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Phase II trial of carboplatin and paclitaxel in non-small cell lung cancer patients previously treated with chemotherapy

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Toxicity

Summary The purpose of this phase II trial was to evaluate the efficacy and toxicity of carboplatin plus paclitaxel in the treatment of advanced non-small cell lung cancer (NSCLC) previously treated with chemotherapy. Patients with a performance status (PS) of 0 or 1 who had received one or two previous chemotherapy regimens for advanced NSCLC were eligible. Paclitaxel 200 mg/m^2 was infused over 3 h and followed by carboplatin (area under the curve 6) infusion over 1 h, once every 3 weeks. Thirty patients were enrolled. A complete response was observed in 1 patient and a partial response in 10 patients, for an overall response rate of 36.7%. The median time to progression was 5.3 months. The median survival time was 9.9 months, and the 1-year survival rate was 47%. Hematological toxicity in the form of grade 3/4 neutropenia occurred in 54%, but grade 3 febrile neutropenia developed in only 3%. Non-hematological grade 3 toxicities were less frequent. There were no treatment-related deaths. The combination of carboplatin plus paclitaxel is an active and well-tolerated regimen for the treatment of NSCLC patients who have previously been treated with chemotherapy and have a good PS.

1. Introduction

Lung cancer remains a major cause of death from cancer in many countries. More than half of all patients diagnosed with non-small cell lung cancer (NSCLC) have advanced stage IIIB or IV disease at presentation, and patients with advanced NSCLC are candidates for systemic chemotherapy. Platinum-based chemotherapy is considered the standard first-line treatment for patients with advanced NSCLC, and prolongs survival, palliates symptoms, and improves quality of life [1,2]. Many patients with good performance status (PS) when progression occurs after first-line chemotherapy are suitable candidates for second-line chemotherapy [3].

The taxanes are an important class of new agents for the treatment of advanced NSCLC. Paclitaxel, in combination with carboplatin, is the most common regimen

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used as first-line chemotherapy for advanced NSCLC, and this combination has a more favorable toxicity profile and is more convenient to administer than other platinum-based regimens [4,5]. Docetaxel has been investigated more extensively than any other agent for second-line treatment of advanced NSCLC, and the results of two randomized phase III trials of second-line chemotherapy in patients with advanced NSCLC demonstrated that docetaxel monotherapy significantly improved survival compared with best supportive care or other single agents (vinorelbine or ifosfamide) [6,7].

Belani et al. recently reported that results of a phase III trial comparing a carboplatin plus paclitaxel regimen with a cisplatin plus etoposide regimen for first-line treatment of advanced NSCLC [8]. Carboplatin plus paclitaxel yielded a higher response rate (23% versus 15%), time to progression (121 days versus 111 days), and overall quality of life benefit than cisplatin plus etoposide, but the median survival time was better in the cisplatin plus etoposide arm than in the carboplatin plus paclitaxel arm (274 days and 233 days, respectively [$P = 0.086$]). The authors reported that a substantially greater proportion of patients in the cisplatin plus etoposide arm received second-line chemotherapy with a taxane-containing regimen than in the carboplatin plus paclitaxel arm, and suggested that treatment with taxanes in a second-line setting may have had an impact on the survival in their study. Remarkably, more than half of the regimens that were used in the second-line setting of their study consisted of paclitaxel alone or carboplatin plus paclitaxel, not docetaxel. While the efficacy of paclitaxel-containing regimens as first-line chemotherapy for advanced NSCLC has been established in many randomized phase III trials [9], the data on the efficacy of paclitaxel-containing regimens in second-line settings are limited [10,11].

Based these considerations we conducted a phase II trial to evaluate the efficacy and toxicity of carboplatin plus paclitaxel in the treatment of advanced NSCLC previously treated with chemotherapy.

2. Patients and methods

2.1. Eligibility criteria

The inclusion criteria were: pathologically confirmed advanced NSCLC patients with measurable disease who had received one or two previous chemotherapy regimens for their disease. Patients were required to submit evidence of failure of prior chemotherapy. Patients who were previously treated with carboplatin or paclitaxel were excluded if the best response was progressive disease (PD). Patients who had received prior radiotherapy were eligible provided that at least 30 days had elapsed between the completion of radiotherapy and entry into the study. Patients were also required to be 20–75 years of age, have an Eastern Cooperative Oncology Group PS of 0 or 1, and have adequate organ function as indicated by the following parameters: absolute neutrophil count $\geq 1500\,mm^{-3}$, platelet count $\geq 100{,}000\,mm^{-3}$, hemoglobin $\geq 9.0\,g/dl$, AST and ALT $\leq 2.0 \times$ the institutional upper normal limits, total bilirubin $\leq 1.5\,mg/dl$, creatinine $\leq 1.5\,mg/dl$, $PaO_2 \geq 65$ Torr. Exclusion criteria were: uncontrolled pleural or pericardial effusion, active concomitant malignancy, prior irradiation to areas encompassing more than a third of the pelvis plus spine, active infection, myocardial insufficiency or myocardial infarction within the preceding 6 months, uncontrolled diabetes mellitus or hypertension, any other condition that could compromise protocol compliance, pregnancy and/or breast-feeding. All patients were required to provide written informed consent before entry into the study. The study was approved by the institutional review board of our institution.

2.2. Treatment plan

Treatment was started within a week of entry into the study. Patients received paclitaxel $200\,mg/m^2$ diluted in 500 ml of 0.9% saline as a 3-h intravenous infusion followed by carboplatin (area under the curve [AUC] 6; Calvert formula) diluted in 250 ml of 5% glucose as a 1-h intravenous infusion, every 3 weeks. All patients were premedicated with dexamethasone (24 mg i.v.), famotidine (20 mg i.v.), and diphenhydramine (50 mg orally) 30 min before the paclitaxel infusion to prevent a hypersensitivity reaction. A 5-HT3-receptor antagonist was intravenously administered as an antiemetic before carboplatin. Therapy was continued for at least two cycles unless the patient experienced unacceptable toxicity or had PD. The maximum number of cycles of chemotherapy was six. In the event of grade 4 leukopenia or thrombocytopenia or of grade 3 neutropenic fever, the dose of carboplatin and paclitaxel was reduced to AUC 5 and $175\,mg/m^2$, respectively, in the following cycle of chemotherapy. The next cycle of chemotherapy was started if the neutrophil count was $\geq 1500\,mm^{-3}$, the platelet count $\geq 100{,}000\,mm^{-3}$, AST and ALT $\leq 100\,IU/l$, total bilirubin $\leq 2.0\,mg/dl$, creatinine $\leq 1.5\,mg/dl$, PS 0 or 1, and the patient was afebrile.

Pretreatment evaluation included a medical history, a physical examination, vital signs, height and body weight, PS, complete blood count, biochemical studies, arterial blood gas analysis, electrocardiogram, chest radiograph and computed tomography scan (CT), abdominal ultrasound or CT, and brain magnetic resonance imaging or CT. A complete blood count, biochemical studies, and chest radiograph were performed weekly during the first cycle of chemotherapy, and 2 weekly starting with the second cycle.

2.3. Response and toxicity assessment

Objective tumor response was assessed as complete response (CR), partial response (PR), stable disease ≥ 8 weeks (SD), or PD according to the Response Evaluation Criteria in Solid Tumors. Measurable lesions were defined as lesions whose longest diameter was ≥ 2 cm. Imaging studies were repeated every 4 weeks until the objective tumor response was confirmed. All responses were reviewed by an independent radiologist. Toxicity was graded using National Cancer Institute-Common Toxicity Criteria version 2.0.

2.4. Statistical analysis

The primary endpoint of this study was the response rate, defined as the proportion of patients whose best response was CR or PR among all enrolled patients in the intent-to-treat analysis. The secondary end points were toxicity and overall and progression-free survival (PFS) from the date of enrollment in this study.

According to Simon's minimax two-stage phase II study design, the treatment program was designed for a minimal response rate of 5% and to provide a significance level of 0.05 with a statistical power of 80% in assessing the activity of the regimen according to a 20% response rate. The upper limit for first-stage drug rejection was no response in 13 evaluable patients. The upper limit for second-stage drug rejection was three responses in 27 evaluable patients. Overall survival time was defined as the interval between enrollment in this study and death or the most recent follow-up visit. PFS was defined as the interval between enrollment in this study and the first documented PD, death, or the most recent follow-up visit. Survival was estimated by the Kaplan–Meier analysis method. All comparisons between proportions were performed by Fisher's exact test.

3. Results

3.1. Patient characteristics

Between October 2002 and November 2003, 30 patients were enrolled in this study, and their characteristics are shown in Table 1. Twenty-six (87%) patients were men, and 21 (70%) patients had adenocarcinoma. Median age was 60 years. The majority of the patients (93%) had received prior platinum-based chemotherapy, and seven (23%) patients had received two prior chemotherapy regimens. The platinum-based chemotherapy regimens that had been used were: cisplatin plus vinorelbine ($n=26$), cisplatin plus gemcitabine ($n=1$), and carboplatin plus gemcitabine ($n=1$). There were 15 (50%) responders to any of the prior chemotherapy regimens and 12 of them had experienced a response (CR/PR) to cisplatin-based chemotherapy. Twenty-one (70%) patients had a treatment-free interval of 3 or more months since the final dose of the prior chemotherapy regimen.

A total of 94 cycles of chemotherapy were administered, and the median number of cycles per patient was three (range, 1–6). Four patients had received only one cycle of treatment either because of toxicity (two patients, grade 3 rash), the patient's refusal (one patient), or PD (one patient).

3.2. Response and survival

Two patients were not evaluable for response because the protocol treatment had been terminated because of toxicity (grade 3 rash) during the first cycle of chemotherapy, and they subsequently received further chemotherapy without PD. There was 1 CR and 10 PRs among the 30 patients, and the objective response rate in the intent-to-treat analysis was 36.7% (95% confidence interval [CI], 19.9–56.1%) (Table 2). Treatment outcomes of all patients are listed in

Table 1 Patient characteristics

Characteristic	No. of patients (%)
Patients enrolled	30
Sex	
Male	26
Female	4
Age, years	
Median	60
Range	39–75
ECOG performance status	
0	7
1	23
Stage	
IIIB	11
IV	19
Histology	
Adenocarcinoma	21
Squamous cell carcinoma	7
Large cell carcnioma	2
Prior treatment	
Platinum-based chemotherapy	28 (93)
Docetaxel	5 (16)
Chest radiotherapy	4 (13)
No. of prior chemotherapy regimens	
1	23
2	7

Table 3. The response rate of patients who experienced a response (CR/PR) to prior cisplatin-based chemotherapy was 43% (6/14), as opposed to 23% (3/13) among the non-response patients ($P=0.41$). The response rate of the patients who had received one prior chemotherapy regimen was 39% (9/23), as opposed to 28% (2/7) among the patients who had received two regimens ($P>0.99$). According to the treatment-free interval since the final dose of the prior chemotherapy regimen, the response rate of patients whose interval was 3 months or more was 33% (7/21), com-

Table 2 Treatment efficacy ($n=30$)

	No. of patients	%
Response		
Overall response rate	11	36.7
Complete response	1	3.3
Partial response	10	33.3
Stable disease	12	40
Progressive disease	5	16.7
Not evaluable	2	6.7
Survival		
Median (months)	9.9	
1 year (%)	47	
Progression-free survival		
Median (months)	5.3	

Table 3 Treatment outcomes of all patients

Patient No.	Prior first-line therapy		Prior second-line therapy		Time from last therapy (months)	CBDCA + PTX, best response	PFS (months)	Survival (months)
	Regimen	Best response	Regimen	Best response				
1	CDDP + VNR	SD	DOC	PD	1.8	SD	1.4	25.2
2	CBDCA + GEM	NE	Gefitinib	PD	0.8	PR	3.8	8.8
3	CDDP + VNR	SD	—		6.8	SD	7.6	18.1
4	CDDP + GEM	PR	—		9.5	PR	7.5	33.8+
5	CDDP + VNR	SD	—		4.8	SD	2.8	7.0
6	CDDP + VNR + DOC + RT	PR	—		6.0	PR	8.0	21.6
7	GEM + VNR	SD	—		23.0	PD	1.2	7.8
8	CDDP + VNR + RT	PR	—		13.6	SD	6.7	25.0+
9	CDDP + VNR	SD	—		5.0	SD	2.1	3.7
10	CDDP + VNR	SD	—		5.0	PD	1.2	6.7
11	CDDP + VNR	PR	—		8.9	NE	1.1	3.3
12	CDDP + VNR	SD	Gefitinib	CR	1.9	SD	6.3	6.3
13	CDDP + VNR	PR	—		5.4	NE	1.0	13.4
14	CDDP + VNR	PR	—		1.7	SD	4.8	5.7
15	CDDP + VNR + RT	PR	—		9.3	SD	5.0	15.7
16	CDDP + VNR	SD	—		2.8	PR	3.7	15.8
17	CDDP + VNR	SD	DOC + GEM	SD	3.8	SD	5.3	21.6+
18	CDDP + VNR + DOC + RT	PR	—		3.9	SD	4.5	9.0
19	CDDP + VNR	PR	—		12.9	PR	9.4	16.0
20	CDDP + VNR	PR	—		11.5	CR	24.8+	24.8
21	CDDP + VNR	PD	—		1.1	PR	9.2	23.6+
22	CDDP + VNR	SD	DOC	SD	4.5	PD	2.3	5.5
23	Gefitinib	SD	—		0.9	PR	8.8	12.7
24	CDDP + VNR	PR	—		11.1	PR	5.3	10.2
25	CDDP + VNR	PR	Gefitinib	PR	4.4	PR	5.5	9.9
26	CDDP + VNR	NE	—		11.7	PR	7.0	12.2
27	CDDP + VNR	PR	—		5.4	SD	6.2	9.4
28	CDDP + VNR	SD	—		0.8	PD	1.4	2.5
29	CDDP + VNR	PR	—		4.4	PD	0.2	8.4
30	Gefitinib	PD	CDDP + VNR	PD	0.9	SD	3.1	3.3

CBDCA, carboplatin; PTX, paclitaxel; PFS, progression-free survival; CDDP, cisplatin; VNR, vinorelbine; GEM, gemcitabine; DOC, docetaxel; RT, chest radiotherapy; SD, stable disease; NE, not evaluable; PR, partial response; PD, progressive disease; CR, complete response.

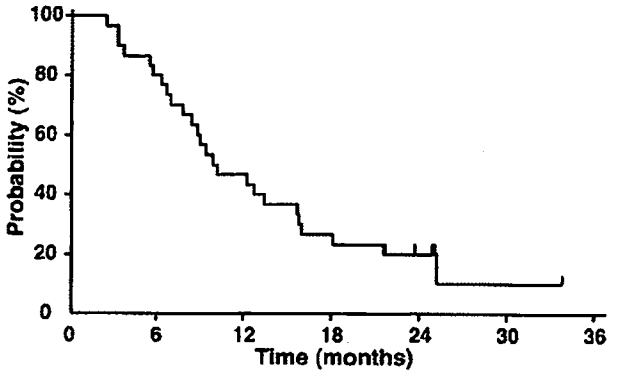

Fig. 1 Kaplan–Meier curve for overall survival.

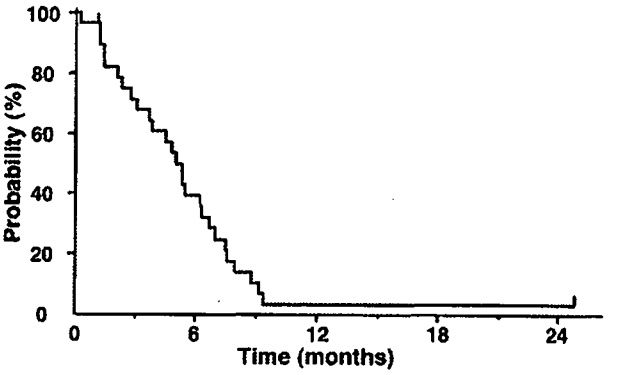

Fig. 2 Kaplan–Meier curve for progression-free survival.

pared with 44% (4/9) in patients in whom it was less than 3 months ($P = 0.68$).

The median follow-up time was 24 months. The median survival time (MST) was 9.9 months (range, 2.5–33.8 months), and the 1-year survival rate was 47% (95% CI, 29–65%). The median PFS was 5.3 months. The Kaplan–Meier curve for overall survival and for PFS is shown in Figs. 1 and 2, respectively. Nineteen patients (63%) received at least one subsequent chemotherapy regimen, and their regimens are shown in Table 4. Fourteen of them were treated with gefitinib, and a PR was achieved in three of them.

3.3. Toxicity

The common toxicities associated with carboplatin plus paclitaxel are listed in Table 5. Grade 3/4 neutropenia occurred in 54% of the patients in our study, but grade 3 febrile neutropenia developed in only 3%. Grade 3/4 anemia and thrombocytopenia were observed in five patients (16%) and two patients (13%), respectively. Non-hematological grade 3 toxicities were less frequent. Grade 3 hyponatremia was observed in five (16%) patients, but they were all asymptomatic. Grade 2 neuropathy occurred in 33% of the patients. There were no treatment-related deaths.

Table 4 Post-study chemotherapy

Regimen	No. of patients	Responder (%)
Gefitinib	14	3 (21)
Docetaxel	9	0
Gemcitabine plus vinorelbine	1	0

4. Discussion

Docetaxel, pemetrexed, and erlotinib have been approved for second-line treatment of advanced NSCLC on the basis of the results of phase III trials [6,7,12,13]. Hanna et al. reported a phase III study comparing 3-weekly pemetrexed 500 mg/m^2 with 3-weekly docetaxel 75 mg/m^2 as second-line treatment for advanced NSCLC. The overall response rate with pemetrexed and docetaxel was 9.1% and 8.8%, respectively, and MST was 8.3 months and 7.9 months, respectively. Although efficacy in terms of the outcome as measured by survival time and response rate was similar for both treatments, the pemetrexed group experienced less grades 3–4 hematological toxicity and alopecia of all grades [12]. In the trial reported by Shepherd et al. 731 NSCLC patients previously treated with chemotherapy were randomized to receive either erlotinib at a dose of 150 mg daily or placebo, and the response rate in the erlotinib group was 8.9%. MST was 6.7 months in the erlotinib group and 4.7 months in the placebo group ($P < 0.001$). The results of their trial showed that erlotinib significantly prolonged the survival of patients with advanced NSCLC who had previously been treated with chemotherapy [13]. Despite the positive results of these phase III trials, the response rate of advanced NSCLC to second-line chemotherapy remains low, and the life expectancy of advanced NSCLC patients remains short. Alternative effective chemotherapy option is needed for second-line treatment of advanced NSCLC.

The combination of carboplatin plus paclitaxel has proved effective as one of the standard platinum-based doublet regimens for first-line treatment of advanced NSCLC [4,5,14]. However, since the efficacy of carboplatin plus paclitaxel used in a second-line setting had hardly been assessed, in the present study we evaluated the efficacy and toxicity of carboplatin plus paclitaxel in the second- or third-line treatment of advanced NSCLC. The results in the 30 patients with advanced NSCLC previously treated with chemotherapy indicated that the combination of carboplatin plus paclitaxel yielded an objective response rate of 36.7% and an MST of 9.9 months, with a 1-year survival rate of 47%. Our study had not included patients who were treated with the platinum/taxane combination chemotherapy. Most of the toxicity observed in our study was hematological. Grade 3/4 neutropenia, anemia, or thrombocytopenia occurred in 54, 16, or 13% of the patients in our study, respectively. Hematological toxicity of carboplatin plus paclitaxel used in first-line treatment for Japanese patients with advanced NSCLC has been reported that grade 3/4 neutropenia, anemia, or thrombocytopenia occurred in 88, 15, or 11% of the patients [15]. The toxicity observed in our study appeared similar to that of carboplatin plus paclitaxel, which was administered as the first-line treatment, although the number of patients in our study was not large. The combination of carboplatin plus paclitaxel seems to be effective and tolerable, not only as first-line therapy for advanced NSCLC but

Table 5 Hematological and non-hematological toxicity ($n = 30$)

Toxicity	NCI-CTC Version 2.0, grade							
	0–1		2		3		4	
	n	%	*n*	%	*n*	%	*n*	%
Leukopenia	11	37	10	33	9	30	0	0
Neutropenia	10	33	4	13	14	47	2	7
Anemia	7	23	18	60	3	10	2	7
Thrombocytopenia	27	90	1	3	2	7	0	0
Febrile neutropenia	29	97	–		1	3	0	0
Nausea	27	90	3	10	0	0	–	
Fatigue	30	100	0	0	0	0	0	0
Neuropathy	20	67	10	33	0	0	0	0
Arthralgia	21	70	8	27	1	3	0	0
Rash	28	93	0	0	2	6	0	0
Infection	29	97	0	0	1	3	0	0
Arrhythmia	29	97	0	0	1	3	0	0
Alopecia	21	70	9	30	–		–	
AST/ALT	29	97	1	3	0	0	0	0
Hyponatremia	25	83	–		5	17	0	0

as second-line therapy as well if the patients had not been previously treated with the platinum/taxane combination chemotherapy.

Hotta at al. reported a meta-analysis based on abstracted data to compare the effect of carboplatin-based chemotherapy with that of cisplatin-based chemotherapy on overall survival, response rate, and toxicity in the first-line treatment of patients with advanced NSCLC [16]. The results indicated that combination chemotherapy consisting of cisplatin plus a third generation agent produced a significant survival benefit compared with carboplatin plus a third generation agent, although the toxicity profiles of the two modalities were quite different. Recently, Pignon et al. reported a pooled analysis from five randomized clinical trials of cisplatin-based chemotherapy in completely resected NSCLC patients [17]. Their analysis suggested that adjuvant cisplatin-based chemotherapy improved survival in patients with NSCLC. Based on the results of their meta-analysis, cisplatin-based chemotherapy should be recommended as first-line therapy for patients with advanced NSCLC. Moreover, in view of the results of our own study, we speculate that the combination of carboplatin plus paclitaxel may be suitable as second-line treatment for advanced NSCLC patients who had experienced progression after first-line cisplatin-based chemotherapy.

Care must be exercised in interpreting the favorable outcome in our study. One concern is that it was a single-institution phase II study, and therefore patient selection may have influenced the outcome. The responders to any of the prior chemotherapy regimens accounted for 50% of the 30 patients enrolled in this study, and about 80% of the patients had received only one prior chemotherapy regimen. The selection criteria, such as an ECOG PS of 0 or 1, may also have contributed to this favorable outcome. Another concern is that our study had included only five patients who were previously treated with chemotherapy using taxanes. Therefore, the efficacy of carboplatin plus paclitaxel as the secondary therapy after chemotherapy using taxanes is not clear. A further randomized study is warranted to be able to draw definitive conclusions about our results.

Conflict of interest statement

None declared.

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Photodynamic therapy for lung cancers based on novel photodynamic diagnosis using talaporfin sodium (NPe6) and autofluorescence bronchoscopy

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Summary
Background: We had previously developed the possibility of use of a photodynamic diagnosis (PDD) system using a tumor-selective photosensitizer and laser irradiation for the early detection and photodynamic therapy (PDT) for centrally located early lung cancers. Recently, we established the autofluorescence diagnosis system integrated into a videoendoscope (SAFE-3000) as a very useful technique for the early diagnosis of lung cancer.
Patients and methods: Twenty-nine patients (38 lesions) with centrally located early lung cancer received PDD and PDT using the second-generation photosensitizer, talaporfin sodium (NPe6). Just before the PDT, we defined the tumor margin accurately using the novel PDD system SAFE-3000 with NPe6 and a diode laser (408 nm).
Results: Red fluorescence emitted from the tumor by excitation of the photosensitizer by the diode laser (408 nm) from SAFE-3000 allowed accurate determination of the tumor margin just before the PDT. The complete remission (CR) rate following NPe6-PDT in the cases with early lung cancer was 92.1% (35/38 lesions). We also confirmed the loss of red fluorescence

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from the tumors immediately after the PDT using SAFE-3000. We confirmed that all the NPe6 in the tumor had been excited and photobleached by the laser irradiation (664 nm) and that no additional laser irradiation was needed for curative treatment.
Conclusions: This novel PDD system using SAFE-3000 and NPe6 improved the quality and efficacy of PDT and avoided misjudgement of the dose of the photosensitizer or laser irradiation in PDT. PDT using NPe6 will become a standard option of treatments for centrally located early lung cancer.

1. Introduction

Photodynamic therapy (PDT), used as a treatment modality for many cancers, uses a tumor-specific photosensitizer and laser irradiation to induce the production of reactive oxygen species in the cancer cells [1,2]. Since the report of the first modern clinical trial of PDT by Dougherty et al. [3], PDT using the photosensitizer, photofrin, has been applied for the treatment of many cancers, and is approved by the United States Food and Drug Administration (FDA) for the treatment of early stage lung cancer as well as advanced esophageal and lung cancers [1,4–6]. In Japan, PDT is recommended as a treatment option for centrally located early-stage lung cancers in the therapeutic guidelines for lung cancer established by the Japanese Ministry of Health, Labor and Welfare using the principles of evidence-based medicine [7–9]. Recently, the second-generation photosensitizer, mono-L-aspartyl chlorine e6 (taraporfin sodium, laserphyrin, NPe6), which has a major absorption band at 664 nm, was approved for use in the diagnosis/treatment of centrally located early lung cancer by the Japanese government [7–11]. A phase II clinical study using NPe6 and a diode laser for early-stage lung cancer demonstrated excellent antitumor effects and safety, including a significantly lower skin photosensitivity as compared to that observed with photofrin [12]. The Japanese government approved the use of NPe6 for PDT in 2003, and the product has been available in the Japanese market from June 2004.

Furukawa et al. reported that it was important to accurately define the tumor extent and depth before PDT, based on their analysis of the histopathological features, in particular, the depth of invasion of the bronchial wall by the cancer, of the recurrent lesions after CR in 114 cases of centrally located early lung cancers who underwent PDT [13]. It has been reported that PDT is associated with a high success rate only when the tumor is confined to the mucosa or submucosa, and that an important correlation exists between the response rate and the tumor size [14–16].

Sutedja et al. reported that autofluorescence bronchoscopy improved staging of radiographically occult lung cancer and had an impact on therapeutic strategy [17]. We have reported the usefulness of a photodynamic diagnosis (PDD) system based on autofluorescence bronchoscopy and the photosensitizer photofrin for the diagnosis of centrally located early-stage lung cancer [7,8,10,11]; red fluorescence emitted from the tumor following excitation of the photosensitizer by laser irradiation, e.g., using the Lung Imaging Fluorescence Endoscopy (LIFE) system, was detected in the cases with cancer, whereas green fluorescence was detected from the normal mucosa. Recently, an autofluorescence diagnosis system integrated into a videoendoscope (SAFE-3000, Pentax, Tokyo) was established [18]. SAFE-3000 consists of a color CCD videoendoscopy-based autofluorescence system with two light sources, namely, a xenon lamp for white light and a diode laser (408 nm) as an autofluorescence mode excitation light source. Ikeda et al. reported the feasibility of use of SAFE-3000 as a very useful laser system for the early diagnosis of centrally located early cancers [18].

In this study, we examined the usefulness of this new PDD system using SAFE-3000 and NPe6 to accurately define the margin of the tumor prior to PDT and improve the efficacy of PDT for lung cancer. In cases with large tumors or anatomical problems that make laser irradiation difficult, it is often difficult to decide whether or not additional laser irradiation might be needed. Therefore, we compared the red fluorescence emitted from the tumor excited by SAFE-3000 with a diode laser (408 nm) just before and after the PDT, and conducted a dosimetric analysis to determine the appropriate dose of the laser irradiation and of the photosensitizer.

2. Materials and methods

2.1. Patient selection

Between June 2004 and March 2006, 29 patients with 38 centrally located early lung cancer lesions who were provided written informed consent, have been treated by PDT using NPe6 and a diode laser (664 nm), and also been examined immediately before and after the PDT by PDD using SAFE-3000. This study was approved by the Ethical Research Committee of Tokyo Medical University.

2.2. Criteria for the diagnosis of centrally located early lung cancer

Lung cancers located no distal to the segmental bronchi and determined histologically to be carcinoma in situ or carcinoma showing only limited invasion with no evidence of invasion beyond the bronchial cartilage and classified as squamous cell carcinoma are defined as centrally located early lung cancers [7,8,19]. We always determined the tumor depth by EBUS, and tumors did not invade the bronchial wall beyond the cartilage and were confined to the basal membrane of the mucosa, submucosa or intracartilagious layers of the bronchial wall. In 2003, the Japan Photodynamic Association and Japanese Society of Laser Surgery and Medicine established the following therapeutic criteria for PDT in cases with centrally located early lung cancer [7,8]: patients with (1) endoscopically assessable early lung cancer, (2) normal chest X-ray and CT imaging,

(3) no metastasis to lymph nodes or no distant metastasis as revealed by routine clinical diagnostic methods including fluorodeoxyglucose position emission tomography (FDG-PET) for staging.

2.3. Autofluorescence bronchoscopy (SAFE-3000)

The newly developed videoendoscopy-based autofluorescence bronchoscopy (AF) system is referred to as SAFE-3000 [18]. In this system, normal bronchial tissue emits intense green autofluorescence when excited by blue light from a diode laser (408 nm), whereas abnormal tissue lacks the green autofluorescence due to the differences in the tissue structure, metabolic state, and blood flow.

2.4. Procedures of PDD and PDT and follow-up

PDT was performed using NPe6 and an aluminum gallium indium phosphorus (AlGaInP) diode laser system (PD laser, Panalas6405, Matsushita Electric Industrial Co., Ltd., Osaka, Japan) [7,8,12]. Laser irradiation (664 nm) for the PDT was performed via a quartz fiber inserted through the biopsy channel of the endoscope, 4 h after the administration of the photosensitizer talaporfin sodium (40 mg/m^2). The total energy of the laser irradiation was 100 J/cm^2, 150 mW/cm^2. Just before the PDT, we performed PDD using SAFE-3000 with a diode laser (408 nm) to define the tumor margin, based on the red fluorescence emitted from the tumor. Immediately after the NPe6-PDT, we again performed PDD using SAFE-3000 to determine the change in the intensity of the red fluorescence emitted from the tumor as compared with that observed just before the PDT.

Cytologic and histologic examinations via fiberoptic bronchoscopy were performed at 1, 2 and 3 months and thereafter at 3-month intervals in the first year and 6-month intervals after the second years after PDT. The antitumor effect of the initial treatment was rated based on endoscopic measurement of the tumor size using forceps and morphologic appearance, and the findings on histopathologic examination of biopsy specimens, according to the general rules of the Japan Lung Cancer Society and the Japan Society of Clinical Oncology [7,8]. The antitumor effect was again evaluated at 3 months after the PDT. The tumors were then classified as showing complete response (CR) (no microscopically demonstrable tumor in brushing and/or biopsy specimens over period of 4 weeks)

Table 1 Characteristics of cases

Total number of patients (lesions)	29 patients (38 lesions)
Age (years)	Mean (72.3)
Gender	Male: 29 patients
Histology	Squamous cell carcinoma: 38 lesions
Endoscopic findings	
Thickened type	35 lesions (C-stage 0: 34 lesions) (C-stage I: 1 lesion)
Polypoid type	2 lesions (C-stage 0: 2 lesions)
Nodular type	1 lesion (C-stage 0: 1 lesion)

3. Results

3.1. PDD using SAFE-3000 and NPe6

Between July 2004 to March 2006, 29 patients (38 lesions) had centrally located early lung cancer and underwent NPe6-PDT and PDD based on the therapeutic criteria. All were male with a median age of 73 years. The histological type of the cancer was squamous cell carcinoma and the clinical stage 0 (including carcinoma in situ) in 37 lesions, C-stage IA (T1N0M0) in 1 lesion (Table 1). In accordance with the therapeutic criteria for PDT of lung cancer, we determined the tumor depth and tumor extent by endobronchial ultrasonography (EBUS) [16,20,21], optical coherence tomography (OCT) [22,23] and SAFE-3000 [18,24], to evaluate the suitability of the patients as candidates for PDT. The Japanese Lung Cancer Society classified centrally located early lung cancers, based on the endoscopic findings, as the thickened type (Fig. 1), polypoid type (Fig. 2) or the nodular type (Fig. 3) [18]. The thickened type, which is characterized by superficial lesions showing subtle mucosal changes of the

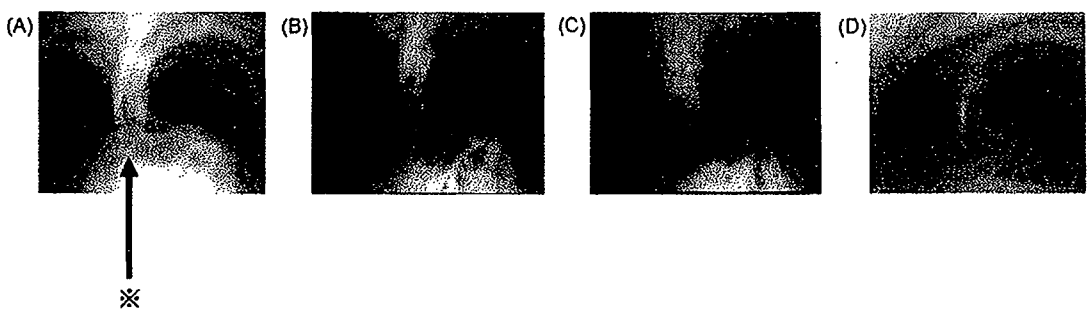

Fig. 1 A 63-year-old man with centrally located early stage lung cancer. (A) Fiberoptic bronchoscopy revealed a superficial lesion. A thickened-type squamous cell carcinoma can be visualized at the carina (the bifurcation of right and left main bronchus). (B) Photodynamic diagnosis using SAFE-3000 and NPe6 was conducted before the PDT. The red fluorescence excited by the diode laser (408 nm) from the SAFE-3000 system revealed the cancerous lesion. (C) Loss of the red fluorescence from the tumor was confirmed by PDD using SAFE-3000 immediately after the NPe6-PDT. (D) CR was achieved 3 months after PDT.

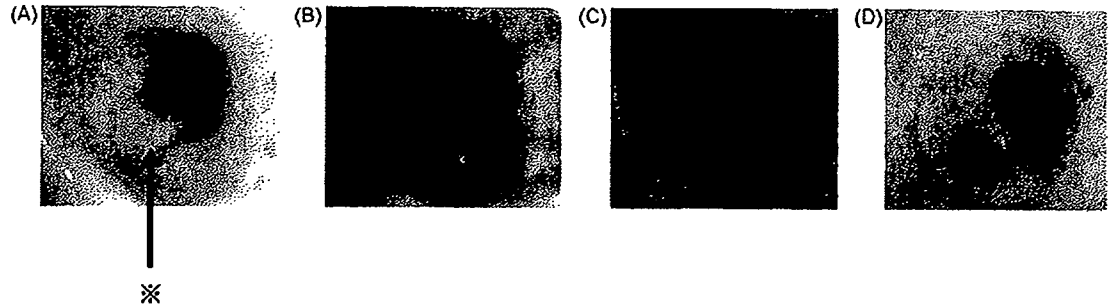

Fig. 2 A 83-year-old man with centrally located early stage lung cancer. (A) Fiberoptic bronchoscopy revealed a polypoid lesion. A polypoid-type squamous cell carcinoma can be visualized at left B^3. (B) Photodynamic diagnosis using SAFE-3000 and NPe6 was conducted before the PDT. The red fluorescence excited by the diode laser (408 nm) from the SAFE-3000 system revealed the cancerous lesion. (C) Loss of the red fluorescence from the tumor was confirmed by PDD using SAFE-3000 immediately after the NPe6-PDT. (D) CR was achieved 3 months after PDT.

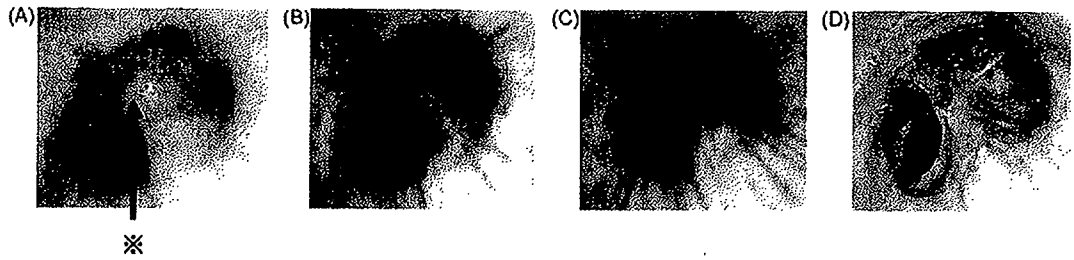

Fig. 3 A 65-year-old man with centrally located early stage lung cancer. (A) Fiberoptic bronchoscopy revealed a nodular lesion. A nodular-type squamous cell carcinoma can be visualized at right upper bronchus. (B) Photodynamic diagnosis using SAFE-3000 and NPe6 was conducted before the PDT. The red fluorescence excited by the diode laser (408 nm) from the SAFE-3000 system revealed the cancerous lesion. (C) Loss of the red fluorescence from the tumor was confirmed by PDD using SAFE-3000 immediately after the NPe6-PDT. (D) CR was achieved 3 months after PDT.

bronchial surface, is the predominant type. In this study, among the 38 lesions, the thickened type accounted for 35 lesions, the polypoid type accounted for 2 lesions, and the nodular type accounted for one lesion (Table 1). In thickened type of lesions, in particular, the malignant lesion showed only slight redness and loss of clarity, which was too subtle to recognize. It was difficult to clearly confirm the tumor extent and to irradiate accurately to the tumor by diode-laser (664 nm) (Fig. 1A). However, just before the NPe6-PDT, we were able to clearly detect red fluorescence from the tumor by the PDD system using SAFE-3000, and therefore, to accurately determine the tumor extent (Figs. 1B–3B). We performed cytological examination from the boarder area, which we observed by SAFE-3000, and we confirmed tumor extent cytologically. Therefore, we were able to irradiate to the tumor thoroughly. No differences in the fluorescence pattern from the tumors were noted among three types (thickened, polypoid and nodular) of tumors mentioned above. In addition, immediately after the PDT, we were able to confirm the loss of the red fluorescence from the tumor lesions (Figs. 1C–3C). The data obtained after the PDT confirmed that all the NPe6, in the tumor had been excited by the laser irradiation (664 nm) and that the red fluorescence of the tumor could, therefore, no longer be observed. Thus, further irradiation by PD laser (664 nm) after the PDT session was not required and the dosimetric assessment in relation of the doses of NPe6 and laser irradiation required for PDT was considered to be appropriate for the cancer treatment.

3.2. The efficacy of NPe6-PDT

The evaluation of the efficacy of PDT is shown in Table 2. The complete response rate of the centrally located early lung cancer lesions to NPe6-PDT was 92.1% (35/38 lesions). Of these, 33 lesions were ≦10 mm and 5 were >10 mm in diameter prior to the PDT, with CR rates of 93.9% and 80.0%, respectively. Two lesions ≦10 mm in diameter in which the

Table 2 Results of PDT for early stage lung cancer

Tumor size (cm)	No. of lesions	CR (rate, %)	PR	Recurrence after CR
≦1.0	33	31 (93.9)	2	3 (9.7%)
1.0<	5	4 (80.0)	1	4 (100%)
Total	38	35 (92.1)	3	7

Table 3 Recurrent cases after CR

Case no.	Age	Tumor size (mm)	Interval of CR (months)	Additional treatment
1	63	8	21	PDT (CR)
2	72	8	10	PDT, radiation
3	63	8	10	PDT, chemotherapy
4	63	15	9	PDT, radiation
5	80	15	15	PDT (CR)
6	78	15	9	PDT, chemotherapy
7	59	25	8	Operation

peripheral margin could not be visualized showed PR. There was no difference in response rates based on depth of cancer nor type of lesions (thickened, polypoid, nodular). We performed chemotherapy and then achieved CR for three cases, which had not obtained CR by PDT. We performed cytological and/or pathological examinations at PDT sites and we confirmed recurrences at PDT sites. Recurrent tumors were local sites, neither lymph nodes metastasis nor distant metastasis.

However, in seven patients (seven lesions) recurrences were encountered after CR was achieved by NPe6-PDT (Table 3). In all the seven cases, the recurrent lesions were thickened types. One patient with a lesion >20 mm in diameter underwent surgery and six patients (six lesions) underwent NPe6-PDT again after reassessing their suitability as candidates for PDT by EBUS, OCT and SAFE-3000. Of these six lesions, CR after the repeat PDT was achieved in only two cases, not achieved in four cases. We performed radiotherapy in two cases, and chemotherapy in two cases. All patients in this study were disease-free and still alive (Table 3).

3.3. Complications of NPe6-PDT

In this study, patients undergoing NPe6-PDT were to required to avoid sunlight for 2 weeks, even though the skin photosensitivity was less strong as compared to that with photofrin-PDT. NPe6-PDT did not cause extensive necrotic changes in the tumors and clean-up bronchoscopy was rarely needed. The period of hospitalization after the laser-irradiation for NPe6-PDT was 7–10 days. These results suggest that NPe6-PDT may be cost-effective and improve patient's quality of life. In two lesions, we observed bronchial stenosis at the site of the tumor, which had spread around the bronchus, necessitating cylindrical laser irradiation.

4. Discussion

In this study, we were able to observe the red fluorescence emitted by the tumor cells because of laser excitation (408 nm) of the NPe6 contained in them, using SAFE-3000 just prior to PDT; this enabled us to clearly determine the tumor margin of the centrally located early lung cancer lesions, independent of the endoscopic findings (Figs. 1B–3B). Thus, this novel PDD technique using the new autofluorescence bronchoscopy system, SAFE-3000, and NPe6, allows more accurate assessment of the tumor margin, and therefore, of the quality and efficacy of PDT. Since tumor lesions are characterized by hypervascularity and increased redness, emission of red fluorescence can be observed from the tumor cells by excitation of a tumor-selective photosensitizer administered prior to the irradiation by blue light (408 nm) from diode laser using SAFE-3000.

We have been examining the feasibility of using photodynamic diagnotic (PDD) systems since 1980 [10,11,25,26]. The potential for the application of hematoporphyrin derivative (HpD) fluorescence using a krypton ion laser for localization of early lung cancer was demonstrated in an animal model by Hayata and Dougherty. The development of PDD systems are fraught with problems including skin photosensitivity and poor selectivity, e.g., of the photosensitizer photofrin, and also autofluorescence interference. In order to overcome the problems, we developed the excimer laser fluorescence image analyzer system [10,11]. The red fluorescence images of the excited photosensitizer and the autofluorescence are amplified separately and then processed by a data analyzer in this system. However, in the R/G fluorescence bronchoscopy system, the rate of false-positive results was found to be very high, i.e., 30%. We also developed a PDD system based on laser-induced fluorescence (originally for lung cancer) endoscopy after administration of a photosensitizer [10,11,27]. The sensitivity of this LIFE-PDD system was more than 90%, however, the false-positive rate remained high.

In this study, we examined a new PDD system developed by us, using SAFE-3000. The advantages of this PDD systems are as follows: increased sensitivity, ability to clearly define the tumor margin, a color CCD videoendoscopy-based AF system equipped with a diode laser and a hand switch to easily switch between the white light and AF modes. There are several kinds of AF systems available for the early detection of cancer lesions [28–32]. However, most are not equipped with a diode laser system to excite the photosensitizer in the tumor. As shown in Figs. 1B–3B, the red fluorescence from the tumor and the border between the normal mucosa and cancer lesion can be clearly visualized with SAFE-3000.

In Figs. 1C–3C, the loss of the red fluorescence from the tumor in the thickened, polypoid as well as the nodular types of cancer immediately after the PDT can be clearly visualized. Invasion of the bronchial cartilage has been reported in 18% and 27% of lesions, respectively, with the nodular and polypoid types of early lung cancer [33], therefore, additional laser-irradiation was often required for these types of early lung cancer. However, our data from the present study suggest that all of the NPe6 in the tumor had been completely excited by the laser irradiation, and

no additional laser irradiation was necessary. The results indicated that the doses of the NPe6 as well as the laser irradiation were appropriate for the treatment of early lung cancer in-dependent upon bronchoscopical findings (thickened, nodular, polypoid types). Therefore, there was no difference in response rates based on type of lesions.

As Furuse et al. reported, the longitudinal extent of the tumor was the only independent predictive factor for CR after PDT [15]; in our study, the tumor size was also an important factor for CR after NPe6-PDT. In two lesions less than 10 mm in diameter, CR was not achieved by NPe6-PDT, even though the peripheral extent of the tumors could be clearly visualized. For these two lesions, we selected cylindrical fiber irradiation, because the tumor extended around the bronchial wall. The reason for the inability to achieve CR in these cases with lesions less than 10 mm could be explained by possible inappropriate estimation of the tumor margin and insufficient laser irradiation.

In this study, the CR rate was high and all patients were alive. However, Furukawa et al. reported that the majority of patients who underwent PDT, were at an advanced age, with poor cardiopulmonary function and died from other disease except for lung cancer, and 5-year survival rate was relatively low. Therefore, we have to follow up all patients and have to analyze the relationship between CR rate and 5-year survival rate after PDT using talaporfin sodium in the future. Moreover, in order to improve the efficacy of PDD and PDT, we should develop the fibers for irradiation of the tumors that could facilitate irradiation of areas that are normally difficult to irradiate.

Conflict of interest statement

There was no financial support for the authors nor does any author have a financial relationship with a commercial entity that has an interest in this manuscript.

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Letter to the Editor

Pleural sarcomatoid malignant mesothelioma consisting of histiocytoid cells

To the Editor:

Pleural sarcomatoid malignant mesothelioma (PSMM) consists of spindle cells arranged in fascicles or having a haphazard fashion. The pattern often resembles fibrosarcoma, but marked anaplasia and bizarre multinucleated tumor cells may result in a picture closely mimicking that of malignant fibrous histiocytoma.[1] We herein report an extremely rare case of PSMM consisting of histiocytoid cells without any inflammatory infiltrate. We discuss its differential diagnosis and the key to establishing an accurate diagnosis.

A 76-year-old Japanese woman, a non-smoker, consulted Osaka Prefectural Medical Center for Respiratory and Allergic Diseases complaining of left chest pain that had persisted for the previous 2 weeks. She had worked as a spinning-mill worker for more than 30 years and occupational exposure to asbestos was highly supported. She had a past history of pleuritis and pericarditis of unknown origin. Chest X-ray demonstrated two fist-sized subpleural nodules in the left mediastinal and parietal costal pleura without any pulmonary lesions. There was a large amount of bloody pleural effusion on the left side. Pleural effusion cytology did not demonstrate any neoplastic findings. The course of the disease was rapidly progressive. A tentative clinical diagnosis of pleuritis carcinomatosa was made and percutaneous needle biopsies were obtained from the subpleural lesions. Histologically, most tumor cells infiltrated into a myxoid stroma and grew as a diffuse pattern (Fig. 1a). Sheets and nests of cells were absent. Inflammatory infiltrate was slight. The tumor cells were mainly composed of histiocytoid cells (Fig. 1b). These histiocytoid cells were ovoid or polyhedral in shape, with palely eosinophilic or foamy cytoplasm. Their nuclei were medium to large, ovoid or angulated, contained fine chromatin, and inconspicuous nucleoli (Fig. 1c). Multinucleated giant tumor cells were rarely recognized. Mitoses were 2/10 high-power fields (HPF). Necrosis and hemorrhagic areas were absent. Reactive histiocytes were sometimes present among these neoplastic cells. Immunohistochemical studies showed that these tumor cells were positive for AE1/AE3 (Fig. 2a), D2-40 (Fig. 2b), HBME-1, vimentin and WT-1 (Fig. 2c), but negative for BerEP4, CA125, calretinin, CAM5.2, CD3, CD15, CD30, CD34, CD45RB, CD79α, CEA, cytokeratin 5/6, cytokeratin 7, desmin, epithelial membrane antigen, napsin A, PE-10, PG-M1, S-100, Smooth-muscle actin, thrombomodulin and thyroid transcription factor-1. MIB-1 was positive in 20% of tumor cells. PAS reaction was positive in some tumor cells and was partly digested with diastase. Alcian blue stain was positive in stroma digested with hyaluronidase and negative in the cytoplasm. Colloidal iron stain was positive in the stroma and digested with hyaluronidase. Taken together, we made a diagnosis of pleural sarcomatoid malignant mesothelioma consisting of histiocytoid cells.

Recognition of this rare variant of malignant mesothelioma is important because of the possibility of confusing it with lymphohistiocytic variant of anaplastic large cell lymphoma (LHALCL), sarcomatoid carcinoma (SC) of the lung or thymus, inflammatory malignant fibrous histiocytoma (IMFH), inflammatory myofibroblastic tumor (IMT) or lymphohistiocytoid mesothelioma (LHM). In LHALCL, large-sized atypical cells with immunohistochemical CD30 positivity are admixed with histiocytes and plasma cells.[2] However, in this tumor, immunohistochemical studies showed that the atypical cells were negative for CD30. As for SC, lesions of the lung and anterior mediastinum were absent, and spindle or giant atypical cells were not apparent histologically in the present case. IMFH is composed of xanthogranulomatous inflammation with scattered atypical large cells with prominent nucleoli. The present tumor lacked xanthogranulomatous inflammation, and histiocytoid cells were not so large and nucleoli were inconspicuous. IMT is composed of a variable mixture of collagen, inflammatory cells and cytologically bland spindle cells having myofibroblastic differentiation. However, in the present case, collagen and these bland spindle cells were absent. LHM is a variant of sarcomatoid malignant mesothelioma, characterized by diffuse proliferation of large, ovoid histiocyte-like and spindle cells, uniformly intermixed with a prominent lymphocytic or lymphoplasmacytic infiltrate.[3,4] But in the present case the lymphocytic or lymphoplasmacytic infiltrate was subtle.

We made a diagnosis of PSMM consisting of histiocytoid cells based on percutaneous needle biopsy specimens. The keys to accurate diagnosis were as follows: (i) recognition of serosal tumor; (ii) immunohistochemical positivity for AE1/AE3, vimentin and mesothelial markers including D2-40,[5] WT1 and HBME-1; and (iii) being aware of the varied histopathological manifestations of PSMM.

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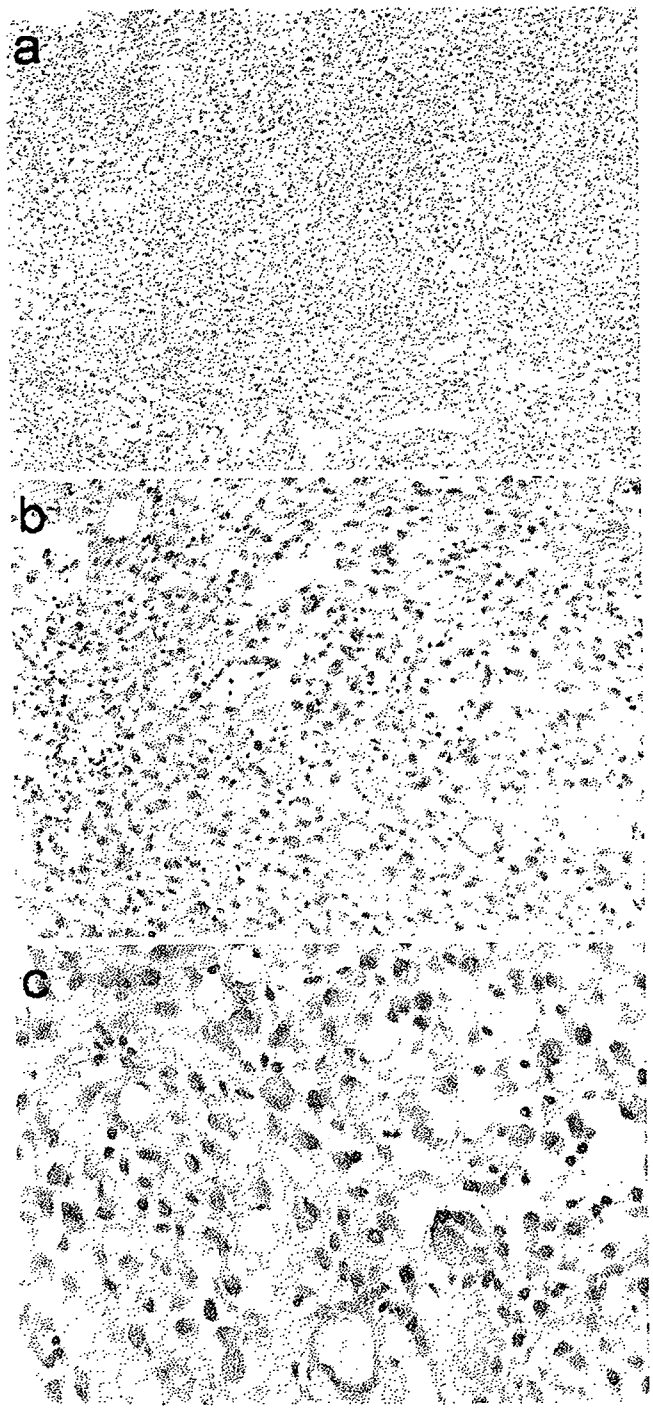

Figure 1 Microscopic view of the tumor. (a) Tumor cells infiltrating diffusely in a myxoid stroma. (b) Tumor cells mainly composed of histiocytoid cells. (c) The tumor cells were ovoid or polyhedral in shape with palely eosinophilic or foamy cytoplasm. Stain: HE.

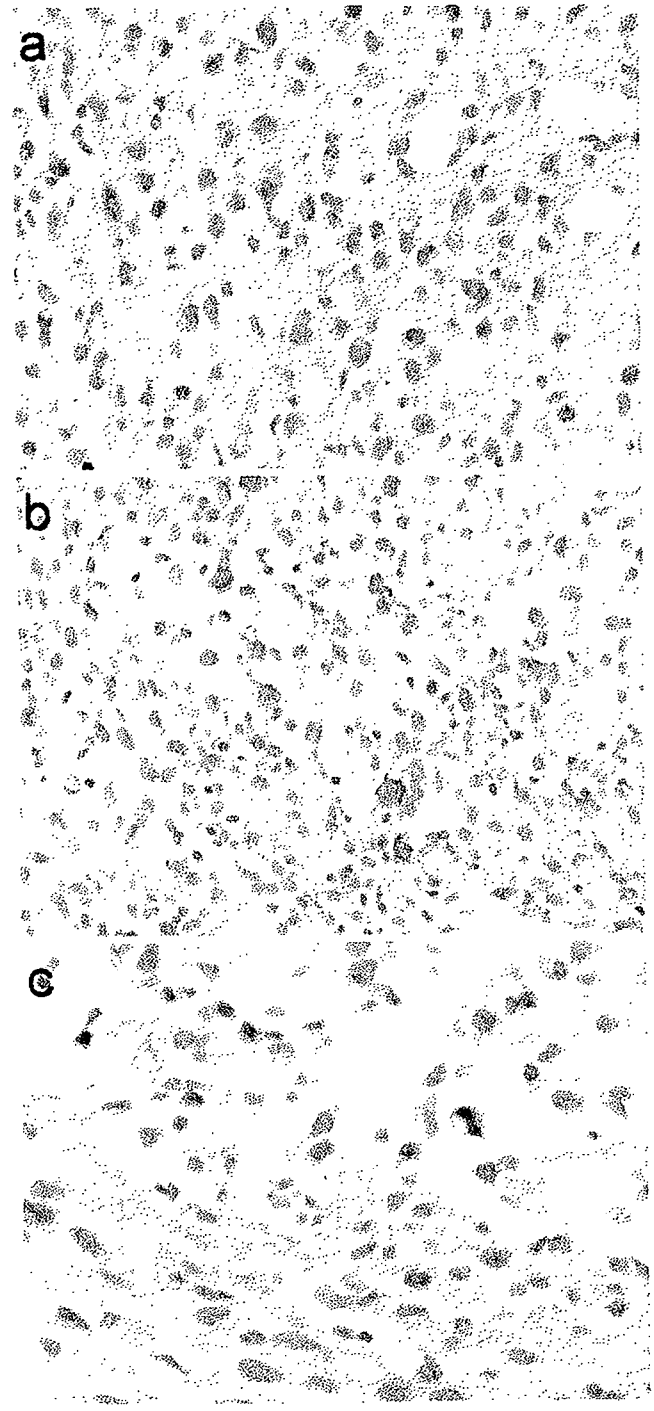

Figure 2 Immunohistochemical staining of the tumor. The histiocytoid cells had positive staining for (a) AE1/AE3, (b) D2-40 and (c) WT1.

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Polymorphisms, Mutations, and Amplification of the *EGFR* Gene in Non-Small Cell Lung Cancers

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Abbreviations: AI, allelic imbalance; CA-SSR1, CA simple sequence repeat 1; *EGFR, epidermal growth factor receptor*; FISH, flourescence in situ hybridization; HBEC, human bronchial epithelial cell; LAD, longer allele dominant; NSCLC, non-small cell lung cancer; PBMC, peripheral blood mononuclear cell; SAD, short allele dominant; SNP, single nucleotide polymorphism; TK, tyrosine kinase; TKI, tyrosine kinase inhibitor; WT, wild-type

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ABSTRACT

Background

The *epidermal growth factor receptor (EGFR)* gene is the prototype member of the type I receptor tyrosine kinase (TK) family and plays a pivotal role in cell proliferation and differentiation. There are three well described polymorphisms that are associated with increased protein production in experimental systems: a polymorphic dinucleotide repeat *(CA simple sequence repeat 1 [CA-SSR1])* in intron one (lower number of repeats) and two single nucleotide polymorphisms (SNPs) in the promoter region, –216 (G/T or T/T) and –191 (C/A or A/A). The objective of this study was to examine distributions of these three polymorphisms and their relationships to each other and to *EGFR* gene mutations and allelic imbalance (AI) in non-small cell lung cancers.

Methods and Findings

We examined the frequencies of the three polymorphisms of *EGFR* in 556 resected lung cancers and corresponding non-malignant lung tissues from 336 East Asians, 213 individuals of Northern European descent, and seven of other ethnicities. We also studied the *EGFR* gene in 93 corresponding non-malignant lung tissue samples from European-descent patients from Italy and in peripheral blood mononuclear cells from 250 normal healthy US individuals enrolled in epidemiological studies including individuals of European descent, African–Americans, and Mexican–Americans. We sequenced the four exons (18–21) of the TK domain known to harbor activating mutations in tumors and examined the status of the *CA-SSR1* alleles (presence of heterozygosity, repeat number of the alleles, and relative amplification of one allele) and allele-specific amplification of mutant tumors as determined by a standardized semiautomated method of microsatellite analysis. Variant forms of SNP –216 (G/T or T/T) and SNP –191 (C/A or A/A) (associated with higher protein production in experimental systems) were less frequent in East Asians than in individuals of other ethnicities ($p < 0.001$). Both alleles of *CA-SSR1* were significantly longer in East Asians than in individuals of other ethnicities ($p < 0.001$). Expression studies using bronchial epithelial cultures demonstrated a trend towards increased mRNA expression in cultures having the variant SNP –216 G/T or T/T genotypes. Monoallelic amplification of the *CA-SSR1* locus was present in 30.6% of the informative cases and occurred more often in individuals of East Asian ethnicity. AI was present in 44.4% (95% confidence interval: 34.1%–54.7%) of mutant tumors compared with 25.9% (20.6%–31.2%) of wild-type tumors ($p = 0.002$). The shorter allele in tumors with AI in East Asian individuals was selectively amplified (shorter allele dominant) more often in mutant tumors (75.0%, 61.6%–88.4%) than in wild-type tumors (43.5%, 31.8%–55.2%, $p = 0.003$). In addition, there was a strong positive association between AI ratios of *CA-SSR1* alleles and AI of mutant alleles.

Conclusions

The three polymorphisms associated with increased EGFR protein production (shorter *CA-SSR1* length and variant forms of SNPs –216 and –191) were found to be rare in East Asians as compared to other ethnicities, suggesting that the cells of East Asians may make relatively less intrinsic EGFR protein. Interestingly, especially in tumors from patients of East Asian ethnicity, *EGFR* mutations were found to favor the shorter allele of *CA-SSR1*, and selective amplification of the shorter allele of *CA-SSR1* occurred frequently in tumors harboring a mutation. These distinct molecular events targeting the same allele would both be predicted to result in greater EGFR protein production and/or activity. Our findings may help explain to some of the ethnic differences observed in mutational frequencies and responses to TK inhibitors.

The Editors' Summary of this article follows the references.

Introduction

Epidermal growth factor receptor (*EGFR*, also known as *ERBB1*) belongs to the *ERBB* gene family of receptor tyrosine kinases (TKs), and is a major regulator of several distinct and diverse signaling pathways [1–3]. It is frequently overexpressed in many malignancies including non-small cell lung cancer (NSCLC), and overexpression may be associated with a negative prognosis [4,5]. A recent finding that mutations of the gene in lung cancers predict, somewhat imprecisely, response to TK inhibitors (TKIs) has generated much interest [6–10]. Mutations are limited to the first four exons of the TK domain, and occur more often in individuals with adenocarcinoma histology, East Asian origin, female gender, and never smoker status. However, exceptions exist to the correlation between mutation status and response to TKIs, suggesting that other factors may play a role. Recently, *EGFR* amplification has been identified as a further factor that may predict response to therapy [11,12]. Experimental evidence indicates that polymorphisms of the gene may also regulate protein expression.

CA simple sequence repeat 1 (CA-SSR1) is a highly polymorphic locus containing 14–21 CA dinucleotide repeats and is located at the 5′ end of the long intron one of the *EGFR* gene, lying upstream and in close proximity to a second enhancer [13,14]. The allele size distribution of *CA-SSR1* demonstrates ethnic differences, with East Asians having longer repeats than individuals of European descent or African-Americans [15]. By interacting with the second or downstream enhancer, a lower *CA-SSR1* repeat number was found to modulate *EGFR* transcription in vivo and in vitro, and to be correlated with increased transcription and protein expression [13,14].

The relationship between *CA-SSR1* repeat length and *EGFR* overexpression has been extensively studied in breast cancers [16,17]. Localized amplification of the *CA-SSR1* repeat, usually limited to the shorter allele, occurs frequently in breast cancers, is related to *EGFR* expression, and demonstrates a field effect, indicating that it is an early event during multistage pathogenesis [18]. In head and neck cancer, patients with a lower number of *CA-SSR1* repeats (total of both alleles < 35 repeats) had a statistically significantly increased likelihood of responding to erlotinib [19].

In addition to *CA-SSR1*, two kinds of single nucleotide polymorphisms (SNPs) in the promoter region may correlate with increased promoter activity and expression of *EGFR* mRNA. One of the SNPs is located −216 bp upstream from the initiator ATG (adenine as +1), and the change of nucleoside is guanine to thymine. This is an important binding site for the transcription factor SP1 that is necessary for activation of *EGFR* promoter activity [20]. The variant forms, −216 G/T or T/T, are more frequent in individuals of European descent and African-Americans than in Asians [21]. The other SNP, −191 C/C, is located in the *EGFR* promoter region near one of four transcription regions (−214 to −200) [22]. This SNP may also be associated with increased protein expression, and the minor forms, −191 C/A or A/A are also rare among Asians [21].

For the reasons discussed above, we investigated the distribution of these SNPs in lung cancer patients and healthy individuals of various ethnicities, the length and allelic imbalance (AI) of *CA-SSR1* in lung cancer patients, and the relationship between AI of *CA-SSR1* and allele-specific amplification in lung cancer patients with mutations of the *EGFR* gene.

Methods

Because of the multiple, complex studies performed in this report, we summarize the salient investigations and their results in Table 1.

Human Bronchial Epithelial Cell and Lung Cancer Cell Lines

All cancer cell lines were cultured in RPMI 1640 (Life Technologies, Rockville, Maryland, United States) supplemented with 5% fetal bovine serum and incubated in humidified air and 5% CO_2 at 37 °C. Most cell lines were established by us at one of two locations. The prefix NCI indicates cell lines established at the National Cancer Institute, and the prefix HCC indicates cell lines established at the Hamon Center for Therapeutic Oncology Research of the University of Texas Southwestern Medical Center.

Human bronchial epithelial cells (HBECs) from healthy individuals or those with lung cancer were immortalized and cultured by us as previously described [23,24]. The cells were cultured in K-SFM medium (Life Technologies) and included 5 ng/ml EGF.

Clinical Samples

A total of 556 samples of primary lung cancers including adenocarcinomas ($n = 345$, 62%), squamous cell carcinomas ($n = 182$, 33%), adenosquamous carcinomas ($n = 16$, 3%), and large cell carcinomas ($n = 10$, 2%) were obtained from four countries, the US, Australia, Japan, and Taiwan, and included 336 (60%) tumors from East Asians and 220 (40%) from other ethnicities (97% of whom were of European descent). None of the cases had prior treatment with TKIs. Samples of tumor containing relatively high percentages of tumor (>70%) were selected and analyzed without microdissection.

Corresponding non-malignant lung tissues were available from 450 of the samples. We also obtained 93 DNA samples from non-malignant lung tissue of European-descent patients with lung cancer in Italy and 250 DNA samples of peripheral blood mononuclear cells (PBMCs) from healthy individuals of European descent ($n = 75$), African-Americans ($n = 75$), and Mexican-Americans ($n = 100$) enrolled in ongoing epidemiological studies in the US for investigation of frequencies of the polymorphisms (Table 2). Institutional Review Board permission and informed consent were obtained at each collection site.

DNA Extraction

Genomic DNA was isolated from cell lines, frozen primary tumors, and non-malignant tissues by digestion with 100 μg/ml proteinase K (Life Technologies) followed by standard phenol-chloroform (1:1) extraction and ethanol precipitation [25].

EGFR Gene Mutations

Details about *EGFR* mutation types and methodologies for mutation detection have been published elsewhere [9]. Briefly, we sequenced exons 18–21 of the TK domain of *EGFR* in tumor and corresponding non-malignant tissues. The overall frequency of mutation was 20%, and there were