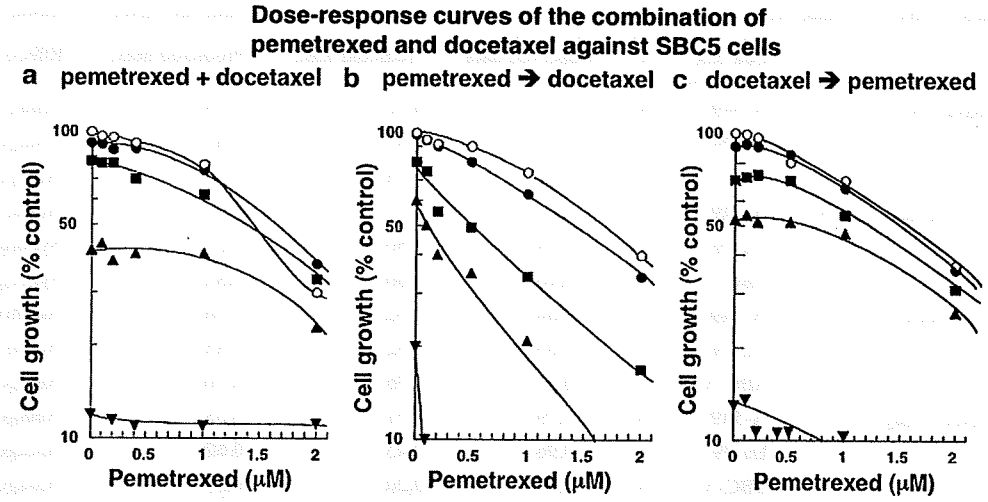

Fig. 3 Schedule dependence of the interaction between docetaxel and pemetrexed in SBC-5 cells. Cells were exposed to these two drugs simultaneously for 24 h (**a**), pemetrexed first for 24 h followed by docetaxel for 24 h (**b**), and vice versa (**c**). The cell number after 5 days was measured using the MTT assay and was plotted as a percentage of the control (cells not exposed to drugs). The concentrations of docetaxel are shown on the abscissa. The concentrations of pemetrexed were 0 (*open circle*), 0.2 (*filled circle*), 0.5 (*filled square*), 1.0 (*filled triangle*) and 2.0 (*filled inverted triangle*) μM, respectively. Data are mean values for three independent experiments; SE was < 25%

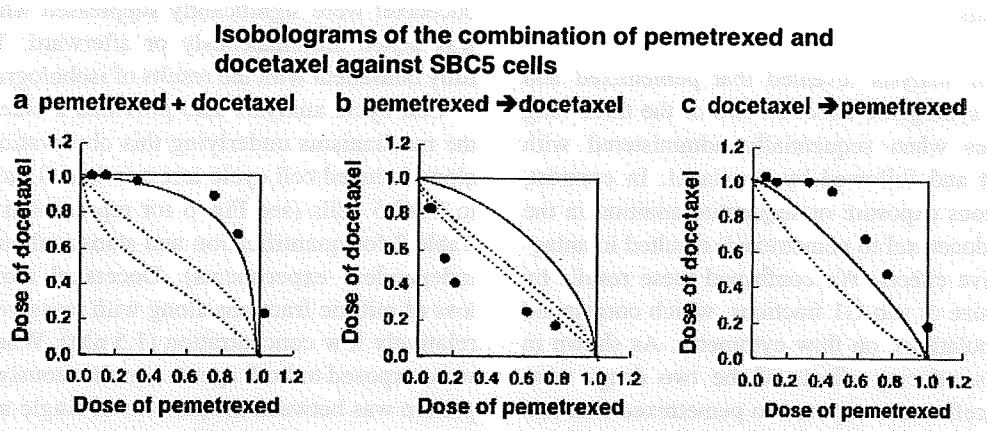

Fig. 4 Isobolograms of simultaneous exposure to docetaxel and pemetrexed for 24 h in SBC-5 cells (**a**). The combined data points fell in the areas of subadditivity and protection. Data are mean values for at least three independent experiments; SE was <25%. Isobolograms of sequential exposure to pemetrexed (24 h) followed by docetaxel (24 h) in SBC-5 cells (**b**). All data points of the combinations fell in the area of supraadditivity. Data are mean values for at least three independent experiments; SE was <20%. Isobolograms of sequential exposure to docetaxel (24 h) followed by pemetrexed (24 h) in SBC-5 cells (**c**). All data points of the combinations fell in the areas of subadditivity and protection. Data are mean values for at least three independent experiments; SE was <25%

of the predicted minimum values (0.60) (Table 1). The difference was significant ($P < 0.05$), indicating synergistic effects. Quite similar effects were observed in Lu-99 cells (Table 1, isobolograms not shown), while additive effects were observed in A-549 cells (Table 1, isobolograms not shown).

Sequential exposure to docetaxel for 24 h followed by pemetrexed for 24 h

Figure 4c shows isobolograms of SBC-5 cells exposed first to docetaxel, followed by pemetrexed. The combined data points mainly fell in the area of subadditivity. The mean values of the observed data were larger than those of the predicted maximum values ($P < 0.02$) (Table 1), indicating antagonistic effects. For A-549 and Lu-99 cells, most combined data points fell within the envelope of additivity and the mean values of the observed data were between those of the predicted minimum and maximum values (Table 1, isobolograms not shown), indicating an additive effect of this schedule.

Simultaneous exposure to pemetrexed and docetaxel for 5 days

For all three cell lines, combined data points fell in the areas of subadditivity and protection, indicating antagonistic effects (Table 1, isobolograms not shown).

Table 1 Mean values of observed data, predicted minimum, and predicted maximum of pemetrexed and docetaxel in combination at IC_{50} level

Schedule	Cell line	n^a	Observed data	Predicted min.[b]	Predicted max.[c]	Effects
Pemetrexed + docetaxel (24 h)	A-549	8	0.72	0.31	0.55	Antagonism ($P < 0.02$)
	Lu-99	6	>1.0	0.41	0.62	Antagonism ($P < 0.05$)
	SBC-5	6	0.71	0.33	0.60	Antagonism ($P < 0.05$)
Pemetrexed (24 h) → docetaxel (24 h)	A-549	7	0.63	0.31	0.92	Additive
	Lu-99	7	0.29	0.50	0.67	Synergism ($P < 0.02$)
	SBC-5	7	0.46	0.60	0.82	Synergism ($P < 0.02$)
Docetaxel (24 h) → pemetrexed (24 h)	A-549	8	0.64	0.32	0.86	Additive
	Lu-99	8	0.63	0.32	0.85	Additive
	SBC-5	7	0.87	0.36	0.70	Antagonism ($P < 0.02$)
Pemetrexed + docetaxel (5 day)	A-549	6	0.79	0.51	0.68	Antagonism ($P < 0.05$)
	Lu-99	6	0.96	0.45	0.62	Antagonism ($P < 0.05$)
	SBC-5	4	0.73	0.20	0.57	Antagonism ($P < 0.05$)

[a] Number of data points

[b] Predicted minimum value for an additive effect

[c] Predicted maximum value for an additive effect

Cell cycle analysis

The isobologram analysis revealed that pemetrexed and docetaxel had a synergistic effect on two of the three lung cancer cell lines when sequentially administered with pemetrexed first and followed by docetaxel. In contrast, either simultaneous exposure or sequential addition in the reversed order (docetaxel to pemetrexed) resulted in antagonistic or additive effects. We confirmed these results by calculating the size of sub-G1 fractions, which correspond to apoptotic populations, on flow cytometry. As shown in Fig. 5, apoptosis-inducing effects of the two drugs were strongest when cells were exposed to pemetrexed first and followed by docetaxel. In contrast, the cytotoxic effects of docetaxel were significantly suppressed when pemetrexed was added simultaneously or afterward. These data are fully consistent with the results of isobologram analysis.

Cell cycle analysis also provided a clue to understand the mechanisms underlying this observation. Pemetrexed alone induced cell cycle arrest in late G1 to early S phase in SBC-5 cells (see Fig. 6 for representative results, and Table 2 for quantification and statistical analysis of three independent experiments). Docetaxel alone caused the loss of mitotic fractions along with massive apoptosis at a relatively low concentration (1.5 nM). When SBC-5 cells were exposed to both agents simultaneously, the cell cycle pattern was between the patterns of single-agent exposure, and the size of sub-G1 fractions was substantially

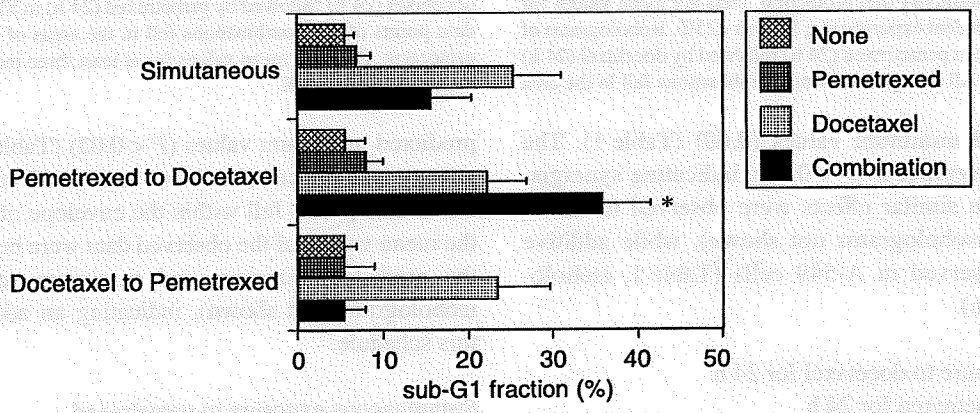

Fig. 5 SBC-5 cells were cultured in the absence (None) or presence of either 5.0 μM pemetrexed (Pemetrexed) or 1.5 nM docetaxel (Docetaxel) alone for 24 h; or in the presence of both drugs for 24 h (Simultaneous); or treated with pemetrexed for 24 h, followed by docetaxel for 24 h (Pemetrexed to Docetaxel); or treated with docetaxel for 24 h, followed by pemetrexed for 24 h (Docetaxel to Pemetrexed). After 72 h, DNA histograms were obtained to calculate the size of sub-G1 fractions as described in "Materials and methods". Data shown are the means ± SD of three independent experiments. The statistical difference was determined by one-way ANOVA with Bonferroni multiple comparison test. An *asterisk* denotes $P < 0.01$

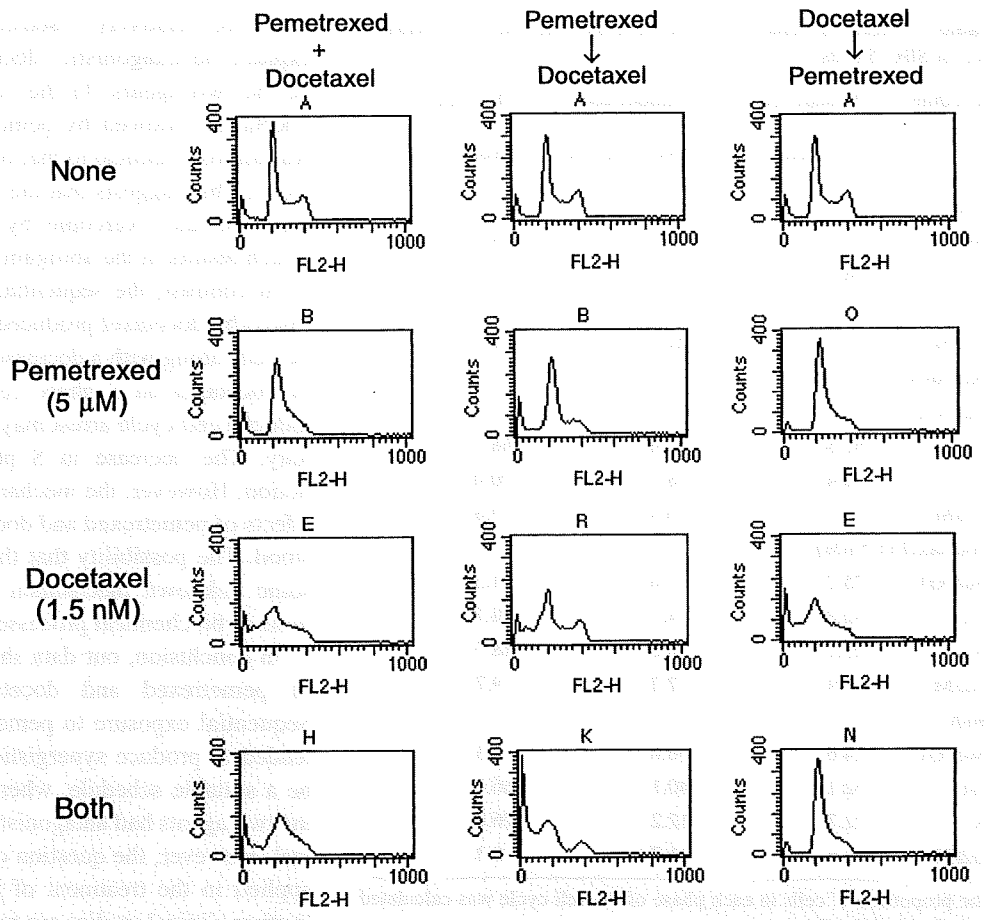

Fig. 6 Cell cycle analysis of SBC-5 cells treated with docetaxel and pemetrexed. *Left column* SBC-5 cells were treated with no drug, 5.0 μM pemetrexed, 1.5 nM docetaxel, or both drug simultaneously for 24 h. *Middle column* SBC-5 cells were treated with 5.0 μM pemetrexed for 24 h, followed by 1.5 nM docetaxel for 24 h. *Right column* SBC-5 cells were treated with 1.5 nM docetaxel for 24 h, followed by 5.0 μM pemetrexed for 24 h. Cells were harvested at 72 h and DNA histogram was obtained as described in "Materials and methods"

reduced. When SBC-5 cells were treated with docetaxel first and followed by pemetrexed, the cell cycle profile was almost identical to that of single exposure to pemetrexed, suggesting that the cell cycle effect of pemetrexed is dominant over that of docetaxel. As a result, the apoptosis-inducing effect of docetaxel was almost completely cancelled in the presence of pemetrexed. In contrast, when SBC-5 cells were treated with pemetrexed first and followed by docetaxel, the proportion of cells in sub-G1 phase was larger than that of cells treated with either pemetrexed or docetaxel alone. This was accompanied by a decrease in S-phase cells. Overall, the results of cell cycle analysis are fully consistent with those of isobologram analysis, and provide the molecular basis of the sequence-dependent differences in cytotoxic interactions between the two agents.

Discussion

In this study, we investigated the effects of pemetrexed in combination with docetaxel on lung cancer cell lines to determine the optimal schedule for this combination. Analysis of the drug–drug interaction effects was carried out using the isobologram method of Steel and Peckham [18], which provides a fundamental basis for assessing whether cytotoxicity induced by combinations of anticancer agents is greater, equal to, or smaller than would have been expected for the individual agents.

We demonstrated that a cytotoxic interaction between pemetrexed and docetaxel is schedule-dependent. Simultaneous exposure to pemetrexed and docetaxel for 24 h and 5 days showed antagonistic effects in all cell lines studied. Sequential exposure to pemetrexed for 24 h followed by docetaxel for 24 h showed synergistic effects in Lu-99 and SBC-5 cells, while it showed additive effects in A-549 cells. Sequential exposure to docetaxel followed by pemetrexed showed additive effects in A-549 and Lu-99 cells, but antagonistic effects in SBC-5 cells. We also used SW620 colon cancer cells for the study, and the combined effects for these schedules were quite the same as those of SBC-5 cells (data not shown).

These findings suggest that the sequential administration of pemetrexed followed by docetaxel may be more cytotoxic to cancer cells and optimal for this combination, while the simultaneous administration of pemetrexed and docetaxel may be less cytotoxic and suboptimal. It should be noted that the sequential administration of pemetrexed

Table 2 Effects of pemetrexed and docetaxel on cell cycle distribution of SBC-5 cells

Schedule	Pemetrexed + Docetaxel (%)	Pemetrexed ↓ Docetaxel (%)	Docetaxel ↓ Pemetrexed (%)
None			
Sub-G1	5.4	4.7	4.7
G1	48.4	51.3	51.3
S	24.9	22.3	22.3
G2/M	21.3	21.7	21.7
Pemetrexed (5 μM)			
Sub-G1	5.5	9.9	2.2
G1	62.8	61.6	68.2
S	28.4	18.1	20.0
G2/M	3.3	10.4	9.6
Docetaxel (1.5 nM)			
Sub-G1	25.2	17.6	21.3
G1	42.8	4.7	50.7
S	27.1	20.0	18.3
G2/M	4.9	17.7	9.7
Both			
Sub-G1	14.6	36.0	2.3
G1	52.1	40.1	66.4
S	22.7	12.2	26.0
G2/M	3.6	11.7	5.3

The proportion of cells in each phase of the cell cycle was calculated with the ModFitLT 2.0 program

followed by docetaxel might be more toxic for normal cells. Since, however, toxicity profiles of both agents are different, increasing overlapping toxicity would likely be mild.

Previously, we evaluated the cytotoxic effects of pemetrexed in combination with paclitaxel in vitro using A-549 cells, breast cancer MCF7, ovarian cancer PA1, and colon cancer WiDr cells in vitro [17]. The results were similar to those of the present study. Although slight differences are present, this would be due to the very strict definitions of synergism and antagonism in the isobologram method (Steel and Peckham). Our previous and present findings suggest that the simultaneous administration of pemetrexed and taxanes is less cytotoxic than the sequential administration of pemetrexed followed by taxanes, and latter schedule should be assessed in clinical trials for the treatment of lung cancer and other solid tumors.

In general, it is difficult to clarify the mechanisms underlying the cytotoxic effects of drug combinations. In this study, however, cell cycle analysis provided a clue to the molecular basis of schedule-dependent synergism and antagonism. The exposure of SBC-5 cells to pemetrexed led to synchronization of most cells that were in late G1 phase to the early S phase of the cell cycle, during which cells are relatively insensitive to docetaxel. This may explain the antagonistic effects of the simultaneous addition of the two agents. In the case of sequential exposure to docetaxel followed by pemetrexed, the cell cycle pattern was almost identical to that of cells treated with pemetrexed alone. This suggests that the cell cycle effect of docetaxel is transient and overcome by the addition of pemetrexed, which results in the abrogation of its cytotoxicity.

In contrast, the sequential exposure to pemetrexed followed by docetaxel produced a striking increase in apoptotic cells along with a decrease in cells in S phase. The effect of docetaxel on S phase cells no longer in pemetrexed-induced cell cycle arrest may cause the synergistic cytotoxicity. The decrease in S phase is compatible with this notion. However, the mechanisms underlying the cytotoxic effects of pemetrexed and docetaxel are still not well understood. The possibility that the drug interactions are due to some unknown mechanism related to complex perturbations of biochemical processes cannot be excluded.

In conclusion, our data show that the antitumor activity of pemetrexed and docetaxel is schedule-dependent. Sequential exposure to pemetrexed followed by docetaxel tended to produce synergistic effects, and would therefore be a suitable schedule, whereas simultaneous exposure to the two agents had antagonistic effects, and may be suboptimal. However, the question of how far these results can be applied in the treatment of patients remains unanswered. Further clinical studies are necessary to clarify whether the therapy sequence alters the antitumor effect and the toxicity of this combination. Our findings provide preclinical rationale for a novel, mechanism-based, therapeutic strategy to be tested in lung cancer patients.

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Conflict of interest statement None.

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A Phase II Study of Cisplatin and Irinotecan as Induction Chemotherapy Followed by Accelerated Hyperfractionated Thoracic Radiotherapy with Daily Low-dose Carboplatin in Unresectable Stage III Non-small Cell Lung Cancer: JCOG 9510

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Objective: It is important to find optimal regimens of cisplatin (CDDP)-based third-generation chemotherapy and radiotherapy for patients with unresectable Stage III non-small cell lung cancer (NSCLC).
Methods: This Phase II study was designed to determine the toxicity and efficacy of two courses of chemotherapy (CDDP 80 mg/m^2 on day 1 and irinotecan 60 mg/m^2 on days 1 and 8) followed by accelerated hyperfractionated thoracic radiotherapy (60 Gy/40 fractions in 4 weeks) combined with daily carboplatin (CBDCA) administration. CBDCA was administered at a target area under the plasma level–time curve of 0.4 × (24 h creatinine clearance + 25), according to Calvert's formula.
Results: Twenty-six patients were enrolled in the study. The patients' median age was 63 years (range 40–74 years) and included 22 males and 4 females. Seven patients were Stage IIIA and 19 were Stage IIIB. Twenty had a performance status (PS) of 1 versus six with a PS of 0. There was one treatment-related death due to sepsis and pneumonia associated with Grade 4 neutropenia and diarrhea during chemotherapy. Grade 3 or 4 neutropenia and diarrhea were observed in 14 and 5 patients, respectively. Toxicity of the radiotherapy was mild. There were 0 complete response and 13 partial responses, giving a response rate of 50.0%. Median survival time and 2-year survival were 16.4 months and 21.5%, respectively. This study was designed with Simon's two-stage design, and the response rate did not meet the criteria to proceed to the second stage and the study was terminated early.
Conclusions: This regimen might be inactive for patients with unresectable Stage III NSCLC.

Key words: cisplatin – irinotecan – carboplatin – chemoradiotherapy – non-small cell lung cancer

INTRODUCTION

Over the past 2 decades, a great number of clinical trials have gradually proven the benefits of a chemotherapeutic approach for treatment of unresectable non-small cell lung cancer (NSCLC) (1,2). In unresectable Stage III NSCLC, in which the tumor is apparently confined to the chest but is surgically unresectable, several randomized trials have shown that combinations of chemotherapy and thoracic radiotherapy have improved survival compared with radiotherapy alone (3–6). It is important to find optimal regimens of combined chemotherapy and radiotherapy and to evaluate the feasibility and efficacy of those combinations.

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