months with an incidence of grade 3 or higher nonhematological adverse events of less than 5% and tolerable myelosuppression. The regimen also showed a high antitumor activity in a subset of 21 patients 75 years or older (ORR = 38%) [9].

TS-1 (Taiho Pharmaceutical Co., Tokyo, Japan) is a new oral anticancer agent that is composed of tegafur, 5-chloro-2, 4-dihydroxypyridine (CDHP), and potassium oxonate in a molar ratio of 1:0.4:1. The 5-FU concentrations in blood and tumors achieved by TS-1 are much higher and longer-lasting than those by UFT [11]. In a phase II trial of TS-1 monotherapy in previously untreated patients with advanced NSCLC, the ORR was 22% and the median survival time was 10.2 months [12]. A phase I/II trial of TS-1 plus gemcitabine was conducted to further enhance the efficacy of the combination of UFT plus gemcitabine, while maintaining a mild level of toxicity in the treatment of elderly patients.

2. Patients and methods

2.1. Patient eligibility

Patients were registered at the central data center when the following eligibility criteria were confirmed: cytologically or histologically confirmed NSCLC; stage IIIB disease without any indications for radiotherapy or stage IV disease; no prior treatment; age 70 years of age or older; and an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 or 1. The criteria for organ function included: neutrophil count $\geq 2000/\mu L$; platelet count $\geq 100,000/\mu L$; hemoglobin level ≥ 9.5 g/dL; serum bilirubin concentration ≤ 1.5 mg/dL; serum aspartate aminotransferase and alanine aminotransferase concentrations ≤ 100 IU/L; creatinine level ≤ 1.3 mg/dL; creatinine clearance rate ≥ 30 mL/min (≥ 60 mL/min for the phase II portion); and arterial oxygen saturation $\geq 90\%$.

Patients were excluded from the study if they had either interstitial pneumonia or pulmonary fibrosis on chest X-ray films, any severe concomitant disease (severe cardiac disease, uncontrolled diabetes mellitus, severe infection), concomitant malignancy, pleural effusion necessitating treatment, or symptomatic cerebral involvement. Written informed consent was required from all patients. The protocol was approved by the institutional review committee of each of the participating institutions.

2.2. Evaluation for enrollment

All patients were required to undergo a computed tomography (CT) scan of the thorax and the upper abdomen, either CT or magnetic resonance imaging (MRI) of the brain and a radioisotopic bone scan for stage assessment. A complete blood cell count and a blood chemistry panel were also obtained at enrollment. After protocol treatment was started, the blood examinations and chest radiography were performed at least once per week. CT or MRI examinations were repeated every 6 weeks to evaluate the target lesions. The tumor response was assessed with the Response Evaluation Criteria in Solid Tumors, and toxicity was assessed with the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0.

2.3. Phase I portion

The primary endpoint for the phase I trial was to determine the maximum tolerable dose (MTD) and dose-limiting toxicity (DLT). The doses were escalated in each successive cohort of 3 or more new patients. TS-1 was administered orally twice daily after meals on days 1–4. Gemcitabine was administered intravenously in 30 min or less on days 8 and 15. The schedule was repeated every 4 weeks for more than 3 cycles, unless disease progression or unacceptable

toxicity occurred. Satisfaction of the entry eligibility criteria regarding the organ function was required before the next cycle could be started.

Three dose levels were evaluated with the following doses: level 1, 30 mg/m² (60 mg/m²/day) of TS-1 and 800 mg/m² of gemcitabine; level 2, 30 mg/m² of TS-1 and 1000 mg/m² of gemcitabine; and level 3, 40 mg/m² (80 mg/m²/day) of TS-1 and 1000 mg/m² of gemcitabine. Gemcitabine was administered when the leukocyte count was $\geq 2000/\mu L$, the thrombocyte count was $\geq 75,000/\mu L$, and nonhematological toxicities were no greater than grade 1.

The dose level was escalated on the basis of the toxicity during the first cycle of chemotherapy and was not escalated for each individual. A DLT was defined as any of the following: (i) grade 4 neutropenia; (ii) grade 3 febrile neutropenia; (iii) grade 4 thrombocytopenia; (iv) grade 3 nonhematological adverse events (except anorexia and fatigue); (v) a delay of gemcitabine infusion on day 15 for more than 7 days; and (vi) a delay of administration of the next course for more than 2 weeks. If DLT occurred in 1 or 2 of the 3 initial patients at a particular dose level, then 3 additional patients were treated at the same dose level. If DLT developed in all 3 patients or in 3 of 6 patients, then enrollment was stopped at this dose level, which was defined as the MTD. The preceding dose level was designated as the recommended dose (RD) for the phase II portion.

2.4. Phase II portion

The primary endpoint for the phase II study was the ORR. The patients were enrolled until the number of those treated with RD, including the patients who received the RD in the phase I portion, reached the predetermined sample size. The treatment schedule used in phase I was also followed in the phase II portion.

2.5. Statistical analysis

The phase II portion was designed to detect the difference between the ORRs of 0.10 and 0.30 with more than 90% power (exact binomial test for one sample proportion, 1-sided $\alpha = 0.05$). The new regimen was to be considered worthy of further investigation if >7 responses were observed in a 37-patient cohort treated at the RD. The Kaplan–Meier method was used to estimate the median values of time-to-event variables, such as overall survival (OS) and progression-free survival (PFS), and their confidence intervals (CIs) were calculated with the Brookmeyer and Crowley method [13]. All analyses were performed with the SAS software package, version 9.1 (SAS Institute, Cary, NC).

3. Results

Forty-nine patients were enrolled from May 2005 through December 2006. The phase I portion had 22 patients. Thirty-seven patients, including 10 patients from the phase I portion who were treated with the RD level, were enrolled in phase II. The median age of all patients in the study was 77 years (range, 70–85 years). Thirty-two (65%) patients had an ECOG PS of 1, 28 (57%) patients had adenocarcinoma, and 32 (65%) patients had stage IV disease (Table 1).

3.1. MTD and DLT in the phase I portion

The phase I portion included 22 patients. At level 1, 1 of 6 patients had a DLT (grade 3 infection). Then, the dose was escalated to level 2 where 6 patients were enrolled and treated. However, 3 of them were not evaluable with regard to the DLT of TS-1/gemcitabine combination; 1 patient experienced sudden death which was unrelated to TS-1 on day 2 of the first cycle, and 2

Table 1
Patients characteristics.

	Phase I	Phase II	Total
Number of patients	22	37	49
Age (years)			
Median	76	77	77
Range	70–85	70–85	70–85
Sex			
Male	18 (82%)	27 (73%)	37 (76%)
Female	4 (18%)	10 (27%)	12 (24%)
ECOG PS			
0	7 (32%)	14 (38%)	17 (35%)
1	15 (68%)	23 (62%)	32 (65%)
Histologic type			
Adenocarcinoma	13 (59%)	21 (57%)	28 (57%)
Other	9 (41%)	16 (43%)	21 (43%)
Stage			
IIIB	8 (36%)	12 (32%)	17 (35%)
IV	14 (64%)	25 (68%)	32 (65%)

ECOG PS, Eastern Cooperative Oncology Group Performance Status.

patients stopped the chemotherapy before the first infusion of gemcitabine (1 refused the protocol treatment, and 1 had grade 3 allergic dermatitis). The Independent Data Monitoring Committee (IDMC) reviewed the reports from investigators on these results and requested the enrollment of 3 additional patients at the level 2 cohort for the evaluation of MTD. However, in enrolling the ninth patient at the level 2, the data center had two simultaneous new registrations from two different hospitals. Therefore, a total of 10 patients were enrolled into the level 2 cohort. Seven of these patients received at least the first gemcitabine infusion and were used in the evaluation; 2 patients at this level had DLTs (1 case of grade 3 infection, 1 case of grade 3 stomatitis, and 1 case of grade 3 skin toxicity). On the basis of these results, the IDMC permitted

the dose escalation to level 3. At level 3, 3 of 6 patients had DLTs (2 cases of grade 4 neutropenia, 1 case of grade 3 leukocytopenia, 1 case of grade 3 febrile neutropenia, 1 case of grade 3 infection, 2 cases of grade 3 nausea, 2 cases of grade 3 diarrhea, 2 cases of grade 3 skin toxicity, and 1 case of grade 3 dyspnea). The MTD and RD were then determined to be level 3 and level 2, respectively. Table 2 lists all adverse events observed during the phase I portion.

The eligibility criterion for the creatinine clearance rate was modified to be ≥60 mL/min in the phase II rather than the rate of ≥30 mL/min in the phase I portion because skin toxicities were more often observed in patients with a creatinine clearance rate of less than 60 mL/min.

3.2. Treatment cycle for patients treated at the RD

Of 37 patients treated at the RD level (TS-1, 60 mg/m^2/day, and gemcitabine, 1000 mg/m^2), 19 (51%) received more than 3 cycles, and 6 (16%) continued for more than 6 cycles. The median number of treatment cycles received was 3. The gemcitabine dose was reduced to 800 mg/m^2 in 5 patients.

3.3. Tumor response and overall survival in patients treated at RD

None of the 37 patients treated at the RD had a complete response (CR) and 10 had a partial response (PR). Therefore, the ORR was 27% (90% exact CI, 15–42%), and the null hypothesis for the phase II portion of the study was rejected. Fourteen patients had stable disease (SD), and 9 patients had progressive disease (PD). Four patients were not evaluable for tumor response. The median PFS time was 4.2 months (90% CI, 3.2–5.7 months). The median survival time was 12.9 months (90% CI, 10.4–14.7 months), and the 1-year survival rate was 51% (90% CI, 36–64%; Fig. 1). All patients had PD and 28 death events were observed at 2 years follow-up after the end of patient enrollment.

Table 2
All adverse events in the phase I portion[a].

Grade	Level 1 (n = 6)					Level 2 (n = 10)					Level 3 (n = 6)				
	1	2	3	4	3–4	1	2	3	4	3–4	1	2	3	4	3–4
Neutropenia	1	5	1		1	1	3	1		1		1	1	2	3
Leukopenia	2	1	1		1	3		1		1	1	2	1	1	2
Anemia		2	1		1	2	1				1	1			
Thrombocytopenia	1	1				1	1	1		1	1	2	2		2
Febril neutropenia													1		1
Infection			1		1			1		1			1		1
Billirubin	1	1				2	1				1	1			
AST/ALT	2/3					3/0	0/2				1/1				
Blood urea nitrogen	1														
Creatinine															
Na/K	1/0					1/1									
Ca	3										1				
Fever	1					1					1				
Fatigue	1					2	1						1		1
Anorexia	2					2		1		1	1	2			
Vomiting	2						1				1				
Nausea	2												2		2
Diarrhea	2										1		2		2
Constipation						2									
Skin		1				4	1			1			2		2
Stomatitis							1			1		1			
Edema	1					2									
Dyspnea	1										1		1		1
Cough	1					1	1								
Pain	1														
Allergic dermatitis								1		1					
Dose-limiting toxicity	Infection					Infection, skin, stomatitis					Neutropenia, leukocytepenia, febrile neutropenia, infection, nausea, diarrhea, skin, dyspnea				

[a] The worst grade during the first cycle was summarized. At the level 2 dose, one patient experienced death which was not drug-related.
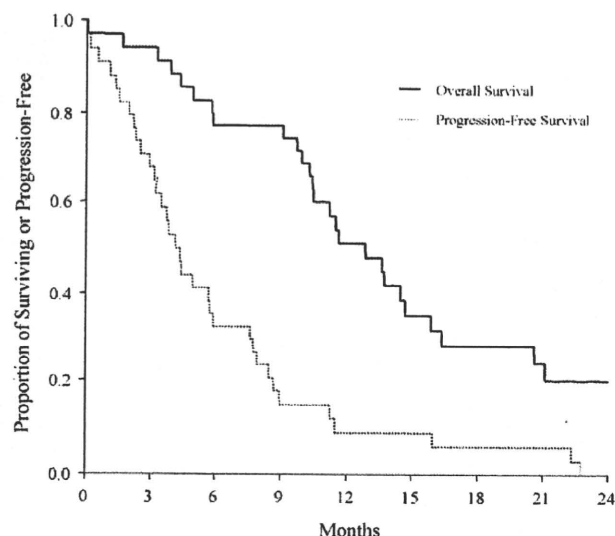
Fig. 1. Overall survival and progression-free survival curves for 37 patients treated with the RD. The curves were constructed using the Kaplan–Meier method.

3.4. Adverse events in patients treated at the RD

The worst grade adverse events that occurred during the treatment of the 37 patients at the RD level are summarized in Table 3. Grade 3 or higher neutropenia and thrombocytopenia were observed in 45.9% and 13.5% of the patients, respectively. In addition, 2 cases each of grade 3 pneumonitis and skin toxicity and 1 case each of grade 3 anorexia, fatigue, stomatitis, infection, and allergic dermatitis were observed.

4. Discussion

Oncologists currently prefer single-agent chemotherapy with either gemcitabine, vinorelbine, or docetaxel for the treatment of elderly patients with advanced NSCLC [2–4]. Although the results of subset analyses in previous phase III studies also suggest the efficacy of carboplatin-based combination including a third-generation agent, such as gemcitabine or a taxane [14,15], the validity of platinum-based doublets for elderly patients continues to be a topic of debate. The greatest concern for the use of a doublet regimen in the elderly is the trade-off between toxicity and survival benefit. In fact, the primary reason that the combination of gemcitabine plus vinorelbine has failed to show efficacy for elderly patients is the increase of severe toxicities leading to poor compliance with chemotherapy [3]. Therefore, the development of combination therapies without an increased rate of severe adverse effects is particularly needed for this population.

Combination chemotherapy of UFT plus gemcitabine has been evaluated for previously untreated patients with advanced NSCLC [9,10]. These trials were performed on the basis of the potential synergism of the two drugs; gemcitabine and 5-FU are both antimetabolites, but they inhibit DNA synthesis via different pathways. Gemcitabine is a substrate for 5 of the nucleoside transporters found in humans. 5-FU leads to an increase in cell surface human equilibrative nucleoside transporter 1 (hENT1) [16,17]. An increase in hENT1 can potentially augment the effect of gemcitabine because this agent enters the cell via hENT1 [18]. On the other hand, adding gemcitabine to 5-FU has been suggested to increase the blood concentration of 5-FU [19]. These results together suggest that the combination of the two drugs have synergistic effects and previous studies have confirmed both the promising efficacy and mild toxicity of the combination. TS-1 is an oral 5-FU designed to enhance

Table 3
Grade 3–4 adverse events for 37 patients at the RD level[a].

	Grade 3	Grade 4	Grades 3–4 (%)
Neutrophils	12	5	45.9
Leukocytes	10	0	27.0
Hemoglobin	4	1	13.5
Platelets	5	0	13.5
Pneumonitis	2	0	5.4
Skin	2	0	5.4
Anorexia	1	0	2.7
Fatigue	1	0	2.7
Stomatitis	1	0	2.7
Infection	1	0	2.7
Allergic dermatitis	1	0	2.7

[a] The worst grade during the treatment was summarized. One patient experienced death which was not drug-related.

anticancer activity much more than UFT, while preventing gastrointestinal toxicity through the deliberate combination of the components [11]. The present phase II study was designed on the basis of the availability of TS-1 and the results for UFT plus gemcitabine. The treatment schedule was based on *in vitro* studies that the sequence of 5-FU followed by gemcitabine is more cytotoxic than the reverse [20].

The ORR of 27% in the current study was considerably higher than the previously reported response rates for vinorelbine (9.9%) [4] and gemcitabine (17.3%) in elderly patients with advanced NSCLC [3]. In addition, of the grade 4 events in the 37 patients of the phase II portion of our study, 5 were cases of neutropenia, and none were cases of thrombocytopenia or febrile neutropenia. This result suggests a potential advantage of our doublet regimen, because the frequencies of grade 4 adverse events were much lower than for monotherapy with vinorelbine or docetaxel [4] and were comparable to those for gemcitabine [3]. Skin toxicity was observed more frequently with the combination of TS-1 plus gemcitabine than with either TS-1 or gemcitabine monotherapy or with other combination regimens. However, grade 3 or higher events remained at a frequency of 2.7%, which was thus considered to be manageable.

Our previous phase II trial of UFT plus gemcitabine showed it was less toxic than TS-1 plus gemcitabine, while both regimens seemed to provide a similar efficacy. However, the frequency of female patients who had adenocarcinoma was substantially lower in the TS-1 study. Since they have been recognized to be a large population with a good prognosis in lung cancer, the TS-1 study had a more unfavorable background for efficacy than the UFT study. Nevertheless, the similarity of efficacy in both studies suggests that TS-1 plus gemcitabine may establish more promising prognostic effect. Considering the acceptable toxicity of TS-1 plus gemcitabine, we consider that it deserves a further evaluation.

In conclusion, the current results suggest that combination chemotherapy with TS-1 and gemcitabine warrants phase III investigations for the treatment of elderly patients with NSCLC. In addition, future studies should also determine whether this combination regimen is equally effective in ethnic groups other than the Japanese.

Conflicts of interest statement

Acknowledgements

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Case report - Thoracic oncologic

Ciliated muconodular papillary tumour of the lung: a newly defined low-grade malignant tumour

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Abstract

We present two cases of ciliated muconodular papillary tumour (CMPT) in this report. CMPT is a newly defined low-grade malignant tumour with ciliated columnar epithelial cells, occurring in the peripheral lung. Both patients underwent pulmonary resection due to an enlarged solitary pulmonary nodule. Pathological findings in both cases confirmed a papillary tumour with a mixture of ciliated columnar and goblet cells. The tumours were rich in mucous and had spread along the alveolar walls, as observed in bronchioloalveolar carcinoma. Nuclear atypia was mild, and no mitotic activity was observed. Immunohistochemically, tumour cells stained positive for carcinoembryonic antigen, thyroid transcription factor-1 and cytokeratin 7 but not for cytokeratin 20. The immunohistochemical staining patterns were almost identical to those of pulmonary adenocarcinoma. We definitively diagnosed as CMPT. Both patients remained relapse-free.
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Keywords: Ciliated muconodular papillary tumour; Low-grade malignancy; Peripheral lung

1. Introduction

Lung tumours with ciliated cells are considered benign, and most of these tumours occur in the central airway [1, 2]. Herein we report two cases of ciliated muconodular papillary tumour (CMPT), which is considered a low-grade malignant tumour with ciliated epithelial cells, occurring in the peripheral lung.

2. Case presentation

2.1. Case 1

A 67-year-old man, a smoker (20 pack/year) having no remarkable medical history, was referred to us with flu-like symptoms. Computed tomography (CT) revealed a lung tumour, 5 mm in diameter in the right S3 segment. Follow-up CT 18 months later revealed that the tumour had slightly enlarged to 6 mm in diameter. The patient desired follow-up observation. Follow-up CT 10 months later revealed that the tumour had further enlarged to 9 mm in diameter with ground glass opacity (GGO) surrounding the tumour (Fig. 1a). Primary lung cancer was suspected, and video-assisted right S3 partial resection was performed. Frozen sections revealed papillary tumours with a mixture of ciliated epithelial and goblet cells. We considered it a low-grade malignant tumour because of the presence of ciliated cells and performed partial resection with wide tumour-free

margins. Microscopic analysis revealed that the tumour cells were composed of ciliated columnar and goblet cells with papillary growth (Fig. 1b,c). Some tumour cells had spread along the alveolar walls, as observed in bronchioloalveolar carcinoma. Nuclear atypia was mild, and no mitotic activity was observed. Immunohistochemically, the tumour cells stained positive for carcinoembryonic antigen (CEA), thyroid transcription factor-1 (TTF-1) and cytokeratin 7 (CK7) but not for cytokeratin 20 (CK20) (Fig. 1d,e). These immunohistochemical findings were almost identical to those observed for adenocarcinoma, which is a typical primary lung cancer of the peripheral lung. We diagnosed the tumour as CMPT. After surgery, the patient remained relapse-free for 10 months.

2.2. Case 2

Following a medical check-up, a 59-year-old woman with no previous medical and smoking history was referred to our institution for further examination of GGO in the right S9 segment, which was revealed by chest CT. We suspected the lesion to be a benign lung tumour and placed the patient under observation. Follow-up CT six months later revealed a tumour 7 mm in diameter with a central cavity (Fig. 1f). We suspected primary lung cancer and performed video-assisted right S9 partial resection. Analysis of the frozen sections revealed a papillary tumour with a mixture of ciliated epithelial and goblet cells, similar to case 1. Based on these findings, we suspected CMPT and performed partial resection. Light microscopic and immunohistochemical findings were also similar to case 1 (papillary tumour
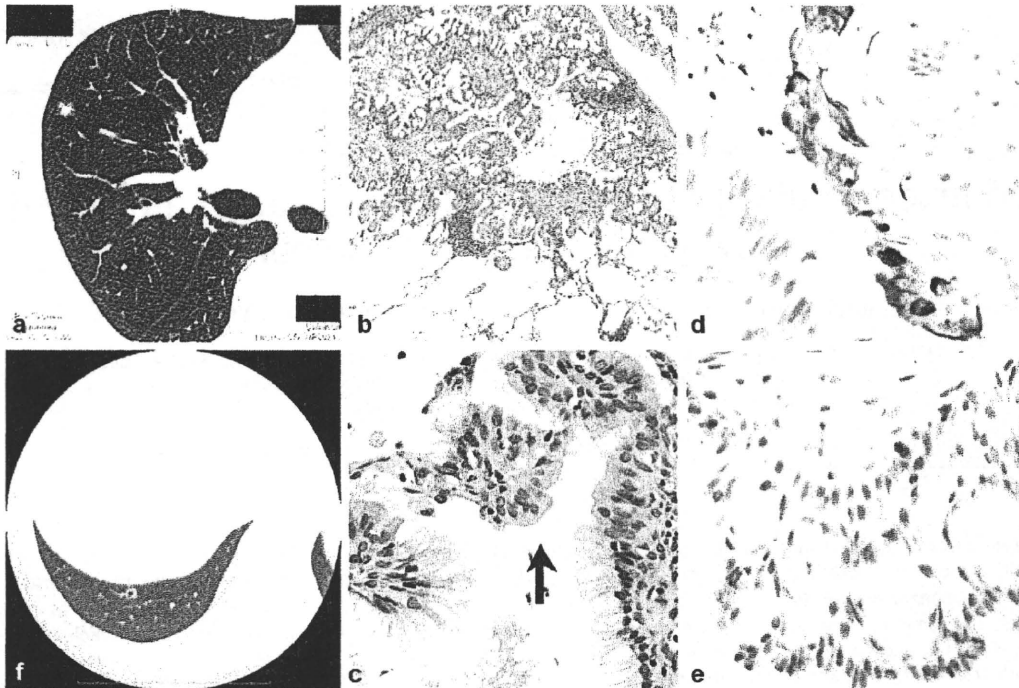
Fig. 1. Computed tomographic and histopathological findings. (a) CT findings of case 1 after 16 months. The detected tumour shows a high-density nodule with surrounding GGO in the right S3 segment. (b) Low-power histological view of case 1 showing papillary findings with mucous. (c) High-power histological view of case 1 showing a mixture of ciliated columnar, goblet and basal cells (arrow: cilia). (d, e) Immunohistochemical staining of case 1 showing CEA-positive (Fig. 1d) and TTF-1-positive cells (Fig. 1e). (f) CT findings of case 2 after seven months. The detected tumour shows cavity-like area in the centre with surrounding GGO in the right S9 segment. CT, computed tomography; GGO, ground glass opacity; CEA, carcinoembryonic antigen; TTF-1, thyroid transcription factor-1.

with ciliated columnar and goblet cells, mild nuclear atypia, lack of mitotic activity and positive staining for CEA, TTF-1 and CK7 but not for CK20). Therefore, this tumour was also confirmed to be CMPT. There was no local and distant failure 18 months after surgery.

3. Discussion

CMPT is a papillary tumour with cilia occurring in the peripheral lung. It is a rare tumour, with few reported cases [3, 4]. The tumour is not classified according to the 2004 fourth edition of the World Health Organization classification pathology and genetics of tumors of the lung, pleura, thymus, and heart [5]. Lung tumours with cilia are rare and are considered benign. Most of them occur in the central airway [1, 2, 6]. A unique feature of CPMT, besides developing in the peripheral lung, is the presence of columnar ciliated epithelial cells. Additionally, although morphological findings revealed few malignant characteristics, immunohistochemical findings suggested it to be malignant. The two cases reported here have mild nuclear atypia and no mitotic activity. Immunostaining was positive for CEA, TTF-1 and CK7 and negative for CK20, and these findings were similar to those observed in case of peripheral lung adenocarcinoma.

The differential diagnoses were (i) hyperplasia, (ii) papilloma and (iii) extremely well-differentiated adenocarcinoma. Hyperplasia is unlikely in CPMT because histology of

Table 1. Summary of clinical and immunohistochemical findings of previously reported and present cases of CMPT

Author	Age/gender	smk	Location	CT finding		Preoperative diagnosis	Size (mm)	Immunohistochemical findings							rec	prg
				GGO	Central density			CEA	TTF-1	Ki-67	CK7	CK20	MUC1	MUC5AC		
Ishikawa [3]	50 F	+	RUL	n/a	n/a	+ (Pappilloma)	15	+	n/a	n/a	n/a	n/a	n/a	n/a	−	10 years alive
Harada [4]	62 M	+	LLL	N	High	−	9	+	−	+	+	−	n/a	−	−	Two years alive
Present case	67 M	+	RLL	Y (margin)	High	−	8	+	+	+ (10%)	+	−	+	+	−	10 months alive
Present case	59 F	−	RUL	Y (pure)	Low	−	5	+	+	+ (3%)	+	−	+	−	−	One year Six months alive

CMPT, ciliated muconodular papillary tumour; CT, computed tomography; smk, smoking; GGO, ground glass opacity; CEA, carcinoembryonic antigen; TTF-1, thyroid transcription factor-1; CK, cytokeratin; MUC, mucin; rec, recurrence; prg, prognosis; n/a, not applicable; RUL, right upper lobe; RLL, right lower lobe; LLL, left lower lobe.

the background lung was normal. Papilloma, particularly solitary glandular papilloma, is a rare and benign lung tumour that generally occurs in the central airway [6]. Recently, Aida et al. reported solitary glandular papilloma of the peripheral lung [7]. Although light microscopy revealed that glandular papilloma is most similar to CMPT, immunohistochemical details of malignant characteristics were unclear, and the authors evaluated the glandular papilloma to be benign. Nakamura et al. reported an extremely well-differentiated adenocarcinoma with ciliated epithelial and goblet cells [8]. However, nuclear atypia and mitosis were prominent in this adenocarcinoma.

There are four reported cases of CMPT including the two cases presented here [3, 4]. The clinical and immunohistochemical features are shown in Table 1. The incidence rate has not been described previously. At our institution, only two (0.05%) among 4200 cases of lung cancer or suspected lung cancer have been diagnosed as CMPT in the past 20 years. In all four cases, the patients were in their 50s and 60s. Additionally, the tumour might occur in any pulmonary lobe, and there was no correlation of tumour occurrence with smoking. CT findings revealed pure GGO to high-density depending on the amount of mucous and fibrosis. Preoperative pathological diagnosis was not available because the tumour was small and located in the peripheral lung region, except in one case diagnosed with papilloma, where the tumour had spread to the subsegmental bronchus [4]. Immunohistochemically, all cases were positive for CEA. The immunostaining findings of these cases, including our cases, were almost similar to the staining pattern of adenocarcinoma, which is a representative lung cancer of the peripheral lung. All reported cases survived without recurrence. Based on these clinical and pathological observations (light microscopy and immunohistochemistry), we consider that CMPT is a newly defined well-differentiated pulmonary tumour with malignant potential, although it has ciliated epithelial cells.

Malignant tumours with cilia have been reported in other organs [9, 10]. Ciliated carcinoma is a variant of endometrial carcinoma and a representative malignant tumour with cilia. This tumour is also a low-grade malignant tumour with a good prognosis; however, careful distinction from benign lesions must be made because of the presence of myometrial or lymphatic invasion [10].

For practical purposes, differential diagnosis of CMPT from peripheral lung cancer is important. Surgical intervention, in particular, would be necessary to distinguish CMPT from mucous-rich bronchioloalveolar carcinoma showing high-density on CT. CMPT should be considered in cases where ciliated tumour cells are recognized in frozen sections.

Based on previous reports and our observations, for tumours with low-grade malignant features and no recurrences, partial resection with wide tumour-free margins seem an appropriate treatment. We have reported two cases of CMPT. Further investigation is required to clearly determine the clinical and pathological features of this tumour.

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doi:10.1016/j.ijrobp.2009.03.069

THE IMPACT OF RADIATION DOSE AND FRACTIONATION ON OUTCOMES FOR LIMITED-STAGE SMALL-CELL LUNG CANCER

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Purpose: To review the treatment outcomes of limited-stage small-cell lung cancer (LS-SCLC) patients and to compare the outcomes among three groups in which the total radiation doses were 45 Gy with accelerated hyperfractionation (AHF), <54 Gy with standard fractionation (SF), and ≥54 Gy with SF.
Methods and Materials: LS-SCLC patients that had been treated with chemoradiotherapy between 1997 and 2007 at Aichi Cancer Center Hospital were reviewed in this study. Of the 127 eligible patients, there were 37 patients in the AHF group, 29 in the SF <54 Gy group, and 61 in the SF ≥54 Gy group.
Results: Fifty-five patients (43%) were alive at the time of this analysis, and the median follow-up time of the surviving patients was 33 months. The median survival times were 30.0 months (95% confidence interval [CI] 16.3–43.7) for the AHF group, 14.0 months (CI 6.6–21.4) for the SF <54 Gy group, and 41.0 months (CI 33.9–48.1) for the SF ≥ 54 Gy group. As for the local control rates, and the overall and progression-free survival rates, all outcomes were significantly lower in the SF <54 Gy group than in the other two groups, although no significant difference was found between the AHF and SF ≥54 Gy groups.
Conclusions: These results suggest the importance of a high dose of radiation when using once-daily regimen. This study will support future prospective studies to establish optimal radiation doses and fractionation. © 2010 Elsevier Inc.

Small-cell lung cancer, Radiation therapy, Radiation dose, Fractionation, Accelerated hyperfractionation.

INTRODUCTION

Chemoradiotherapy is currently the standard treatment for limited-stage small-cell lung cancer (LS-SCLC) (1). Although thoracic radiotherapy (TRT) has been established as an integral component of the treatment platform for LS-SCLC, some questions regarding the optimal radiotherapy approach have also arisen. With regard to fractionation, Turrisi *et al.* determined that accelerated hyperfractionation (AHF) is superior to standard fractionation (SF) in an Intergroup Phase III study (2). However, despite the significant improvement in long-term survival, a pattern of care study found that only 10% of patients with LS-SCLC received a twice-daily regimen because of the inconvenience of twice-daily treatment sessions and the increased rate of severe esophageal toxicity seen with this regimen, whereas more than 80% received once-daily TRT (3). Although traditionally modest doses of TRT (45–50 Gy) are often used in once-daily 1.8- to 2-Gy fractions (4, 5), the optimal total dose for a once-daily regimen has not been proven. In addition, it is also still unclear whether twice-daily TRT of

45 Gy in 3 weeks is superior to a higher total dose than traditional modest doses delivered with a once-daily regimen. In this study, we reviewed the treatment outcomes of LS-SCLC patients that were treated with chemoradiotherapy at Aichi Cancer Center Hospital and compared the outcomes among three groups in which the total radiation doses were 45 Gy with a twice-daily regimen, less than 54 Gy with a once-daily regimen, and equal or greater than 54 Gy with a once-daily regimen.

METHODS AND MATERIALS

Patient selection

LS-SCLC patients that had been treated with chemoradiotherapy between 1997 and 2007 at Aichi Cancer Center Hospital and who met the eligibility criteria were enrolled into this retrospective study. The diagnosis of SCLC was confirmed by histologic or cytologic findings in all cases. Limited-stage was defined as disease confined to one hemithorax with or without bilateral supraclavicular node metastasis. The eligibility criteria consisted of no previous treatment and an Eastern Cooperative Oncology Group performance status

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of 0–2. Additional eligibility criteria were as follows: they had not undergone surgery for LS-SCLC nor had been treated with a radiation field, not including elective nodal irradiation, because the significance of omitting elective nodal irradiation remains unclear (6). Written informed consent was obtained from all patients before treatment. Each patient underwent the following studies: chest radiography and fiberoptic bronchoscopy, complete blood count and biochemical tests, a computed tomography (CT) scan of the thorax and abdomen, a CT scan or magnetic resonance imaging of the brain, and a radionuclide bone scan, or positron emission tomography. Positron emission tomography was used for a few patients (7%) who were treated after 2001 according to the physician's preference. Bone marrow aspiration or biopsy was performed in cases of neutropenia and thrombocytopenia.

Radiation therapy technique

TRT was carried out with linear accelerators, and the energy of 6–10 MV photons was used. The TRT fields were changed from anteroposterior-posteroanterior fields to parallel opposed oblique fields after 30 Gy in the twice-daily regimen and 36–40 Gy in the once-daily regimen. Most patients (83%) that were eligible for this study were treated using conventional fluoroscopic simulation techniques at the start of the TRT, and CT simulation techniques were used only for the planning of the boost fields. The other 17% were treated using CT simulation techniques throughout the entire TRT. The other planning techniques were similar to those in our previous report on non–small-cell lung cancer (7). TRT was administered twice daily (1.5 Gy per fraction, with a 6 h or more interval between fractions) for a total dose of 45 Gy in 3 weeks or once-daily (1.8–2.0 Gy per fraction) for a total dose of 39.6–66 Gy in 4–7 weeks. After the TRT, prophylactic cranial irradiation (PCI) was administered to the patients who had a complete or near-complete response (10). The PCI consisted of 24 Gy in 2 Gy per fractions or 25 Gy in 2.5 Gy per fractions once daily, 5 days per week.

All patients who entered the clinical trial were treated with the AHF regimen. However, there were no adequate rationale for a decision about a patient's TRT dose and fractionation. The TRT dose and fractionation was decided according to the physician's preference.

Chemotherapy

In principle, the patients were treated with four cycles of chemotherapy and received at least one cycle of chemotherapy concurrent with TRT. The chemotherapy was given in a 28-day cycle in the concurrent phase and a 21-day cycle in the sequential phase. The most commonly used regimens were cisplatin/etoposide, carboplatinum/etoposide, and cisplatin/irinotecan. As a general rule, the cisplatin/etoposide regimen consisted of cisplatin (80 mg/m^2 intravenously) on day 1 and etoposide (100 mg/m^2 intravenously) on Days 1, 2, and 3. The carboplatinum/etoposide regimen consisted of carboplatinum (area under the blood concentration-time curve: 5 intravenously) on Day 1 and etoposide (100 mg/m^2 intravenously) on Days 1, 2, and 3. The cisplatin/irinotecan regimen was only performed sequentially with TRT and consisted of cisplatin (80 mg/m^2 intravenously) on Day 1 and irinotecan (60 mg/m^2 intravenously) on Days 1, 8, and 15.

Study design and statistical analysis

All available radiation records and charts were reviewed to assess patient and tumor characteristics and the details of treatment and outcome. Tumor response was classified in accordance with the

Response Evaluation Criteria in Solid Tumors criteria (9). Complications were graded in accordance with the National Cancer Institute's Common Toxicity Criteria, version 3.0 (10). The date of the last follow-up was defined as the last recorded information available for the patient. Only 3 patients were lost to follow-up. Survival was measured from the start date of any treatment to the date of the last follow-up or death from any cause. Local failure, defined as locoregional progression on CT (including the primary tumor and the bilateral mediastinal and ipsilateral hilar lymph nodes), was measured from the start date of any treatment to the date of the first evidence of locoregional disease progression. Concurrent local and distant failures were scored as local failures for the first failure sites. Progression-free survival was measured from the start date of any treatment until the date of local or distant failure.

Overall survival (OS), overall local, and overall progression-free survival were calculated using Kaplan-Meier estimates. Subgroup analysis was used to compare the outcomes among the three groups, in which the total radiation doses were 45 Gy with AHF, <54 Gy with SF, and ≥54 Gy with SF, using the log–rank test. Moreover, sex, age at diagnosis, performance status, disease stage (I, II, vs. III), PCI (yes vs. no), total chemotherapy cycles (<3 vs. ≥3), concurrent chemotherapy (yes vs. no), and the duration of TRT (<40 days vs. ≥40 days) were also assessed for their impact on OS using the log–rank test. Fisher's exact test was used for comparisons of categorical data. Cox's proportional hazards model was used for multivariate analysis. $p < 0.05$ was considered significant.

RESULTS

Patient and treatment characteristics

A total of 127 patients were enrolled into the study. The median total dose of TRT with the once-daily regimen was 54 Gy; therefore, we divided the patients that had been treated with the once-daily regimen into two groups using the median total dose of 54 Gy for the subgroup analysis. The characteristics of the 127 eligible patients are shown in Table 1. Fifteen patients (40%) from the AHF group entered a clinical trial, but no patients from the other two groups did. The baseline characteristics were balanced in terms of sex, performance status, stage, and chemotherapy cycles. However, there was a slight imbalance in age; the patients in the AHF group tended to be younger than those in the other two groups, and the rate of patients older than age 75 years was lower than in the other two groups, but these differences were not significant ($p = 0.15$). There were significant differences in the rate of patients that received concurrent chemotherapy and PCI among the three groups ($p = 0.012$, $p < 0.001$, respectively). Fifty-five (43%) patients were alive at the time of this analysis, and the median follow-up time of the surviving patients was 33 months (range, 2–118 months). The median follow-up time of the surviving patients was 34 months (range, 16–96 months) for the AHF group, 67 months (range, 12–91 months) for the SF <54 Gy group, and 22 months (range, 2–118 months) for the SF ≥54 Gy group. There were no significant differences in the median follow-up time of the surviving patients among the three groups ($p = 0.32$).

As a result, 84% received four or more cycles of chemotherapy. Eight percent received three cycles, and 8% received less than two cycles either because the patient refused continuation

Table 1. Patient and tumor pretreatment characteristics

	Prescription group			
Characteristic	AHF group (*n* = 37)	SF <54 Gy group (*n* = 29)	SF ≥54 Gy group (*n* = 61)	*p* value*
Age (y)	58 (40–68)	70 (51–82)	66 (29–81)	
≥75 (%)	0 (0%)	3 (10%)	5 (8%)	0.15
Sex (%)				0.59
Male	30 (81%)	25 (86%)	54 (82%)	
Female	7 (19%)	4 (14%)	7 (18%)	
Performance status				0.29
0	13 (35%)	7 (25%)	20 (33%)	
1	24 (65%)	20 (68%)	36 (59%)	
2	0 (0%)	2 (7%)	5 (8%)	
Stage				0.20
I	0 (0%)	1 (3%)	6 (10%)	
II	4 (11%)	0 (0%)	4 (7%)	
IIIA	22 (59%)	11 (38%)	24 (39%)	
IIIB	11 (30%)	17 (59%)	27 (44%)	
CHT cycles	3.9 (2–5)	3.7 (1–6)	3.9 (1–6)	0.72
≥3 cycles	33 (89%)	27 (93%)	56 (92%)	
Concurrent CHT	37 (100%)	23 (79%)	56 (92%)	0.012*
Total dose (Gy)	45 (45)	50 (39.6–52.2)	56 (54–63)	<0.001*
Duration of TRT (days)	21 (19–27)	41 (30–56)	43 (36–59)	<0.001*
PCI	24 (65%)	6 (21%)	18 (30%)	< 0.001*

Abbreviations: AHF = accelerated hyperfractionation; SF = standard fractionation; CHT = chemotherapy; TRT = thoracic radiotherapy; PCI = prophylactic cranial irradiation.

Age and total dose data are presented as the median value. CHT cycles data are presented as the mean value. The numbers in square brackets indicate the range of age and CHT cycles.

* Fisher's exact test.

of the chemotherapy or their leukocyte or platelet counts or renal function did not return to levels at which chemotherapy could be performed. Most patients (91%) received at least one cycle of concurrent chemotherapy with TRT, whereas the remaining 9% only received sequential chemotherapy because the radiation field sizes of these patients were too large as the primary tumor was located in the inferior lobe or the primary tumor was so bulky that concurrent chemoradiotherapy was considered to carry a high risk of severe radiation pneumonitis (11). All patients received at least one cycle of platinum-based agents/etoposide regimen regardless of the TRT regimen. The cisplatin/irinotecan regimen was only performed sequentially with

TRT for 24% of patients in the AHF group and 16% of the SF ≥54 Gy group.

Tumor response

Table 2 shows the tumor response in each group. The overall response rate was 94% (95% confidence interval [CI] 91–98%, 58% complete response rate [CI 50–67%], and 36% partial response rate [CI 28–45%]) for all eligible patients. There was a significantly lower rate of complete response in the SF <54 Gy group than in the AHF and SF ≥54 Gy groups (*p* = 0.018 and 0.0062, respectively). There was a significantly higher rate of complete response in the AHF group than in the SF ≥54 Gy group (*p* = 0.042).

Table 2. Tumor response in each group

	Prescription group						
	AHF (*n* = 37)		SF <54 Gy (*n* = 29)		SF ≥54 Gy (*n* = 61)		
Response	No.	%	No.	%	No.	%	*p* value*
Overall response	34	92%	27	93%	59	97%	0.32
CR	27	73%	11	38%	36	59%	0.02*
PR	7	19%	16	55%	23	38%	
SD	0	0%	1	3%	2	3%	
PD	3	8%	1	3%	0	0%	

Abbreviations: AHF = accelerated hyperfractionation; SF = standard fractionation; CR = complete response; PR = partial response; SD = stable disease; PD = progressive disease.
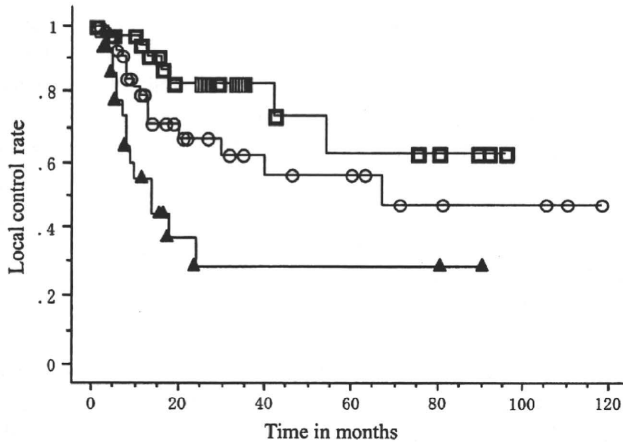
* Fisher's exact test.

Fig. 1. The local control rates for patients with a total dose of 45 Gy with accelerated hyperfractionation (□), <54 Gy with standard fractionation (▲), and ≥54 Gy with standard fractionation (○).

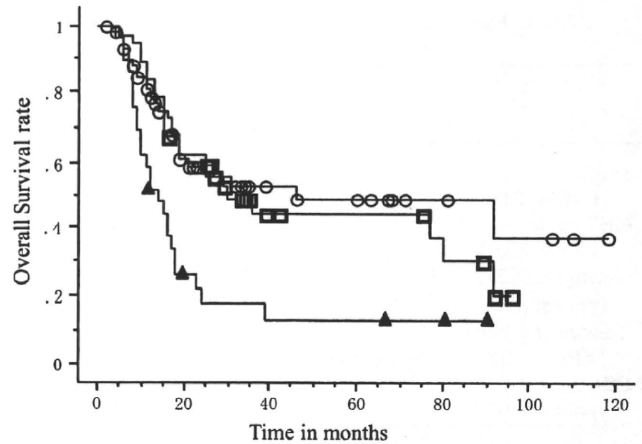Fig. 2. Overall survival for patients with a total dose of 45 Gy with accelerated hyperfractionation (□), <54 Gy with standard fractionation (▲), and ≥54 Gy with standard fractionation (○).

Local control and progression-free survival

Figure 1 shows the local control rates for each group. The 3-year local control rates were 61.1% (CI 50.3–71.9%) for all eligible patients, 81.3% (CI 67.2–95.5%) for the AHF group, 27.7% (CI 5.0–50.4%) for the SF <54 Gy group, and 61.2% (CI 44.8–77.6%) for the SF ≥54 Gy group. The local control rate was also significantly lower for the SF <54 Gy group than the AHF and SF ≥54 Gy groups ($p = 0.0016$ and 0.011, respectively). Local control for the AHF group tended to be superior to that for the SF ≥54 Gy group, although no statistically significant difference was found ($p = 0.096$).

The 3-year progression-free survival rates were 28.1% (CI 19.5–36.7%) for all eligible patients, 37.5% (CI 21.5–53.5%) for the AHF group, 7.5% (CI 0–17.5%) for the SF <54 Gy group, and 33.2% (CI 19.7–46.7%) for the SF ≥54 Gy group. Progression-free survival was also significantly lower for the SF <54 Gy group than for the AHF and SF ≥54 Gy groups ($p = 0.015$ and 0.013, respectively). Progression-free survival was similar in the AHF group and the SF ≥54 Gy group ($p = 0.80$).

Overall survival

Figure 2 shows the survival curves for each group. The median survival time of all eligible patients was 24.0 months (CI 18.1–29.9 months). The median survival times were 30.0 months (CI 16.3–43.7 months) for the AHF group, 14.0 months (CI 6.6–21.4 months) for the SF <54 Gy group, and 41.0 months (CI 33.9–48.1 months) for the SF ≥54 Gy group. The 3-year survival rates were 41.2% (CI 31.6–50.8%) for all eligible patients, 44.1% (CI 26.5–61.7%) for the AHF group, 13.8% (CI 0–27.3%) for the SF <54 Gy group and 53.1% (CI 38.6–67.6%) for the SF ≥54 Gy group. There was a significantly lower rate of OS in the SF <54 Gy group than in the AHF and SF ≥54 Gy groups ($p = 0.0018$ and 0.00036, respectively). OS for the SF ≥54 Gy group seemed to be slightly superior to that for the AHF group, although no statistically significant difference was found ($p = 0.64$).

Factors associated with overall survival

Table 3 shows the effects of patient characteristics, disease factors, and treatment parameters on OS according to univariate analysis. To evaluate further the independent effects of disease stage, chemotherapy cycles, concurrent chemotherapy, PCI,

Table 3. Factors associated with overall survival according to univariate analysis

Factors	No. of patients	3-year OS (95% CI)	p value
Sex			0.367
Male	109	40.8% (33.1–48.5)	
Female	18	41.8% (36.1–47.5)	
Age (y)			0.652
<75	119	40.6% (33.2–48.0)	
≥75	8	48.6% (43.6–53.6)	
PS			0.546
0, 1	120	42.0% (34.6–49.4)	
2	7	28.6% (23.6–33.6)	
Stage			0.016*
I, II	15	80.0% (78.3–81.7)	
III	112	35.7% (28.3–43.1)	
CHT cycle			0.033*
<3	11	43.4% (35.8–51.0)	
≥3	116	20.0% (14.2–25.8)	
Concurrent CHT			0.026*
Yes	116	43.7% (36.1–51.3)	
No	11	20.0% (17.3–22.7)	
Treatment group			<0.001*
AHF	37	44.1% (26.5–61.7)	
SF ≥54 Gy	29	53.1% (38.6–67.6)	
SF <54 Gy	61	13.8% (0–27.3)	
Duration of RT (days)			0.821
<40	66	41.3% (31.0–51.6)	
≥40	61	40.0% (29.8–50.2)	
PCI			0.089
Yes	48	48.1% (36.6–59.6)	
No	79	35.6% (31.6–39.6)	

Abbreviations: CI = confidence interval; PS = performance status; CHT = chemotherapy; NA = not applicable; AHF = accelerated hyperfractionation; SF = standard fractionation; RT = radiation therapy; PCI = prophylactic cranial irradiation.

  * Statistically significant.

Table 4. Factors associated with overall survival according
to multivariate analysis

Factors	Hazard ratio of death (95% CI)	*p* value
Stage (I, II vs. III)	0.24 (0.074–0.78)	0.017*
CHT cycle (<3 vs. ≥3)	0.50 (0.24–1.07)	0.073
Concurrent CHT (yes vs. no)	0.61 (0.29–1.31)	0.20
Treatment group (AHF vs. SF ≥54 Gy SF 54 Gy)	NA	0.033*
PCI (yes vs. no)	0.75 (0.43–1.31)	0.31

Abbreviations: CHT = chemotherapy; NA = not applicable; AHF = accelerated hyperfractionation; SF = standard fractionation.
* Statistically significant.

and treatment group on OS, a multivariate Cox proportional hazards regression analysis was performed. This analysis included those factors that had displayed a *p* value <0.10 in the univariate analysis. As a consequence, disease stage and treatment group remained significant factors in the multivariate analysis (Table 4).

Toxicity

Documentation concerning toxicity data was not available for 6 patients (2 in each group), which left 121 patients assessable for toxicity. Only late toxicity ≥Grade 2 was assessed from the available information of each chart. There were only 2 treatment-related deaths (one in the SF <54 Gy group and the other in the SF ≥54 Gy group). Both patients died of radiation pneumonitis. Five patients developed Grade 2 radiation pneumonitis, 4 in the SF <54 Gy group and 1 in the SF ≥54 Gy group. Apart from the toxicities described, no other information about late toxicity was noted in the charts.

DISCUSSION

In our study, the comparison of overall, progression-free, and local control survival rates and the rate of complete response suggested that TRT administered with a total dose of <54 Gy by once-daily regimen was more disadvantageous than TRT treated with a total dose of ≥54 Gy in a once-daily regimen or a total dose of 45 Gy administered using the AHF regimen, and the difference was statistically significant for all outcomes. These results clearly demonstrate that radiation intensification improves the complete response rate and local control and that improved local control translates into improved OS. Furthermore, these results also suggest the importance of a high dose of radiation when using a once-daily regimen.

Because SCLC has high radiation sensitivity (12), recent pattern of care studies have shown that the traditional modest doses of TRT that are used in once-daily 1.8- to 2-Gy fractions are also widely used for LS-SCLC in Japan and Turkey (4, 5). However, although response rates are high, local control rates have been poor in this TRT setting. Intensifying the radiotherapy effect by accelerating its delivery was one of the initial strategies explored in prospective trials. Turrisi *et al.* (2) randomly assigned 471 LS-SCLC patients to either 45 Gy in 5 weeks (1.8 Gy once-daily for 25 fractions) or 45 Gy in 3 weeks (1.5 Gy twice-daily for 30 fractions) beginning with the first of four cycles of PE. The 5-year survival rate was 26% with accelerated TRT compared with 16% for the conventional TRT (*p* = 0.04), and the accelerated TRT arm was also superior to conventional TRT in local tumor control (*p* = 0.06). These data strongly suggest that attempts designed at improving local tumor control can favorably impact on the long-term outcome of patients with LS-SCLC. However, despite the significant improvement in long-term survival, only 10% of patients with LS-SCLC received a twice-daily regimen (3). Moreover, a second trial performed by the North Central Cancer Treatment Group reported negative results with a twice-daily regimen, although overall treatment times and total radiation doses were identical in each arm of the North Central Cancer Treatment Group trial (13). Therefore, different strategies were considered that might increase the local control rate with chemoradiotherapy. Accelerated fractionation via the concomitant boost technique uses once-daily irradiation early in the course of treatment and then twice-daily irradiation toward the end. Komaki *et al.* (14) reported a Phase 1 study (Radiation Therapy Oncology Group 97-12) using this regimen to improve local control by increasing the dose of TRT given with concurrent cisplatin/etoposide without causing acute severe esophagitis. They found that 61.2 Gy was the maximum tolerated dose, and there was a suggestion of improvement in the estimated short-term survival rate (18 months) by dose escalation from 50.4 Gy to 61.2 Gy (25% and 82%, respectively). Roof *et al.* (15) also showed a clear dose–response curve between 54 and 63 Gy with a once-daily regimen, although they did not find a significant difference in outcome because of their small sample size of 54 patients.

Obviously, there are problems that limit the interpretation of a single institutional retrospective review. We recognize the imbalance among our three groups. The rates of patients receiving PCI and concurrent chemotherapy were significantly different among the three groups (Table 1), although the multivariate analysis proved that these factors were not associated with OS. Another difficulty associated with retrospective reviews is the accurate assessment of toxicity. The rate of Grade 2-4 toxicities in our study was lower than those reported elsewhere. Although the patient charts were thoroughly scrutinized, the documentation concerning complications may not have been as thorough as it would have been in a prospective trial.

Whether twice-daily TRT to 45 Gy in 3 weeks is superior to a higher total dose than traditional modest doses delivered with a once-daily regimen is still unclear. Our results did not find a significant difference in outcome between the AHF group with a total dose of 45 Gy and the SF group with a total dose of ≥54 Gy. However, there was a significantly higher

rate of complete response in the AHF group compared with the SF ≥54 Gy group ($p = 0.042$), and the local control for the AHF group tended to be superior to that for the SF ≥54 Gy group. These results indicate that in the once-daily regimen much more than 54 Gy is necessary to achieve local control at the same level as 45 Gy with the AHF regimen. Despite the significantly higher rate of complete response and local control in the AHF group compared with the SF ≥ 54 Gy group, progression-free survival and OS were similar between the AHF group and the SF ≥54 Gy group ($p = 0.80$ and 0.64, respectively). We think that the reason was our small sample size. On the other hand, these data indicated that patients in the AHF group died from systemic disease as did those in the SF ≥54 Gy group, despite the better local control in the AHF group compared with the SF ≥54 Gy group. Therefore, another chemotherapy strategy such as integrating newer chemotherapy agents (16) or dose-intense regimens using either growth factors or stem-cell support (17) may be necessary for the platform of the curative approach to LS-SCLC.

Further intensification of TRT regimens such as a Phase III trial is under development by the Cancer and Leukemia Group B and the Radiation Therapy Oncology Group (18). This randomized trial is designed to compare three TRT approaches with four cycles of PE, with the three regimens being 45 Gy in 3 weeks (1.5 Gy twice-daily for 30 fractions), 70 Gy in 7 weeks (2.0 Gy once-daily for 35 fractions), and 61.2 Gy in 5 weeks (1.8 Gy accelerated fractionation via concomitant boost). We think that this trial will demonstrate the optimal method of radiation dose intensification. However, at this time, 45 Gy twice-daily TRT should be considered as the standard treatment for LS-SCLC because there are no Phase III once-daily trials that have shown better outcomes than the twice-daily regimen.

In conclusion, this analysis suggests that disease stage and treatment groups that are stratified according to 45 Gy with AHF, <54 Gy with SF, and ≥54 Gy with SF are independent factors associated with improved OS in patients with LS-SCLC and the potential importance of a high dose of radiation when using a once-daily regimen. However, there are problems that limit the interpretation of our single institutional retrospective review. There were some prognostic differences in the three groups compared, especially in the rates of patients receiving PCI and concurrent chemotherapy. A future prospective study of TRT regimens in the setting of chemoradiotherapy for LS-SCLC is needed to establish optimal radiation doses and fractionation, and such a study is under development by the Cancer and Leukemia Group B and Radiation Therapy Oncology Group.

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ORIGINAL ARTICLE

Relationship of mRNA expressions of RanBP2 and topoisomerase II isoforms to cytotoxicity of amrubicin in human lung cancer cell lines

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Abstract

Purpose RanBP2 is a small ubiquitin-like modifier ligase for DNA topoisomerase II (TopoII) and plays a role in maintaining chromosome stability by recruiting TopoII to centromeres during mitosis. Engineered-mice with low amounts of RanBP2 have been reported to form lung adenocarcinomas. Furthermore, in the murine embryonic fibroblasts, formation of chromatin bridges in anaphase, a distinctive feature of cells with impaired DNA decatenation by chemical inhibition of TopoII, has been reported. In this study, we tested whether the association between mRNA expression of the RanBP2 gene and chemosensitivity of a TopoII inhibitor, amrubicin could be seen.

Methods Using a panel of 20 lung cancer cell lines, the mRNA expression levels of the RanBP2, TopoII-alpha and TopoII-beta genes were examined by quantitative real-time reverse transcription PCR. The in vitro cytotoxicity of amrubicin was assessed using a tetrazolium-based colorimetric assay (MTT assay).

Results Although RanBP2 mRNA expression was infrequently downregulated in human lung cancer cell lines, significantly higher RanBP2 transcripts were observed in small cell lung cancer than non-small cell lung cancer. There were no correlations between chemosensitivity of amrubicin and mRNA expression levels of the RanBP2, TopoII-alpha and TopoII-beta genes.

Conclusions Our in vitro results suggest that mRNA expressions of RanBP2 and TopoII isoforms are unlikely to be a predictive biomarker for the sensitivity to amrubicin.

Keywords SUMO ligase · Topoisomerase II inhibitor · Predictive biomarker · Chromosomal instability · Lung cancer

Introduction

Lung cancer is a leading cause of cancer mortality in the United States and in Japan [13, 29]. Lung cancer has two main types: small cell lung cancer (SCLC) and non-SCLC (NSCLC). The major histological subtypes of NSCLC include adenocarcinoma, squamous carcinoma and large cell carcinoma. About 15% of lung cancers are SCLC. SCLC spreads rapidly and widely forming additional large tumors in lymph nodes, bones, adrenal glands, liver and brain. Because of its aggressive nature the overall survival of SCLC is worse than that of NSCLC and only 5–10% at 5 years. Survival of patients with either SCLC or NSCLC is strongly correlated with the stage of disease. For patients with advanced tumors, the prognosis is dismal because the available treatment regimens such as chemotherapy and radiation therapy are essentially palliative and primarily serve only to prolong survival. In fact, combination chemotherapy with etoposide plus cisplatin or irinotecan plus cisplatin for extensive-stage (ES) SCLC as well as the common first-line platinum-based combination regimens for advanced NSCLC only produced a median survival time of about 1 year [18, 19, 23]. Thus, new treatment approaches are clearly required.

Amrubicin, developed and approved in Japan for the treatment of SCLC and NSCLC, is a totally synthetic

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anthracycline anticancer agent and a potent TopoII inhibitor [14]. Amrubicin monotherapy with 45 mg/(m^2 day) for 3 consecutive days by intravenous administration produced response rates of 75.8 and 27.9% for previously untreated patients with ES-SCLC and advanced NSCLC, respectively. A phase II study of the combination of 60 mg/m^2 cisplatin and 40 mg/(m^2 day) amrubicin for 3 days has been reported to show response rate of 87.8%, the MST of 13.6 months and 1-year survival rate of 56.1% against ES-SCLC. Based on this result, Japan Clinical Oncology Group (JCOG) is conducting a randomized phase III study to compare the combinations of cisplatin plus amrubicin and cisplatin plus irinotecan for previously untreated ES-SCLC.

To improve clinical outcomes in advanced lung cancer, clinical integration of molecular biomarkers that predict responses to chemotherapeutic agents may be indispensable [16]. Recently, RanBP2 has been reported to act as a small ubiquitin-like modifier (SUMO) ligase for DNA TopoII and play an important role in targeting TopoII to centromeres during mitosis and in maintaining chromosome stability [5]. Embryonic fibroblasts derived from the engineered mutant mice with low expression of RanBP2 have been reported to show formation of chromatin bridges in anaphase, a distinctive feature of cells with impaired DNA decatenation by chemical inhibition of TopoII [4], suggesting that low expression of RanBP2 may have an analogous effect of TopoII inhibitors. In addition, RanBP2 has a tumor suppressor function since these mutant mice succumbed to a range of cancers, primarily lung carcinomas, and were also susceptible to chemically-induced tumorigenesis. Based on these observations, we hypothesized that RanBP2 expression might be involved in chemosensitivity of a TopoII inhibitor, amrubicin.

The identification of molecular biomarkers with the potential to predict treatment outcomes is essential for individualizing the most beneficial chemotherapy. As one of the multiple approaches to establishing predictive biomarkers, we evaluated whether there would be associations between mRNA expression of the RanBP2 gene as well as the TopoII-alpha and beta genes and chemosensitivity to amrubicin using human lung cancer cell lines.

Materials and methods

Cell lines and drug

Fifteen NSCLC and five SCLC cell lines used were described previously [24]. These cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum. Amrubicin was kindly provided by Sumitomo pharmaceuticals Company, Osaka, Japan.

Cytotoxicity assay

Cytotoxicity was evaluated using an MTT assay as described previously [11]. Suspensions of exponentially growing cells were dispensed into wells of 96-well tissue-culture plates. After incubation at 37°C for 24 h, the solutions of amrubicin at various concentrations were added, and then incubated for 3 days. The effects of treatment were expressed as percent growth inhibition using untreated cells as the uninhibited control and assessed by IC50 (drug concentrations inducing a 50% reduction of cell survival) which was calculated from dose–response curves.

RNA preparation and RT-PCR amplification

Total RNA was extracted and further purified as described previously [24]. The RNAs were stored at −80°C until use. Total RNA (50 ng) extracted from each cell line was subjected to one-step real-time reverse transcriptase-PCR (RT-PCR) for absolute quantitating mRNA levels of the RanBP2, TopoII-alpha, TopoII-beta and beta-actin genes as described previously. The PCR primers used were as follows.

RanBP2-S:	5′-CAATGGAAATGGGGAAGACTTT-3′
-AS:	5′-CATCACTTCAGTCCCACCTGTA-3′
TopoII-alpha-S:	5′-GGTGTGGAACTAGAAGGCCTAA-3′
-AS:	5′-TGAATCAGACCAGGGATTTCTC-3′
TopoII-beta-S:	5′-TTTTTCACCATCATTTGGTCTG-3′
-AS:	5′-GGGCTTAGGGACTGTATCTGAA-3′
Beta actin-S:	5′-TTCTACAATGAGCTGCGTGTGT-3′
-AS:	5′-CAGCCTGGATAGCAACGTACA-3′

Linear regression analysis of standard-curves demonstrated a strong correlation for all the genes ($R^2 > 0.99$). The relative gene expression levels were normalized with a house keeping gene, beta-actin.

Western blot analysis

Western blot analysis was done as described previously [11], using the following primary antibodies: anti-RanBP2 (ab2938, Abcam), anti-TopoII-alpha (ab45175, Abcam), anti-TopoII-beta (ab58442, Abcam) and anti-actin (A2268, Sigma-Aldrich) antibodies.

Statistical analyses

The strength of the association between the expression levels of RanBP2, TopoII-alpha and TopoII-beta and chemosensitivity data was calculated by either Pearson's correlation coefficient or linear regression analysis. Correlations were considered significant at $P < 0.05$. For comparison of IC50 values of amrubicin and each gene expression level among histological subtypes, we employed one-way

analysis of variance (ANOVA) followed by Bonferroni post-test. All analysis was performed with the use of Stat View software version 5.0.

Results

Chemosensitivity of amrubicin was examined using 20 human lung cancer cell lines including 15 NSCLC cells and 5 SCLC cells. Cytotoxicity following a 72 h continuous exposure of amrubicin was measured by MTT assay. The IC 50 value of amrubicin in SK-LC-3 was about 9 μM, while the IC 50 values in the other cell lines were less than 1 μM as shown in Table 1. There was no significant difference between histological types (Fig. 1).

The mRNA quantifications of the RanBP2, TopoII-alpha, TopoII-beta genes were carried out in real-time PCR and the expression levels were normalized with beta-actin as an internal control (Table 1). Among 20 cell lines tested, the level of RanBP2 mRNA expression in an H460 cell line was about 20-fold lower than those in non-tumorous lung tissues obtained from two patients with lung cancer. There were statistically significant differences in the RanBP2 expression between SCLC and the other histological subtypes ($p < 0.05$) (Fig. 2a). We checked RanBP2 protein expression in two lung cancer cell lines, SK-LC-2 and H460, representing high and low expression of the RanBP2
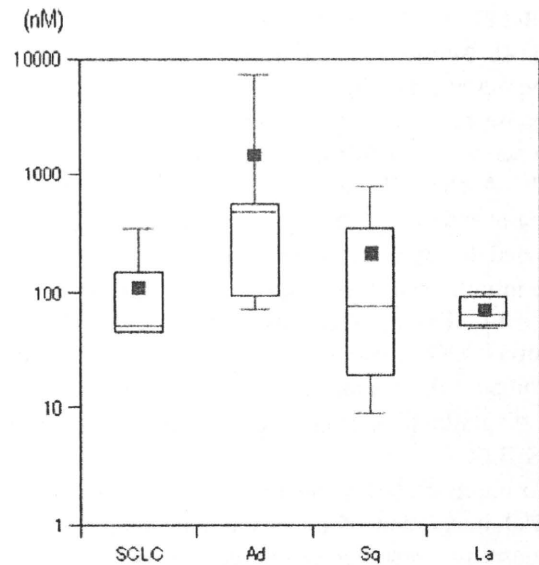

Fig. 1 IC50 values of Amrubicin in lung cancer cell lines. *Box plots* show relationships between IC50 values of Amrubicin and the four histological subtypes of lung cancer. The *horizontal line* within each *box* represents the median value and the closed box shows the mean value, respectively

gene, and found similar mRNA and protein expression patterns (Fig. 2d). We also found statistically higher expression levels of TopoII-alpha in SCLC and adenocarcinoma cell lines compared with those in normal lung tissues,

Table 1 IC50 values for amrubicin and relative mRNA expression for RanBP2, TopoII alpha and TopoII beta in lung cancer cell lines

Cell line	Histology	Amrubicin (μM)	RanBP2	TopoIIa	TopoIIb
ACC-LC-94	Ad	0.0668	0.621	1.324	1.174
ACC-LC-319	Ad	0.579	1.108	1.682	1.437
SK-LC-3	Ad	8.99	2.634	2.009	1.899
A549	Ad	0.131	1.191	1.553	1.665
SK-LU-1	Ad	0.492	1.307	2.930	1.479
VMRC-LCD	Ad	0.0835	4.134	2.660	2.942
RERF-LC-MT	Ad	0.469	0.661	0.8719	0.753
Calu1	Sq	0.203	1.280	2.173	1.750
SK-MES-1	Sq	0.0768	1.160	0.883	0.807
PC-1	Sq	0.009	1.937	1.739	2.888
RERF-LC-A	Sq	0.0222	0.717	1.454	1.036
PC-10	Sq	0.77	0.713	1.049	1.170
NCI-H460	La	0.101	0.043	1.518	2.098
Calu6	La	0.0469	1.467	1.116	1.828
SK-LC-6	La	0.0632	2.362	2.508	4.383
ACC-LC-48	SCLC	0.0512	1.957	1.672	2.044
ACC-LC-49	SCLC	0.0866	2.592	1.975	3.523
ACC-LC-80	SCLC	0.0459	3.953	1.361	1.993
ACC-LC-172	SCLC	0.0439	2.387	4.450	4.207
SK-LC-2	SCLC	0.337	3.662	3.510	4.264
NL 1	Normal lung	NA	1.006	0.158	2.447
NL 2	Normal lung	NA	0.913	0.179	1.937

NL 1 and NL 2: non-tumorous lung tissues obtained from two patients with lung cancer.
Ad adenocarcinoma,
La large cell carcinoma,
SCLC small cell lung cancer,
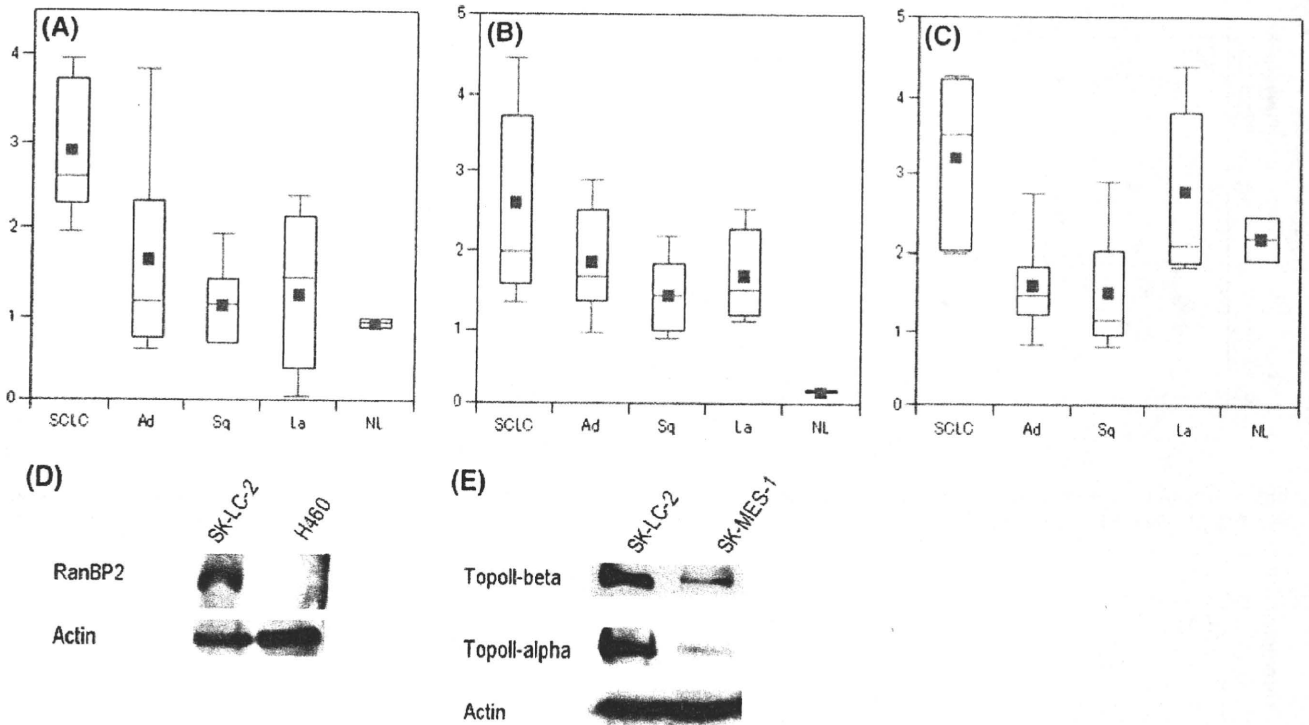*Sq* squamous cell carcinoma.

NA not available

Fig. 2 Relative mRNA expression for (a) RanBP2, (b) TopoII-alpha and (c) TopoII-beta among histological subtypes and normal lung tissues, and protein expression for (d) RanBP2 and (e) TopoII isoforms in representative cell lines. a RanBP2 mRNA expression in SCLC was higher than those in the other histological subtypes of lung cancer. b TopoII-alpha mRNA expression levels in lung cancer cell lines were relatively higher compared to those in normal lung tissues. c The expression levels of TopoII-beta in lung cancer cell lines were similar to those in normal lung tissues. d, e Western blot analyses for RanBP2 and TopoII isoforms in two lung cancer cell lines representing high and low expression, respectively. The expression patterns of protein and mRNA were not different

although there were no significant differences in TopoII-alpha mRNA expression levels among four histological subtypes of lung cancer (Fig. 2b). On the other hand, the expression levels of TopoII-beta in lung cancer cell lines were similar to those of normal lung tissues, although relatively higher expression levels were observed in SCLC and large cell carcinoma (Fig. 2c). In addition, we checked TopoII-alpha and TopoII-beta protein expressions in two lung cancer cell lines, SK-LC-2 and SK-MES-1, representing high and low expression of the two TopoII isoforms, and found that protein expression patterns of these genes were not different with mRNA expression patterns (Fig. 2e).

There were weak but significant positive correlations between RanBP2 and TopoII-alpha mRNA expressions, between RanBP2 and TopoII-beta mRNA expressions and between TopoII-alpha and TopoII-beta mRNA expressions among 20 lung cancer cell lines ($r = 0.532$; $P < 0.05$, Fig. 3a and $r = 0.623$; $P < 0.05$, Fig. 3b, $r = 0.647$; $P < 0.01$, Fig. 3c, respectively). Chemosensitivity data were analyzed in relation to the mRNA expression levels of the RanBP2, TopoII-alpha, TopoII-beta genes using linear regression analysis. No significant associations were observed between the IC50 values of amrubicin and the mRNA expression levels of RanBP2 (Fig. 4a), TopoII-alpha (Fig. 4b) and TopoII-beta (Fig. 4c) among 20 cell lines.

Discussion

RanBP2 has been reported to be involved in both nucleocytoplasmic transport and mitosis and also act as a SUMO ligase for DNA TopoII and play a role in maintaining chromosome stability by recruiting TopoII to centromeres during mitosis [5]. In addition, RanBP2 hypomorphic mice are particularly sensitive to spontaneous and carcinogen-induced lung tumors, indicating that RanBP2 might play a potential tumor suppressor role in human lung cancer. Two previous studies reported that RanBP2 mRNA expression levels are substantially reduced in human non-SCLC [2, 8]. However, the present study showed that RanBP2 transcript levels were infrequently downregulated in human lung cancer cell lines compared with normal lung tissues, although there were statistically significant differences in the RanBP2 expression between SCLC and NSCLC. Consistent with our results, several lines of evidence from publicly available human gene expression data of the Oncomine
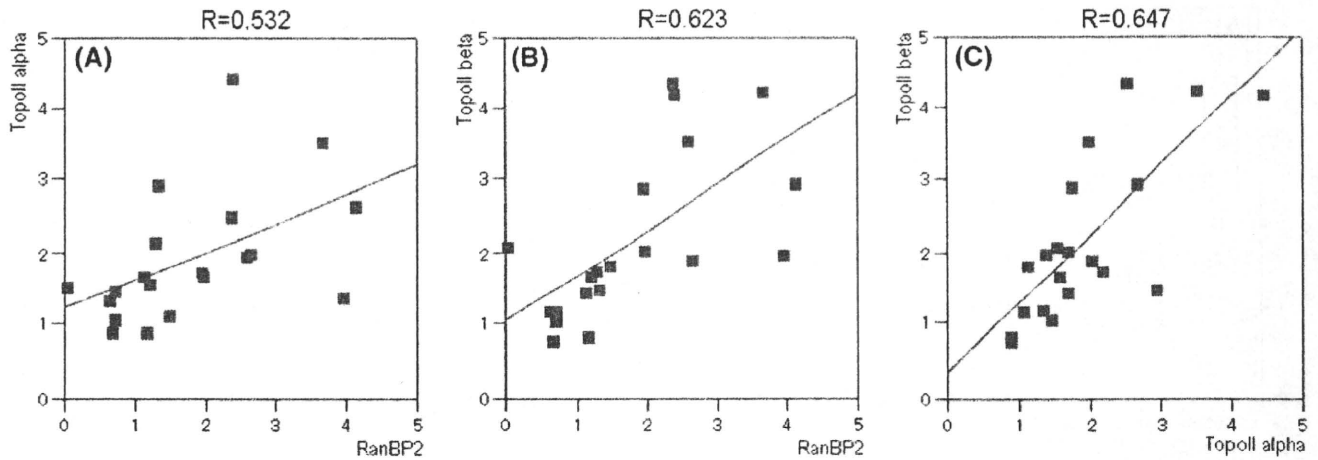
Fig. 3 Correlations between a RanBP2 and TopoII alpha mRNA expression, b RanBP2 and TopoII beta mRNA expression and c TopoII alpha and TopoII beta mRNA expression in lung cancer cell lines
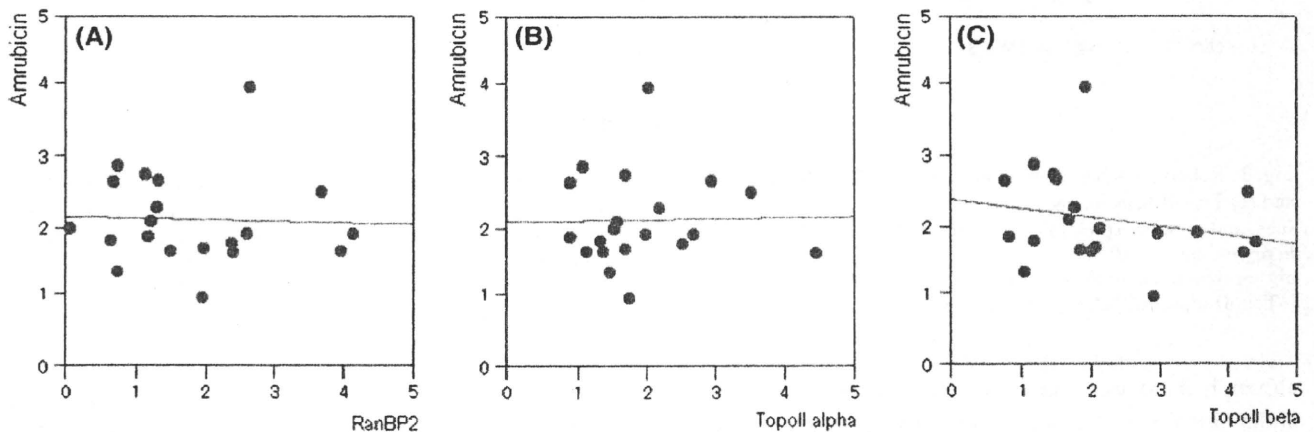

Fig. 4 Associations between relative mRNA expression for (a) RanBP2, (b) TopoII alpha and (c) TopoII beta and chemosensitivity of Amrubicin Log(IC50 in nM)

database (http://www.oncomine.com) and GEO profiles (http://www.ncbi.nlm.nih.gov/geo/) reported that RanBP2 mRNA expression levels are not reduced in NSCLC compared with normal lung tissues [3, 22, 26–28, 30]. In addition, there is a microarray study showing that RanBP2 expression levels are similar to those of our data in four overlapping lung cancer cell lines [9]. The concordance and discordance between our findings and previous works might be caused by the difference between cell lines and resected human lung tumors as well as the different experimental conditions used. Thus, further studies are warranted to establish the role of RanBP2 as a tumor suppressor gene in human lung carcinogenesis.

In RanBP2 hypomorphic murine embryonic fibroblasts (MEFs), formation of chromatin bridges in anaphase, a distinctive feature of cells with impaired DNA decatenation by mutation or chemical inhibition of TopoII-alpha [4], was observed, while spindle structure, kinetochore–microtubule

interactions, and localization of kinetochore and spindle assembly checkpoint proteins appeared normal [5]. Therefore, the low expression of RanBP2 may have an analogous effect of TopoII inhibitors, although the inhibitors are able to cause an inevitable consequence of DNA damage at high doses [4, 21]. Then, we speculated that there might be an association between RanBP2 mRNA expression and chemosensitivity of a TopoII inhibitor, amrubicin and tested whether we could see it using human lung cancer cell lines. However, we did not find any associations, suggesting that cytotoxicity of amrubicin might come mainly from DNA damage response induced at high doses and that formation of chromatin bridges in anaphase caused by low expression of the RanBP2 gene might not have additional effects on amrubicin-induced DNA damage response.

The two isozymes, TopoII-alpha and TopoII-beta function to unknot and decatenate covalently closed circles of DNA, although functional differences of these isozymes

and their differential spliced variants as well as precise role of their homodimerization and heterodimerization are unknown [20, 21]. There are several lines of evidence indicating a close relationship between TopoII-alpha levels and drug sensitivity in cell lines made resistant to TopoII inhibitors [7, 17, 25], cell lines with reduced expression of TopoII [1] and a VP-16-resistant breast cancer cell line infected with adenovirus containing TopoII-alpha [32]. Another study has shown the relationship between TopoII expression and multidrug sensitivity including TopoII inhibitors using eight human lung cancer cell lines [10]. There is also some evidence that TopoII-beta may be related with resistance to TopoII inhibitors [6, 15]. However, we did not find any association between expression levels of TopoII isoforms and chemosensitivity of amrubicin. Consistent with our results, a previous report of unselected human lung cancer cell lines also showed no clear association between TopoII-alpha protein expression and in vitro sensitivity to TopoII inhibitors [31]. Another study also failed to show importance of the enzyme using a panel of cell lines [12]. Although the behavior of cell lines in vitro may differ from the in vivo situation, and depend on the experimental conditions, these contradictory findings may require further investigation.

Amrubicin is highly active and one of the most potent anticancer drugs against SCLC and NSCLC [14]. Among the toxicities, hematologic adverse events such as leukopenia and thrombocytopenia are frequent and dose-limiting factors. Although identification of molecular biomarkers with the potential to predict treatment outcomes is essential to eliminate the use of any ineffective agents and to avoid toxic side effects [16], the cellular response to amrubicin is still poorly understood. To predict drug response in lung cancer patients, integrated analyses such as array-based mRNA expression profile, epigenome profiles, proteome analysis would be needed.

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