Patient subset analyses of these randomised phase III trials or retrospective trials (Kaneda et al, 2004; Miller et al, 2004) clearly show the existence of populations that are more likely to respond to gefitinib and erlotinib, including women, patients with adenocarcinoma (especially with bronchial alveolar carcinoma (BAC)), nonsmokers and Asian patients (compared with Caucasians). Somatic mutations in specific regions of exons 18, 19 and 21 of the ATP-binding domain of EGFR have recently been shown to have strong associations with sensitivity to gefitinib or erlotinib (Lynch et al, 2004; Paez et al, 2004; Pao et al, 2004). Consistent with these findings, the frequencies of these EGFR mutations were higher in women, patients with adenocarcinoma, nonsmokers and Asians, all of whom are among the more frequent responders, as mentioned above (Shigematsu et al, 2005). There are two characteristic types of EGFR mutations. One is the presence of in-frame deletions, including the amino acids at codons 746–750 in exon 19, and the other is an amino-acid substitution at codon 858 (L858R) in exon 21. Recent analyses (Bell et al, 2005) of phase II and III trials for EGFR-TKI, in which patients were not selected based on their mutation status, have suggested that EGFR mutations are correlated with response to therapy but are not correlated with overall survival (OS). Furthermore, EGFR gene amplification/copy number (Cappuzzo et al, 2005; Hirsch et al, 2005) or overexpression (Hirsch et al, 2003) has been shown to be a more useful prognostic marker of response to gefitinib treatment. Patient selection according to EGFR mutation status may yield a superior survival rate by excluding patients who are unlikely to respond to gefitinib treatment. However, other populations that might obtain a clinical benefit from gefitinib treatment, even in the absence of EGFR mutation, may exist.

Three Japanese groups (Asahina et al, 2006; Inoue et al, 2006; Yoshida et al, 2007) have reported prospective phase II studies of gefitinib for advanced-stage NSCLC that were designed to consider the EGFR mutation status of the patients. All of these studies have reported a high response rate and extended progression-free survival (PFS) period, compared with historical controls. However, all of these studies had a relatively short observation period, making the data preliminary. Moreover, the original sample size was calculated after patient selection, and a critical consideration of the suitability of the assay used to detect the mutations (which was performed using small paraffin-embedded specimens obtained from bronchoscopic biopsies), and the estimated EGFR-positive rate were lacking. Additionally, all the trials were conducted at single institutions located in one small area of Japan. Thus, the published data may not be representative of the situation found in general clinical practice throughout Japan and therefore may not directly translate to the general feasibility of gefitinib treatment in Japan.

In view of this situation, we performed a multicentre prospective phase II trial of gefitinib for advanced NSCLC harbouring EGFR mutations. We prospectively registered patients from 15 different institutes in Japan at the beginning of EGFR mutation screening using a central database. Whether or not tissue was available from a bronchoscopic biopsy or surgery was not an inclusion criterion. All the clinical samples from the registered patients were delivered to a central laboratory that then determined the EGFR mutation status or the histological BAC features. The analysis of the survival data was based on a minimum observation period of at least 15 months from the time of entry of the last patient.

MATERIALS AND METHODS

Eligibility criteria

Eligible patients had histologically confirmed stage III NSCLC for which thoracic irradiation was not indicated or were stage IV. Chemotherapy-naive patients or those who had previously received up to two prior chemotherapy regimens, including those performed in an adjuvant setting, were eligible. Other eligibility criteria included an age $\geqslant 20$ years, measurable disease, the availability of sufficient amounts of tumour specimen for EGFR mutation analysis, an Eastern Cooperative Oncology Group performance status of 0–2, adequate organ function (WBC $\leqslant 3000\,\mu l^{-1}$, platelets $\geqslant 75\,000\,\mu l^{-1}$, AST and ALT $\leqslant 100\,IU\,l^{-1}$, serum creatinine $\leqslant$ twice the upper limit of the reference range; $P_{aO2} \geqslant 60\,mm\,Hg$). The exclusion criteria included pulmonary fibrosis, the presence of symptomatic brain metastasis, active concomitant malignancy, severe heart disease, active gastrointestinal bleeding and continuous diarrhoea. All the patients signed a written informed consent form. Approval of this study and the gene analyses were obtained from the Institutional Review Board and the Ethics Committee of each hospital.

EGFR gene analysis

Tumour specimens were obtained using bronchial fiberscope or surgical procedures. The specimens were fixed with formalin and embedded in paraffin. Four slices (4–5 μm) from the embedded block were sent to a central laboratory (Mitsubishi Chemical Safety Institute Ltd., Ibaraki, Japan) for genetic analysis. Most of the tumour specimens were available prior to the registration of this study. Genomic DNA was isolated from specimens using QIAamp Micro kits (QIAGEN KK, Tokyo, Japan). The EGFR mutations in exons 18, 19 and 21, as previously reported (Lynch et al, 2004; Paez et al, 2004), were determined using polymerase chain reaction (PCR) amplification and intron–exon boundary primers according to the published method. An EGFR registrant mutation in exon 20, which was reported by Pao et al (2005) was also examined using PCR and the previously reported primers. Polymerase chain reaction was performed using a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA), and the PCR products were confirmed using a Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA, USA), then sequenced directly using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and ABI PRISM 3100 (Applied Biosystems). All sequencing reactions were performed in both forward and reverse directions and were analysed using the Basic Local Alignment Search Tool (BLAST); all the electropherograms were reanalysed by visual inspection to check for mutations. The presence of an EGFR mutation was confirmed using at least three independent PCR.

All sequence data were sent from the central laboratory to Kinki University. A principle investigator then confirmed whether or not the EGFR mutation status was positive, and the results were sent to the West Japan Thoracic Oncology Group (WJTOG) data centre. The data centre then informed each participating centre of the results of the genetic analysis and requested that the eligibility criteria of the patients be rechecked to insure that only EGFR-positive subjects were registered in the trial. Each tumour was categorised according to histology by a pulmonary pathologist (JF). The percentage of area exhibiting a BAC pattern was also examined to determine the WHO pathological category.

Treatment plan

Gefitinib (250 mg day^{-1}) was administered once daily. Treatment was continued uninterrupted until disease progression or intolerable toxicity (grade 4 nonhaematological toxicities, any incidents of interstitial pneumonia or a treatment delay of more than 2 weeks because of adverse effects). Gefitinib administration was delayed if the patient's leukocyte and platelet counts were lower than 1500 and 5000 μl^{-1}, respectively, and was withheld until these counts had recovered. Gefitinib administration was also delayed if grade 3 or greater nonhaematological toxicities without nausea, vomiting or alopecia occurred and was withheld until recovery to grade 2.

Routine clinical and laboratory assessments and chest X-ray assessments were performed weekly or biweekly, where possible; CT examinations of the target lesion were performed every month, and magnetic resonance imaging of the whole brain and a bone scan were performed every 3 months. The objective responses of the patients were evaluated every month using the Response Evaluation Criteria in Solid Tumours (RECIST) guidelines (Therasse et al, 2000). Tumour response was centrally evaluated by independent reviewers at an extramural conference and was performed for the intent-to-treat population. All adverse effects that occurred during gefitinib treatment were reported, and the severity of the effects was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0.

Statistical analyses

The primary end point of this study was the response rate. A one-stage design using the binominal probability was used to determine the sample size. Assuming that a response rate of 50% would indicate potential usefulness, whereas a rate of 25% would be the lower limit of interest, and with $\alpha = 0.10$ (two side) and $\beta = 0.20$, the estimated accrual number was 23 patients. Estimating that the EGFR-positive rate would be about 20%, the screening number required to accrue 23 EGFR-positive patients was 115. After assuming an inevaluability rate of < 10%, the final required screening number was 125.

The secondary end points of this study were toxicity, OS, PFS, 1-year survival (1Y-S) and the disease control rate (DCR). Survival analyses were conducted on the intent-to-treat population using follow-up data available as of 30 April 2007. The survival curves were estimated using Kaplan–Meier plots.

RESULTS

Patient characteristics

Between March 2005 and January 2006, 118 patients were prospectively screened from 15 institutions; 117 of them underwent EGFR mutation analysis (tumour tissue was not available for one patient). The median time required for the EGFR mutation analysis was 12 days (range: 7-28 days). Among the 117 patients, EGFR mutations were detected in 32 patients (27%), 14 of whom had a deletion in or near E746-A750 (including one del E746-T751 ins A, two del L747-T751 and one del L747-T753 ins S) in exon 19. A further 17 had L858R, and one had a L861Q point mutation in exon 21 (Table 1).

Tissue samples from 17 patients (53%) were obtained by transbronchial biopsy. The EGFR detection rates for the surgical specimens and the bronchoscopic biopsy specimens were similar (30 vs 25%). The EGFR mutations were significantly more frequent in women ($P \leqslant 0.02$), in patients with adenocarcinoma ($P = 0.001$) and in people who had never smoked ($P < 0.001$) (Table 2). Finally, 28 patients (14 with deletions in exons 19 and 14 with point mutations in exon 21) were actually registered and received treatment with gefitinib, whereas four patients were dropped from the study as they became ineligible because of tumour progression during the time required for the mutation analysis.

Patient characteristics are listed in Table 3. In the initial screening, there were 56 female patients (48%), 97 patients (83%) with adenocarcinoma and 53 (45%) who had never smoked. The frequency of these characteristics was higher among the patients with EGFR mutations who were actually registered; namely, 18 patients (64%) were women, 27 (96%) had adenocarcinoma and 19 (68%) had never smoked. The median age of the 28 actually registered patients was 68 years; 24 patients (86%) had a good performance status (0-1), 22 (79%) had stage IV diseases and 17

Table 1 Type of EGFR mutations (n = 32)

Characteristics	No. of patients	%
Exon 18	0	0
Exon 19	14	44
del E746-A750	10	32
del E746-T751 ins A	1	3
del L747-T751	2	6
del L747-T753 ins S	1	3
Exon 21	18	56
L858R	17	53
L861Q	1	3

EGFR = epidermal growth factor receptor.

Table 2 Relationship between patient characteristics and EGFR mutation status

Characteristics	EGFR mutation positive (n = 32)		EGFR mutation negative (n = 85)		
	No. of Patients	%	No. of Patients	%	P
Sex					
Male	11	34	50	59	
Female	21	66	35	41	<0.02
Histology					
Adenocarcinoma	31	97	66	78	
Nonadenocarcinoma	1	3	19	22	=0.001
Smoking status					
Never	21	66	31	36	
Current/former	11	34	54	64	<0.001

EGFR = epidermal growth factor receptor.

(61%) were chemotherapy naive. Thoracic irradiation was contraindicated in one patient with stage IIIA disease because of the large irradiation field that would have been required. All five patients with stage IIIB diseases had malignant effusions. Four patients had received adjuvant therapies; five had received platinum doublets or a combination of gemcitabine and vinorelbine as their first-line therapy. Two patients had received two regimens of platinum doublets followed by docetaxel or pemetrexed. One patient had received local radiation for pain control.

Response and survival

The objective tumour responses are listed in Table 4. The overall response rate and DCR were 75% (95% CI: 57.6-91.0%) and 96% (95% CI: 87.0-96.4%), respectively. Five out of ten male patients (50%), six out of nine smokers (67%) and five out of eight male smokers with adenocarcinoma (63%) achieved a PR. One female nonsmoker with squamous cell carcinoma also achieved a PR. Among the registered patients with EGFR mutations, the response rate was no different between current/former smokers and those who had never smoked (67 vs 79%) or between chemotherapy-naive and postchemotherapy patients (77 vs 73%). Female and patients with a mutational deletion in exon 19 tended to have a higher response rate than male (89 vs 50%) and patients with a missense mutation in exon 21 (86 vs 64%), respectively.

The median follow-up time was 18.6 months (range: 13.8-23.4 months). The median PFS time was 11.5 months (95% CI: 7.3 months to -) (Figure 1A). The median OS has not yet been reached, and the 1Y-S was 79% (95% CI: 63.4-93.8%) (Figure 1B).

Table 3 Patient characteristics of all registered patients (n = 28)

Characteristics	No. of patients (%)
Age	
Median	68
Range	49–89
Performance status	
0	11 (39)
1	13 (47)
2	4 (14)
Sex	
Male	10 (36)
Female	18 (64)
Histology	
Adenocarcinoma	27 (96)
Squamous cell carcinoma	1 (4)
Large cell carcinoma	0 (0)
Adenosquamous carcinoma	0 (0)
Other	0 (0)
Smoking status	
Never	19 (68)
Current/former	9 (32)
Stage	
IIIA[a]	1 (3)
IIIB	5 (18)
IV	22 (79)
Prior cancer therapy	
Chemotherapy	
No	17 (61)
One regimen (adjuvant)	4 (14)
One regimen (not adjuvant)	5 (18)
Two regimens	2 (7)
Recurrence after surgery	11 (39)
Radiation	1 (4)

[a]Unresectable, no indication for thoracic radiation because of a large radiation field.

Table 4 Response rate (n = 28)

Response	No. of patients	Response rate (%)	95% CI
Complete response	1	3.6	
Partial response	20	71.4	
Stable disease	6	21.4	
Progressive disease	0	0.0	
Not evaluable[a]	1	3.6	
Overall response	21	75.0	57.6–91.0
Disease control rate	27	96.4	87.0–96.4

CI = confidence interval. [a]One patient was not evaluable because of a poor evaluation of efficacy.

Safety and toxicity

Toxicity was evaluated in all eligible patients (Table 5). The most frequent adverse events were rash, dry skin, diarrhoea, stomatitis and elevated AST/ALT levels. Two patients experienced grade 3 rash and one patient experienced grade 3 keratitis; however, these patients all achieved a PR, and the adverse effects subsided after pausing gefitinib treatment for around 2 weeks. Four patients experienced grade 3 hepatotoxicity; three of these patients had to discontinue treatment for this reason.

One patient developed interstitial lung disease (ILD) (Ando et al, 2006). Ground-glass opacity was detected in the right upper lobe 19 days after the start of gefitinib administration, resulting in the cessation of treatment. However, the lesion enlarged into bilateral lung fields on day 25, and steroid therapy was initiated. Nonetheless, the patient died of respiratory failure on day 48. Two patients also experienced grade 1 ILD. They recovered without steroid administration.

Subsequent treatment after disease progression

Of the 14 patients who become refractory to gefitinib and exhibited disease progression, 10 received chemotherapy as their first treatment regimen after gefitinib (Table 6); 5 patients received platinum doublets and 1 patient received vinorelbine as a second-line treatment; and 3 received docetaxel and 1 received platinum doublet as a third-line treatment. In all, 4 out of the 10 patients (40%) had a PR. Of the nine patients who become refractory to the first treatment regimen after gefitinib, six received chemotherapy as their second regimen after gefitinib, including one who received gemcitabine, one who received docetaxel, and one who was re-treated with gefitinib as a third-line therapy; two other patients received docetaxel and one was re-treated with gefitinib as a fourth-line therapy. Two of the six patients (33%) had a PR. The two patients who received gefitinib re-treatment both had SD.

## BAC features, *EGFR* amplification and T790M mutation in exon 20

A total of 110 tissue samples were available for pathological review, of which 90 were from adenocarcinoma; 33 of these specimens (37%) revealed proportional BAC components in the specimen. Among them, 15 were considered extensive and the remaining 18 were found to have minor BAC components. The 39 surgical specimens included 36 from adenocarcinomas. The *EGFR* mutations were detected in 12 out of the 36 adenocarcinoma specimens. None of the samples with a BAC component, micropapillary pattern or mucin production was associated with an *EGFR* mutation (Table 7).

Data on *EGFR* gene copy numbers were available in only 12 samples. We used the criteria for defining a high *EGFR* gene copy number (gene amplification or high polysomy, as determined using FISH) that were described in a previous report (Cappuzzo et al, 2005). A total of 7 out of the 12 samples had a high gene copy number (FISH positive), and 6 (3 with *EGFR* mutations) out of the 7 samples had proportional BAC components. In all, 5 out of the 12 samples were FISH negative, only 1 (with no *EGFR* mutation) of which had a BAC component. Two patients that were FISH negative, BAC negative and *EGFR* mutation positive had SD when treated with gefitinib.

Another *EGFR* mutation, T790M in exon 20, has been reported to be associated with resistance to gefitinib (Kobayashi et al, 2005; Pao et al, 2005). We checked for this mutation in six patients who did not respond to gefitinib; however, the mutation could not be identified in any of the patients.

DISCUSSION

We performed a multicentre phase II study examining the use of gefitinib for advanced NSCLC in patients with *EGFR* mutations, prospectively recruiting patients at the time of genetic screening and avoiding a selection bias. All patients were registered in a central database. All tissues were delivered from the local participants to the central facility, where they were reviewed by a pathology specialist and the *EGFR* mutation status was evaluated. The median time for the *EGFR* mutation detection analysis was 12 days, which is probably an acceptable time lag before the start of treatment for advanced NSCLC. However, a shorter period would clearly be desirable for routine clinical practice. Indeed, 4 out of the 32 *EGFR*-positive patients were dropped from the study because of disease progression before their actual registration

1178
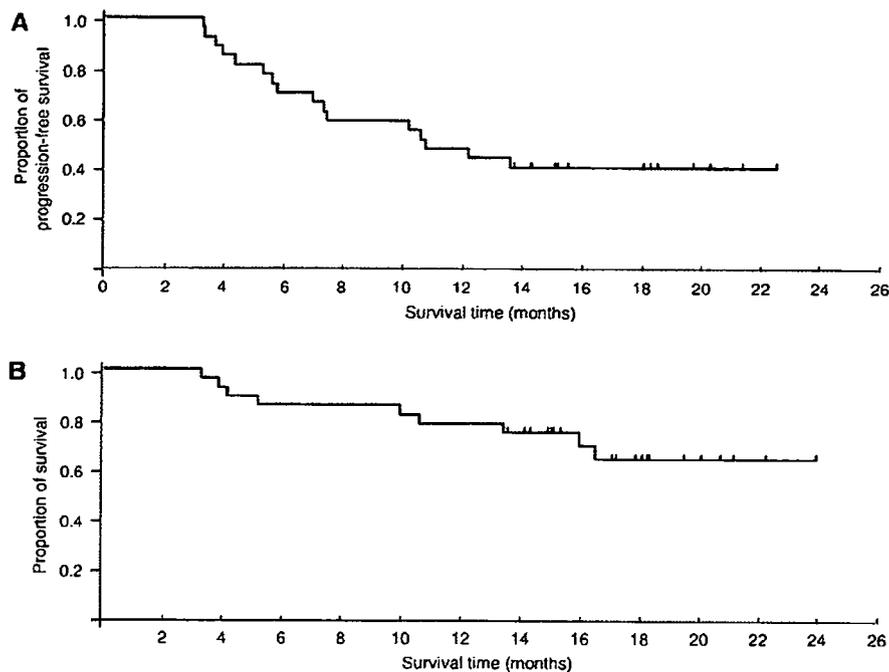
Figure I (**A**) Progression-free survival (PFS) and (**B**) overall survival (OS) of all eligible patients (n = 28). The median PFS was 11.5 months. The median OS has not yet been reached. The 1-year survival rate was 79%.

Table 5 Common adverse events (n = 28)

Adverse events	No. of patients (%)			
	Grade I	Grade 2	Grade 3	Grade 4
Haematologic				
Anaemia	12 (43)	3 (11)	0 (0)	0 (0)
Leucopaenia	4 (14)	1 (4)	2 (7)	0 (0)
Neutropaenia	4 (14)	1 (4)	1 (4)	0 (0)
Thrombocytopaenia	3 (11)	0 (0)	0 (0)	0 (0)
Nonhaematologic				
Rash	10 (36)	11 (39)	2 (7)	0 (0)
Dry skin	9 (32)	10 (36)	0 (0)	0 (0)
Nail changes	5 (18)	2 (7)	0 (0)	0 (0)
Keratitis	0 (0)	0 (0)	1 (4)	0 (0)
Fever	0 (0)	1 (4)	0 (0)	0 (0)
Fatigue	3 (10)	3 (10)	3 (10)	0 (0)
Diarrhoea	7 (25)	1 (4)	0 (0)	0 (0)
Constipation	1 (4)	0 (0)	0 (0)	0 (0)
Stomatitis	8 (29)	1 (4)	0 (0)	0 (0)
Gastritis	1 (4)	0 (0)	0 (0)	0 (0)
Anorexia	2 (7)	1 (4)	0 (0)	0 (0)
Nausea	3 (11)	1 (4)	0 (0)	0 (0)
Vomiting	2 (7)	2 (7)	1 (4)	0 (0)
Dyspnoea	2 (7)	0 (0)	1 (4)	0 (0)
ILD	2 (7)	0 (0)	0 (0)	1 (4)[a]
Vertigo	1 (4)	1 (4)	0 (0)	0 (0)
Dysgeusia	0 (1)	1 (4)	0 (0)	0 (0)
Elevated AST/ALT	10 (36)	2 (7)	4 (14)	1 (4)[a]
Elevated creatinine	2 (7)	1 (4)	2 (7)	0 (0)

ALT = alanine transaminase; AST = aspartate transaminase; ILD = interstitial lung disease. [a]Same patient.

could occur. Yatabe et al (2006) has developed a rapid assay to detect EGFR mutations, and we have decided to use this assay in a phase III trial. The EGFR mutation rates in transbronchial biopsy samples were found to be the same as those in surgical specimens, suggesting that this assay can also accommodate stage IV NSCLC. We detected the two characteristic types of EGFR mutations (in exons 19 and 21) in 44 and 56% of the patients, respectively (Table 1); these percentages are identical to those in previous reports from Japan (Shigematsu et al, 2005; Asahina et al, 2006; Inoue et al, 2006; Yatabe et al, 2006; Yoshida et al, 2007). In summary, we confirmed the feasibility of using the EGFR detection assay in daily practice.

The overall response rate was 75%, which was comparable to those of other phase II studies of gefitinib in patients with EGFR mutations (Asahina et al, 2006; Inoue et al, 2006), despite our study permitting the entry of patients who had previously received up to two chemotherapy regimens. The DCR of 96% was relatively high, and the median PFS of 11.5 months and 1Y-S of 79% were also very promising. In a Korean study, Lee et al (2006) also reported a very promising response rate (56%) and 1Y-S (76%) for gefitinib in a prospective study of selected NSCLC patients with adenocarcinoma and never/light smokers, defined as having smoked no more than 100 cigarettes during one's lifetime. In the screening process for the present study, EGFR mutations were significantly more frequent in women, patients with adenocarcinoma and those who had never smoked. However, among the patients who were selected according to their EGFR mutation status, no differences in response were observed between never smokers and current/former smokers or between chemotherapy-naive and postchemotherapy patients. In a retrospective study, Han et al (2006) directly compared clinical predictors (smoking history, gender and histology) and the EGFR mutation status for their ability to predict response and survival. They showed that female never smokers with adenocarcinoma (three clinical predictors) had a 33% response rate, whereas patients with a positive EGFR mutation status had a 62% response rate. Furthermore, in a multivariate analysis, only a positive EGFR mutation status was associated with an improved OS, suggesting that the EGFR mutation status should be analysed whenever possible to optimise response predictions based on clinical

Table 6 Subsequent treatments after failure to respond to gefitinib (n = 28)

Gefitinib treatment	No. of Patients	1st regimen after gefitinib	No. of patients	2nd regimen after gefitinib	No. of patients
1st line	17	Plt doublet	5	Gem or Doce	2
				Gefitinib[a]	1
		VNR	1	—	-
2nd line[b]	4	Doce	2	Doce	1
		Plt doublet	1	Doce	1
2nd line	5	Doce	1	Gefitinib[a]	1
3rd line	2	—	—	—	—
Total	28		10		
Response			4/10		2/6

Doce = docetaxel; Gem = gemcitabine; Plt = platinum; VNR = vinorelbine. [a]Both patients had an SD response after gefitinib re-treatment. [b]First regimen as systemic chemotherapy after adjuvant treatment.

Table 7 Bronchial alveolar carcinoma (BAC) features and EGFR mutation status

	EGFR mutation		
	+	−	P-value
Surgically resected adenocarcinoma case	12	24	
BAC component			
Yes	8	17	1.0
No	4	7	
Micropapillary pattern			
Yes	4	12	0.18
No	8	12	
Mucin production			
Yes	1	5	1.0
No	11	19	

EGFR = epidermal growth factor receptor.

background factors. In the present study, EGFR mutations were detected in 16 out of 40 (40%) female never smokers with adenocarcinoma who underwent the screening process, and 14 out of these 16 patients (88%) achieved a response after undergoing gefitinib therapy. We could not compare the predictive powers of clinical predictors and the EGFR mutation status with regard to the clinical benefits of gefitinib in this study. Thus, the need for EGFR mutation testing among clinically favourable patients remains uncertain. Decisions regarding the first-line therapy of choice for patients with EGFR mutations or a clinically favourable profile (nonsmoker with adenocarcinoma) must also await the results of an ongoing randomised phase III study in an Asian population (IPASS: Iressa Pan-Asian Study) comparing platinum doublets with gefitinib.

In contrast, 50% of the men, 67% of the smokers and 63% of the men who were smokers achieved a PR in this study. Furthermore, one female nonsmoker with squamous cell carcinoma also responded to gefitinib. The histological type of this tumour was reassigned by a pulmonary pathologist, and the tumour was finally confirmed to be a squamous cell carcinoma. Squamous cell carcinoma harbouring an EGFR mutation is rarely seen but has been previously reported (Asahina et al, 2006). In a Japanese phase II trial of gefitinib for unselected chemotherapy-naive patients (Niho et al, 2006), the response rates among smokers, men, and patients with nonadenocarcinoma were 19, 13 and 10%, respectively. Thus, NSCLC patients who are either smokers, men or have a nonadenocarcinoma histology are unlikely to receive gefitinib treatment as a first-line treatment instead of standard chemotherapies (platinum doublets), which yield a response rate of about 30% (Schiller et al, 2002). Therefore, EGFR mutation screening may

have a higher impact on the selection of responders to gefitinib treatment among these kinds of Asian patient subset (for example, smokers with adenocarcinoma, and nonsmoking men or women with nonadenocarcinoma).

The benefit of chemotherapy in general among patients with EGFR mutations, compared with EGFR mutation-negative patients, remains uncertain. Previous studies (Bell et al, 2005) have suggested that patients with EGFR mutations tend to be more sensitive to chemotherapy than those with wild-type EGFR. In the present study, 40 and 33% of the patients responded to first- and second-line chemotherapy regimens after gefitinib, respectively. These relatively high response rates for refractory NSCLC suggest that patients with an EGFR mutation-positive status are generally sensitive to chemotherapy. Large-scale multivariate analyses, using pooled data from prospective phase II or III trials in which the EGFR mutation status was clearly confirmed, are needed to clarify this point.

The toxicities observed in the present study were mostly tolerable. Most of the common adverse events, like rash, diarrhoea or hepatotoxicity, were mild and subsided after gefitinib administration was paused for a short period. One male smoker with adenocarcinoma died of ILD. Thus, even among patients who are selected based on their EGFR mutation status, men or smokers may still be at risk for developing ILD; therefore, biomarkers to predict ILD are needed.

Patients with exon 19 mutations tended to have a higher response rate than those with a missense mutation in exon 21, consistent with the findings of previous reports (Jackman et al, 2006; Riely et al, 2006). The Spanish Lung Cancer Group also reported on a prospective phase II study of erlotinib in advanced NSCLC patients with EGFR mutations (Paz-Ares et al, 2006). The overall response rate was 82%. They also showed a difference in response rates between patients with mutations in exons 19 and 21 (95 and 67%, respectively). Exon 11 c-kit mutations are more closely correlated with a good prognosis in patients with gastrointestinal stromal tumour, who may benefit from lower doses of imatinib, whereas patients with exon 9 mutations may require higher doses (Debiec-Rychter et al, 2006). In the case of EGFR, functional differences between mutation types may also exist.

We found no discernible associations between the EGFR mutation frequency and the presence of a BAC component. Several reports, including that of Hirsch et al (2005) suggest that a higher EGFR copy number is correlated with BAC histological features. We also found an association between a high EGFR copy number and the presence of a BAC component, even though the number of specimens examined was relatively small. In a study on erlotinib, the presence of a BAC component was clearly associated with EGFR amplification. As the EGFR mutation rate is lower in western populations than in Asian populations, the EGFR gene copy number might be a more useful biomarker in western populations, especially with regard to the use of erlotinib.

In conclusion, gefitinib treatment for patients with advanced NSCLC harbouring an EGFR mutation demonstrated a promising activity in patients with a good performance status. Patient screening according to EGFR mutation status may be a useful tool in daily practice and will likely have a great impact on the selection of patients who are likely to benefit from gefitinib treatment.

REFERENCES

Asahina H, Yamazaki K, Kinoshita I, Sukoh N, Harada M, Yokouchi H, Ishida T, Ogura S, Kojima T, Okamoto Y, Fujita Y, Dosaka-Akita H, Isobe H, Nishimura M, on behalf of the Hokkaido Lung Cancer Clinical Study Group (2006) A phase II trial of gefitinib as first-line therapy for advanced non-small cell lung cancer with epidermal growth factor receptor mutations. *Br J Cancer* 95: 998–1004

Ando M, Okamoto I, Yamamoto N, Takeda K, Tamura K, Seto T, Ariyoshi Y, Fukuoka M (2006) Predictive factors for interstitial lung disease, antitumor response and survival in non-small-cell lung cancer patients treated with gefitinib. *J Clin Oncol* 24: 2549–2556

Bell DW, Lynch TJ, Haserlat SM, Harris PL, Okimoto RA, Brannigan BW, Sgroi DC, Muir B, Riemenschneider MJ, Iacona RB, Krebs AD, Johnson DH, Giaccone G, Herbst RS, Manegold C, Fukuoka M, Kris MG, Baselga J, Ochs JS, Haber DA (2005) Epidermal growth factor receptor mutations and gene amplification in non-small-cell lung cancer: molecular analysis of the IDEAL/INTACT gefitinib trials. *J Clin Oncol* 23: 8081–8092

Cappuzzo F, Hirsch FR, Rossi E, Bartolini S, Ceresoli GL, Bemis L, Haney J, Witta S, Danenberg K, Domenichini I, Ludovini V, Magrini E, Gregorc V, Doglioni C, Sidoni A, Tonato M, Franklin WA, Crino L, Bunn Jr PA, Varella-Garcia M (2005) Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. *J Natl Cancer Inst* 97: 643–655

Debiec-Rychter M, Sciot R, Le Cesne A, Schlemmer M, Hohenberger P, van Oosterom AT, Blay JY, Leyvraz S, Stul M, Casali PG, Zalcberg J, Verweij J, Van Glabbeke M, Hagemeijer A, Judson I, EORTC Soft Tissue and Bone Sarcoma Group, The Italian Sarcoma Group, Australasian GastroIntestinal Trial Group (2006) KIT mutations and dose selection for imatinib in patients with advanced gastrointestinal stromal tumours. *Eur J Cancer* 42: 1093–1103

Fukuoka M, Yano S, Giaccone G, Tamura T, Nakagawa K, Douillard JY, Nishiwaki Y, Vansteenkiste J, Kudoh S, Rischin D, Eek R, Horai T, Noda K, Takata I, Smit E, Averbuch S, Macleod A, Feyereislova A, Dong RP, Baselga J (2003) Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. *J Clin Oncol* 21: 2237–2246

Han SW, Kim TY, Lee KH, Hwang PG, Jeon YK, Oh DY, Lee SH, Kim DW, Im SA, Chung DH, Heo DS, Bang YJ (2006) Clinical predictors versus epidermal growth factor receptor mutation in gefitinib-treated non-small-cell lung cancer patients. *Lung Cancer* 54: 201–207

Hirsch FR, Varella-Garcia M, Bunn Jr PA, Di Maria MV, Veve R, Bremmes RM, Barón AE, Zeng C, Franklin WA (2003) Epidermal growth factor receptor in non-small-cell lung carcinomas: correlation between gene copy number and protein expression and impact on prognosis. *J Clin Oncol* 21: 3798–3807

Hirsch FR, Varella-Garcia M, McCoy J, West H, Xavier AC, Gumerlock P, Bunn Jr PA, Franklin WA, Crowley J, Gandara DR, Southwest Oncology Group (2005) Increased epidermal growth factor receptor gene copy number detected by fluorescence in situ hybridization associates with increased sensitivity to gefitinib in patients with bronchioloalveolar carcinoma subtypes: a Southwest Oncology Group Study. *J Clin Oncol* 23: 6838–6845

Inoue A, Suzuki T, Fukuhara T, Maemondo M, Kimura Y, Morikawa N, Watanabe H, Saijo Y, Nukiwa T (2006) Prospective phase II study of gefitinib for chemotherapy-naïve patients with advanced non-small cell lung cancer with epidermal growth factor receptor gene mutations. *J Clin Oncol* 24: 3340–3346

Jackman DM, Yeap BY, Sequist LV, Lindeman N, Holmes AJ, Joshi VA, Bell DW, Huberman MS, Halmos B, Rabin MS, Haber DA, Lynch TJ, Meyerson M, Johnson BE, Jänne PA (2006) Exon 19 deletion mutation of epidermal growth factor receptor are associated with prolonged survival in non-small cell lung cancer patients treated with gefitinib or erlotinib. *Clin Cancer Res* 12: 3908–3914

Kris MG, Natale RB, Herbst RS, Lynch Jr TJ, Prager D, Belani CP, Schiller JH, Kelly K, Spiridonidis H, Sandler A, Albain KS, Cella D, Wolf MK, Averbuch SD, Ochs JJ, Kay AC (2003) Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small-cell lung cancer. *JAMA* 290: 2149–2158

Kaneda H, Tamura K, Kurata T, Uejima H, Nakagawa K, Fukuoka M (2004) Retrospective analysis of the predictive factors associated with response and survival benefit of gefitinib in patients with advanced non-small cell lung cancer. *Lung Cancer* 46: 247–254

Kobayashi S, Boggon TJ, Dayaram T, Jänne PA, Kocher O, Meyerson M, Johnson BE, Eck MJ, Tenen DG, Halmos B (2005) EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* 352: 786–792

Lee DH, Han JY, Yu SY, Kim HY, Nam BH, Hong EK, Kim HT, Lee JS (2006) The role of gefitinib treatment for Korean never-smokers with advanced or metastatic adenocarcinoma of lung: a prospective study. *J Thorac Oncol* 1: 965–971

Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 350: 2129–2139

Miller VA, Kris MG, Shah N, Patel J, Azzoli C, Gomez J, Krug LM, Pao W, Rizzo B, Tyson L, Venkatraman E, Ben-Porat L, Memoli N, Zakowski M, Rusch V, Heelan RT (2004) Bronchioloalveolar pathologic subtype smoking history predicts sensitivity to gefitinib in advanced non-small-cell lung cancer. *J Clin Oncol* 22: 1103–1109

Niho S, Kubota K, Goto K, Yoh K, Ohmatsu H, Kakinuma R, Saijo N, Nishiwaki Y (2006) First-line single agent treatment with gefitinib in patients with advanced non-small-cell lung cancer: a phase II study. *J Clin Oncol* 24: 64–69

Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M (2004) EGFR mutation in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 304: 1497–1500

Paz-Ares L, Sanchez JM, Garcia-Velasco A, Masuti B, Majem M, Lopez-Vivanco G, Provencio M, Montes A, Amador M, Rosell R (2006) A prospective phase II trial if erlotinib in advanced non-small cell lung cancer (NSCLC) patients with mutations in the tyrosine kinase (TK) domain of the epidermal growth factor receptor (EGFR). *Proc Am Soc Clin Onc* 24(Suppl): abstract 7020

Pao W, Miller VA, Zakowski MF, Doherty J, Politi KA, Sarkaria I, Singh B, Varmus H (2004) EGF receptor gene mutations are common in lung cancers from 'never smokers' and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci USA* 101: 13306–13311

Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG, Varmus H (2005) Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* 2: 1–11

Ranson M, Hammond LA, Ferry D, Kris M, Tullo A, Murray PI, Miller V, Averbuch S, Ochs J, Morris C, Feyereislova A, Swaisland H, Rowinsky EK (2002) ZD1839, a selective oral epidermal growth factor receptor-tyrosine kinase inhibitor, is well tolerated and active in patients with solid, malignant tumors: results of a phase I trial. *J Clin Oncol* 20: 2240–2250

Riely GJ, Pao W, Pham D, Li AR, Rizvi N, Venkatraman ES, Zakowski MF, Kris MG, Ladanyi M, Millar VA (2006) Clinical course of patients with non-small cell lung cancer and epidermal growth factor receptor exon 19 and exon 21 mutations treated with gefitinib or erlotinib. *Clin Cancer Res* 12: 839–844

Schiller JH, Harrington D, Belani CP, Langer C, Sandler A, Krook J, Zhu J, Jhonson DH, Eastern Cooperative Oncology Group (2002) Comparison of four chemotherapy regimens for advanced non-small cell lung cancer. *N Engl J Med* 346: 92–98

Shepherd FA, Rodrigues Pereira J, Ciuleanu T, Tan EH, Hirsh V, Thongprasert S, Compos D, Maoleekoonpiroj S, Smylie M, Martins R, van Kooten M, Dediu M, Findlay B, Tu D, Johnston D, Bezjak A, Clark G, Santabarbara P, Seymour L, National Cancer Institute of Canada Clinical Trials Group (2005) Erlotinib in previously treated non-small cell lung cancer. *N Engl J Med* 353: 123–132

Shigematsu H, Lin L, Takahashi T, Nomura M, Suzuki M, Wistuba II, Fong KM, Lee H, Toyooka S, Shimizu N, Fujisawa T, Feng Z, Roth JA, Herz J, Minna JD, Gazdar AF (2005) Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. *J Natl Cancer Inst* 97: 339–346

Thatcher N, Chang A, Parikh P, Rodrigues Pereira J, Ciuleanu T, von Pawel J, Thongprasert S, Tan EH, Pemberton K, Archer V,

Carroll K (2005) Gefitinib plus best supportive care in previously treated patients with refractory advanced non-small-cell lung cancer: results from a randomised placebo-controlled, multicentre study (Iressa Survival Evaluation in Lung Cancer). *Lancet* 366: 1527–1537

Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG (2000) New guidelines to evaluate the response to treatment in solid tumors. *J Natl Cancer Inst* 92: 205–216

Yatabe Y, Hida T, Horio Y, Kosaka T, Takahashi T, Mitsudomi T (2006) A rapid, sensitive assay to detect EGFR mutation in small biopsy specimens from lung cancer. *J Mol Diagn* 8: 335–341

Yoshida K, Yatabe Y, Young Ji P, Shimizu J, Horio Y, Matsuo K, Kosaka T, Mitsudomi T, Hida T (2007) Prospective validation for prediction of gefitinib sensitivity by epidermal growth factor receptor gene mutation in patients with non-small cell lung cancer. *J Thoracic Oncol* 2: 22–28

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Phase II Trial of Preoperative Chemoradiotherapy Followed by Surgical Resection in Patients With Superior Sulcus Non–Small-Cell Lung Cancers: Report of Japan Clinical Oncology Group Trial 9806

Hideo Kunitoh, Harubumi Kato, Masahiro Tsuboi, Taro Shibata, Hisao Asamura, Yukito Ichonose, Nobuyuki Katakami, Kanji Nagai, Tetsuya Mitsudomi, Akihide Matsumura, Ken Nakagawa, Hirohito Tada, and Nagahiro Saijo

From the Department of Medical Oncology and Division of Thoracic Surgery, National Cancer Center Hospital; Department of Thoracic Surgery, Tokyo Medical University; Japan Clinical Oncology Group Data Center, Center for Cancer Control and Information Services, National Cancer Center; Department of Thoracic Surgery, Cancer Institute Hospital, Tokyo; Department of Chest Surgery, National Kyushu Cancer Center, Fukuoka; Pulmonary Unit, Kobe City Medical Center General Hospital, Kobe; Department of Thoracic Surgery, National Cancer Center Hospital East, Kashiwa; Department of Thoracic Surgery, Aichi Cancer Center Hospital, Nagoya; Department of Surgery, National Hospital Organization Kinki-Chuo Chest Medical Center, Sakai; and Department of Thoracic Surgery, Osaka City General Hospital, Osaka, Japan.

Corresponding author: Hideo Kunitoh, MD, Department of Medical Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan; e-mail: hkkunito@ncc.go.jp.

A B S T R A C T

Purpose

To evaluate the safety and efficacy of preoperative chemoradiotherapy followed by surgical resection for superior sulcus tumors (SSTs).

Patients and Methods

Patients with pathologically documented non–small-cell lung cancer with invasion of the first rib or more superior chest wall were enrolled as eligible; those with distant metastasis, pleural dissemination, and/or mediastinal node involvement were excluded. Patients received two cycles of chemotherapy every 4 weeks as follows: mitomycin 8 mg/m^2 on day 1, vindesine 3 mg/m^2 on days 1 and 8, and cisplatin 80 mg/m^2 on day 1. Radiotherapy directed at the tumor and the ipsilateral supraclavicular nodes was started on day 2 of each course, at the total dose of 45 Gy in 25 fractions, with a 1-week split. Thoracotomy was undertaken 2 to 4 weeks after completion of the chemoradiotherapy. Those with unresectable disease received boost radiotherapy.

Results

From May 1999 to November 2002, 76 patients were enrolled, of whom 20 had T4 disease; 75 patients were fully assessable. Chemoradiotherapy was generally well tolerated. Fifty-seven patients (76%) underwent surgical resection, and pathologic complete resection was achieved in 51 patients (68%). There were 12 patients with pathologic complete response. Major postoperative morbidity, including chylothorax, empyema, pneumonitis, adult respiratory distress syndrome, and bleeding, was observed in eight patients. There were three treatment-related deaths, including two deaths owing to postsurgical complications and one death owing to sepsis during chemoradiotherapy. The disease-free and overall survival rates at 3 years were 49% and 61%, respectively; at 5 years, they were 45% and 56%, respectively.

Conclusion

This trimodality approach is safe and effective for the treatment of patients with SSTs.

J Clin Oncol 26:644-649. © 2008 by American Society of Clinical Oncology

INTRODUCTION

Superior sulcus tumors (SSTs), involving structures at the thoracic inlet, represent a small subtype of non–small-cell lung carcinoma (NSCLC). These SSTs, first described by Henry Pancoast[1,2] and thus also called Pancoast tumors, have posed a challenging problem for surgeons, radiation oncologists, and medical oncologists alike, ever since they were first described.[3]

Preoperative radiotherapy has long been the community standard in the management of SSTs.[4-17] However, both the complete resection rate (approximately 50%) and long-term survival rate

(approximately 30%) have remained poor and unchanged over the last 40 years, since the first treatment strategy was reported in the 1960s. Local control has remained the main problem,[15,17,18] adversely affecting quality of life as well as survival of patients. Presence of mediastinal lymph node metastasis (N2 status) has been reported to be associated with a particularly poor prognosis.[9,18]

However, a series of clinical trials over the last two decades have shown concurrent chemoradiotherapy to be beneficial in the treatment of unresectable stage III NSCLC.[19-21] The addition of chemotherapy to thoracic radiotherapy seems to suppress distant micrometastases,[22,23] and giving

concurrent chemotherapy with radiotherapy has been shown to yield improved local control[19,24] with survival benefit.

Encouraged by the promising data of concurrent chemoradiotherapy for N2 NSCLC, the Southwest Oncology Group (SWOG) applied this modality as preoperative therapy for patients with SSTs (SWOG 9416, Intergroup Trial 0160), and reported favorable results.[25]

The Japan Clinical Oncology Group (JCOG) launched another trial of this preoperative concurrent chemoradiotherapy, or the trimodality approach, for the treatment of SSTs in 1999, before the first report of SWOG 9416 was published. Our study was initiated to evaluate the safety and efficacy of this treatment strategy in this rare subset of patients with NSCLC. As the induction treatment, we used mitomycin, vindesine, and cisplatin (MVP) combination chemotherapy, which has been demonstrated to be safe and effective for concurrent chemotherapy with thoracic radiotherapy in Japanese trials.[19]

PATIENTS AND METHODS

Eligibility Criteria

Patients with untreated histologically or cytologically documented NSCLC involving the superior sulcus with clinical stage T3 or T4 disease were eligible for entry onto this study. T4 diseases included tumor invasion to the spine (including to a transverse process of vertebra), aorta, or superior vena cava; invasion to the chest wall or subclavian vessels was included in T3 disease. Involvement of the superior sulcus was confirmed by computed tomographic (CT) or magnetic resonance imaging (MRI) evidence of tumor invasion of the first rib or more superior chest wall. Patients with pleural or pericardial dissemination, malignant effusion, and/or distant metastasis (M1) were excluded. Those with clinical N2 disease (mediastinal node involvement) were also excluded; all mediastinal nodes measuring ≥ 1.0 cm in size on CT images were required to be biopsied and documented to be negative for metastasis before patient enrollment. However, those with ipsilateral supraclavicular node involvement (N3) were eligible, unless it was accompanied by mediastinal node metastasis. Each patient was required to fulfill the following criteria: 15 to 74 years of age, Eastern Cooperative Oncology Group performance status of 0 to 1; adequate organ function (ie, leukocyte count ≥ 4,000/μL, platelet count ≥ 10^5/μL, hemoglobin ≥ 11.0 g/dL, serum creatinine less than 1.5 mg/dL, creatinine clearance ≥ 60 mL/min, serum bilirubin less than 1.5 mg/dL, serum ALT and AST less than double the upper limit of the institutional normal range, arterial partial pressure of oxygen ≥ 70 mmHg, and predicted postoperative forced expiratory volume in 1 second ≥ 0.8 L. From July 2001, when the protocol was revised after the death of a patient from septic shock during chemoradiotherapy, those patients with systemic use of corticosteroids were excluded.

Patient eligibility was confirmed by the JCOG Data Center before patient registration. This study was approved by the institutional review boards at each participating center, and written informed consent was obtained from all patients.

Treatment Plan

Induction chemotherapy. Patients received two courses of MVP combination chemotherapy with a 4-week interval in between. Mitomycin was administered at 8 mg/m^2 on chemotherapy day 1, and vindesine was administered at 3 mg/m^2 on days 1 and 8; both were administered as bolus injections. Cisplatin was administered at 80 mg/m^2 as a 2-hour infusion on day 1, with ample hydration and antiemetic administration.

The second cycle of chemotherapy was postponed until all the severe toxicities recovered to grade 1 or 0. If the second cycle could not be started within 2 weeks of the due date, it was canceled, and the patient received only preoperative radiotherapy, if possible.

Induction radiotherapy. Thoracic radiotherapy was started with a linear accelerator (≥ 4 MeV) on chemotherapy day 2. The first session was scheduled

to be given with the first chemotherapy cycle at 27 Gy in 15 fractions over 3 weeks. Then the second session was started after a week's interval until day 2 of the second course of chemotherapy. The second session, given with the second cycle of MVP, was administered at 18 Gy in 10 fractions over 2 weeks. The total radiation dose was thus 45 Gy in 25 fractions administered over 6 weeks, including the 1-week split, or interval, between the two sessions; this schedule, including the split, basically followed that of the original method reported by Furuse et al.[19] The radiation field included the primary tumor and the ipsilateral supraclavicular nodes. The mediastinal and hilar nodes were not irradiated, even in cases with hilar node involvement (clinical N1 cases).

Surgery. After the induction chemoradiotherapy, each case was re-evaluated to determine the clinical response and resectability. The resectability of the tumor was determined by the multimodality team of each institution, irrespective of the clinical response (tumor shrinkage). Surgical resection of the tumor was performed 2 to 4 weeks after the completion of the induction therapy. The surgical procedures undertaken included lobectomy or pneumonectomy, with systematic node dissection. Standard systematic node dissection, ND2, includes complete removal of the hilar and mediastinal nodes. Less complete dissection includes ND0 (ie, no systematic dissection with or without lymph node sampling) or ND1 (ie, hilar node dissection with or without mediastinal lymph node sampling).

Boost therapy. For unresected or incompletely resected cases, boost radiotherapy of 21.6 Gy in 12 fractions was given. Those who were judged to have undergone complete resection were followed up without additional therapy until clinical evidence of recurrence.

Patient Evaluation and Follow-Up

Before enrollment onto the study, each patient underwent complete medical history taking and physical examination, blood cell count determinations, serum biochemistry testing, arterial blood gas analysis, chest x-ray, ECG, CT scan of the chest, bronchoscopy, CT scan or ultrasound of the upper abdomen, whole-brain CT or MRI, and an isotope bone scan. Chest MRI was recommended for evaluation of the local tumor status but was not mandatory. Blood cell counts, serum biochemistry testing, and chest x-ray were performed weekly during each course of chemotherapy. Chest CT was performed every 3 to 4 weeks during the induction therapy.

Chemotherapy toxicity was evaluated according to the JCOG Toxicity Criteria,[26] modified from the National Cancer Institute Common Toxicity Criteria version 1. Tumor responses were assessed radiographically according to the standard, two-dimensional WHO criteria[27] and were classified into complete response (CR), partial response, no change, progressive disease (PD), and not assessable. Response confirmation at 4 weeks or longer intervals was not necessitated. After curative resection and/or definitive boost radiotherapy, the patients were followed up with periodic re-evaluation, including with chest CT, as well as a systemic survey every 6 months for the first 3 years.

Central Review

Radiographic reviews for eligibility of the enrolled patients and the clinical responses were performed at the time of the JCOG Lung Cancer Surgical Study Group meeting, held every 3 to 4 months. The study coordinator (H.K., a medical oncologist), the group coordinator (M.T., a surgical oncologist), and a few selected investigators of the group reviewed the radiographic films. The clinical response data presented below were all confirmed by this central review.

Statistical Considerations

The primary end point of the study was the survival rate at 3 years. The sample size calculation was performed, as described in Appendix 1 (online only).

Secondary end points included the objective tumor response to chemotherapy, complete resection rate, and postsurgical morbidity/mortality. Both overall survival (OS) and progression-free survival (PFS) were calculated from the date of enrollment by the Kaplan-Meier method. For exploratory analysis to identify prognostic factors, the OS or PFS of subgroups was compared by two-sided log-rank tests. All analyses were performed with the SAS software version 8.2 (SAS Institute, Cary, NC).

1184

Patient Characteristics

From May 1999 to November 2002, 76 patients from 19 institutions were enrolled onto the study. Three patients were ineligible. One patient was found to have concomitant anemia and did not receive the protocol treatment. Two others were found ineligible by the central review, after completion of the protocol therapy; the tumor was judged not to involve the first rib in one case, and in the other, a mediastinal node was judged to be enlarged on chest CT, without confirmation by mediastinoscopy. These two cases were included in the analysis. Therefore, 75 patients were analyzed to determine the toxicities, response rates, surgical and pathologic results, PFS, and OS. All 76 patients were included in the analysis of the patient characteristics, as shown in Table 1. In each of the T4 cases, the tumor was judged to have involved the spine. Nodal status was clinically determined and was pathologically confirmed in only a few cases.

Induction Therapy Delivery and Toxicity

The study schema with the actual numbers of patients receiving the protocol therapy is shown in Appendix Figure A1 (online only).

The induction therapy could be completed in 71 (95%) of the 75 patients. The treatment was terminated in the remaining four patients after only one course of chemotherapy (owing to the development of adverse events in two cases, patient refusal in one case, and early toxicity-related death in one case).

Table 2 lists the major toxicities of the protocol therapy. They were mainly hematologic, and although more than 80% of the patients experienced neutropenia/leukopenia, they were generally transient and not complicated by infection/fever. Overall, toxicities were well tolerated. There was one toxic death on chemoradiotherapy day 6 as a result of severe myelosuppression and subsequent development of septic shock.

Clinical Response to the Induction Therapy

The clinical responses of the 75 eligible patients to induction therapy were judged radiologically and confirmed by the central review. The responses were as follows: CR, 0 patients; partial response, 46 patients; no change, 22 patients; PD, five patients; not assessable, two patients. The overall response rate was 61% (95% CI, 49% to 72%).

Surgical and Pathologic Results

Thoracotomy was performed in 57 (76%) of the 75 patients who received the induction therapy. The surgical procedures undertaken

Table 1. Patient Characteristics (n = 76)

Characteristic	No. of Patients	%
Sex		
Male	67	88
Female	9	12
Age, years		
Median	57.5	
Range	34-74	
ECOG performance status		
0	30	39
1	46	61
Clinical T stage		
T3	56	74
T4	20	26
Clinical N stage		
N0	59	78
N1	9	12
N2*	1	1
N3	7	9
Smoking history		
No	4	5
Yes	72	95
Median smoking history	1.5 packs for 37 years	
Body weight loss within 6 months		
≤ 5%	61	80
5-10%	7	9
> 10%	5	7
Missing	3	4
Histology		
Adenocarcinoma	34	45
Squamous cell carcinoma	27	36
Others/unclassified	15	20
Primary site		
Right	39	51
Left	37	49

Abbreviation: ECOG, Eastern Cooperative Oncology Group.
*Found ineligible by central review but included in the subsequent analyses.

Table 2. Major Toxicities of Induction Therapy (N = 75) and Postsurgical Complications

Toxicity or Complication	No. of Patients Grade 1/2	No. of Patients Grade 3	No. of Patients Grade 4	% Grade 3/4
Acute toxicity*				
Leukopenia	1/11	37	26†	84
Neutropenia	3/9	26	36†	83
Anemia	19/47	5	0	7
Thrombocytopenia	14/12	9	2†	15
ALT	27/5	2	0	3
Creatinine	18/2	0	0	0
PaO₂	37/6	0	0	0
Emesis	32/25	2	— (not defined)	3
Diarrhea	7/5	1	0	1
Constipation	22/3	1	0	1
Esophagitis	22/9	0	0	0
Infection	10/9	6	1†	9
Neuropathy	8/0	0	— (not defined)	0
Skin toxicity	16/2	1	0	1
Fever	25/19	1	1	3
Postsurgical complications‡				
ARDS	0	1	1 (grade 5)	
Empyema	0	2	0	
Cylothorax	1	1	0	
Pneumonitis	0	1	0	
Late complications‡				
Pneumonitis	0	1	0	
Bleeding	0	0	1 (grade 5)	

Abbreviations: PaO₂, alveolar-arterial difference in partial pressure of oxygen; ARDS, adult respiratory distress syndrome.
*During induction therapy.
†Includes one patient with toxic death owing to septic shock.
‡Report of each complication was evaluated by National Cancer Institute Common Toxicity Criteria version 3.0.

1185

were as follows: lobectomy, 53 patients; partial resection, three patients; exploratory thoracotomy, one patient; none of the cases required pneumonectomy. Combined resection of the chest wall was undertaken in 51 of the 57 patients. Complete mediastinal lymph node dissection (ND2) was performed in 42 patients, and the remaining 15 patients underwent less extensive dissection or sampling (ND0 or ND1).

The results of thoracotomy were as follows: gross residual tumor (R2 resection, including one with probe thoracotomy), three patients; microscopically residual tumor on pathologic review (R1 resection), three patients; complete surgical and pathologic resection (R0 resection), 51 patients. Pathologic downstaging of the tumor as compared with the clinical stage before induction therapy was achieved in 23 patients (40% of the patients who underwent surgery); this is an inherently inaccurate figure and should be interpreted as such, owing to the lack of pathologic confirmation of the c stage at presentation. Pathologic CR, with no residual viable tumor cells in the resected specimens, was achieved in 12 patients (16% of the 75 treated patients). Table 3 lists the surgical and pathologic results according to the initial clinical T factor.

The major postoperative morbidities included adult respiratory distress syndrome (ARDS) in two patients, empyema in two patients,

chylothorax in two patients, and pneumonitis in two patients. One patient died of sudden major bleeding on postoperative day 24. The bleeding was identified at autopsy as being from an intercostal artery. Another patient died of ARDS after off-protocol pneumonectomy. The patient had been judged to have PD in response to the induction therapy as a result of emergence of intrapulmonary metastases. The attending surgeon and the patient agreed to salvage surgery, and the patient developed postoperative ARDS.

Thus the total number of toxic deaths was three, including one caused by septic shock during the induction, one by delayed postoperative bleeding, and one by the development of ARDS after off-protocol, salvage surgery.

Boost Therapy

Boost radiotherapy was given to 15 patients, including 12 of the 15 patients in whom thoracotomy was not performed after the completion of induction chemoradiotherapy. One patient received boost radiotherapy after grossly incomplete resection, and another received boost radiotherapy after gross complete resection with microscopically residual disease. In 12 of the 15 patients, boost radiotherapy was completed with a total dose of 66.6 Gy.

PFS and OS

Figures 1 and 2 show the PFS and OS curves, updated in November 2006. Forty-one patients were alive, with a median follow-up period of 68 months. The median PFS time was 28 months. The PFS rates at 3 and 5 years were 49% and 45%, respectively. The median OS has not yet been reached. The OS at 3 and 5 years were 61% and 56%, respectively. Subset analysis (Appendix Figs A2 through A5, online only) revealed that clinical T stage was a prognostic factor (Appendix Fig A2). Patients with clinical T3 disease had better outcome than those with clinical T4 disease (the survival rates at 3 and 5 years were 69% and 61%, respectively, versus 40% and 40%, respectively; log-rank $P = .031$). The clinical N stage and histologic type of the tumor did not significantly affect the OS (Appendix Figs A3 and A4) or PFS. As expected, the survival rate was good in patients in whom complete resection could be achieved, with a projected 5-year OS of 70% as compared with 24% in whom complete resection could not be

| Table 3. Surgical and Pathologic Results According to Initial Clinical T Stage		
Characteristic	c-T3	c-T4
No. of patients	55	20
No surgery performed		
No.	7	11
%	13	55
Reason for no surgery		
Protocol violation	0	1
Toxic death	0	1
Adverse event	0	1
Progressive disease	2	2
Judged unresectable	0	3
Patient refusal	5	3
Surgical procedures		
Thoracotomy		
No.	48	9
%	87	45
Pneumonectomy	0	0
Lobectomy	45	8
Probe thoracotomy	1	0
Other	2	1
With combined resection	44	7
Rib	38	6
Parietal pleura	4	1
Vertebra	3	3
Major vessel	3	0
Clavicle	1	0
Completeness of resection		
R2 operation	2	1
R1 operation	3	0
R0 operation		
No.	43	8
%	78	40
Pathologic results		
Downstaging	18	5
Pathlogic complete response	9	3
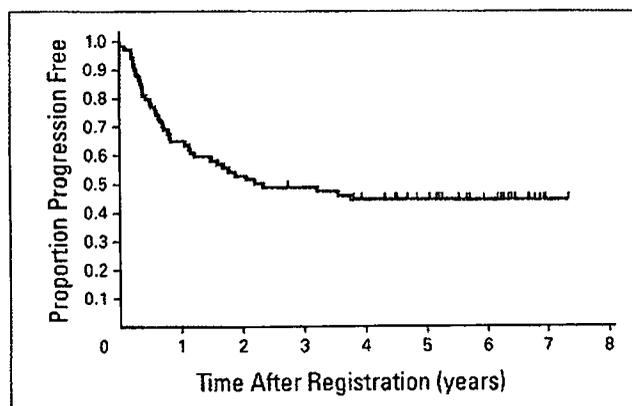
Fig 1. Progression-free survival (PFS) of the 75 eligible patients. PFS at 3 years and 5 years was 49% (95% CI, 38% to 60%) and 45% (95% CI, 34% to 56%), respectively, with a median PFS of 27.7 months.
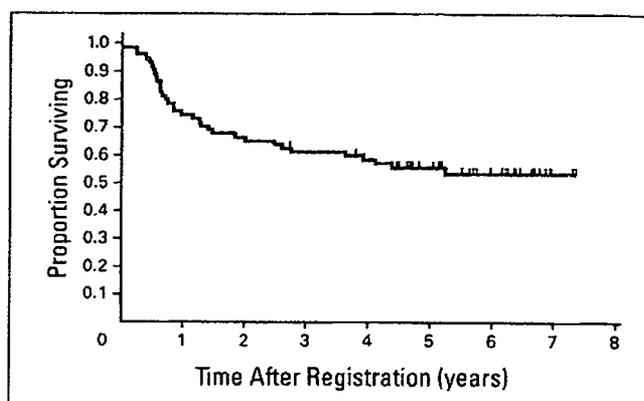
1186

Fig 2. Overall survival (OS) of the 75 eligible patients. OS at 3 years and 5 years was 61% (95% CI, 49% to 71%) and 56% (95% CI, 44% to 66%), respectively. The median OS has not been reached.

achieved (Appendix Fig A5). The survival of the 12 patients with pathologic CR was especially favorable (Appendix Fig A6, online only).

Pattern of Relapse

So far, 39 patients have experienced tumor relapse. Table 4 lists the initial relapse sites, according to the curative extent of the surgical resection. For unresected or incompletely resected cases, locoregional relapse was predominant. To the contrary, for completely resected cases, relapse at distant sites was the most frequent relapse pattern, with some brain-only relapse patients.

DISCUSSION

We conducted a multi-institutional phase II trial of a trimodality approach, namely, preoperative chemoradiotherapy followed by surgical resection, in patients with SSTs. Because of the rarity of this subtype of NSCLC, no randomized trial has been conducted previously.[28] Our report is the second of a large-scale, prospective trial after SWOG 9416/INT 0160 and reproduced its favorable outcomes.[25]

The long-term results of the SWOG 9416/INT 0160 trial were recently published.[29] Although the chemotherapy regimens used were different, a standard classic platinum-based combination was used in both. The preoperative radiotherapy doses were also identical (45 Gy), although a 1-week split (interval between two sessions) was included in our protocol (but not in the SWOG trial). Boost chemotherapy was planned after curative resection in the SWOG trial, but the compliance

Table 4. Initial Relapse Sites			
Relapse Site	Patients With Complete Resection (n = 51)	Patients Without Complete Resection (n = 24)	Total (N = 75)
Locoregional* only	2	8	10
Distant only	14	6	20
Brain only	4	1	5
Both	4	5	9
Total	20	19	39

*Locoregional = surgical margin, within radiation field, hilar lymph nodes, mediastinal lymph nodes, supraclavicular lymph nodes.

rate was poor,[25] as in other perioperative therapy reports; we had anticipated that the majority of the patients would not be fit enough for additional toxic therapy after a major thoracic surgery and did not include it in our protocol.

Despite these minor differences, the results of the two trials were strikingly similar (Table A1, online only). The radiologic response rate was higher, whereas the pathologic CR rate was lower in our trial, but the differences were probably not clinically relevant, considering interobserver differences in the response evaluation and the well-known discrepancy between clinical versus pathologic effects. The intensive trimodality approach was found to be feasible in both reports, with a reasonably low toxic death rate of 4%. The resection rate, which had remained unchanged at approximately 50% for almost 40 years with conventional preoperative radiotherapy, was approximately 70% in both studies. Particularly noteworthy was the reproducibility of the favorable survival data, with a 5-year OS rate of 44% in the United States trial and 56% in our trial, which were clearly superior to the historical value of 30%.[3,25]

A shift in the trend of clinical problems also became clear.[25,28,29] The relapse patterns changed from predominantly locoregional[17,18] to mainly distant recurrences in cases with complete resection,[25,28,29] and a significant number of such patients suffered from metastasis in the brain as the initial site of relapse.[29] To the contrary, complete resection could be achieved in less than half of the patients with c-T4 disease, and neither local control nor long-term survival was satisfactory in those in whom it could not be achieved. It seems that there might be room for improvement in radiotherapy.

Several questions remain unresolved. One is that of management of patients with mediastinal node involvement. These clinical N2 cases have been known to have the poorest prognosis[9,18] and were excluded from both the SWOG and JCOG trials. Although trimodality approaches have been reported in cases with clinical N2 stage NSCLC,[30,31] inclusion of the hilar and mediastinal nodes in the irradiation field increased the toxicity risk to an unacceptable level in our prior phase II trial (JCOG 9805).[32]

In addition to the unresolved questions above, our study also had a critical limitation. Although this was a prospective, large-scale, and multi-institutional trial, no definite conclusions could be obtained from the single-arm phase II study. As repeatedly pointed out, however, a phase III trial would be unrealistic due to the rarity of SSTs. Possibly, clinical questions common with other patient subsets could be tested in a phase III trial targeting a broader patient population; for example, patients with SSTs and other stage III NSCLC could be enrolled onto a phase III trial of prophylactic cranial irradiation after definitive induction therapy.[33]

In conclusion, we report a favorable outcome of preoperative chemoradiotherapy in patients with SSTs, confirming the results of the previous SWOG/Intergroup trial. We believe that this strategy may be acceptable as standard for the treatment of this disease and also serves as a reference for future trials.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

1187

Conception and design: Hideo Kunitoh, Harubumi Kato, Nagahiro Saijo

Financial support: Nagahiro Saijo

Administrative support: Nagahiro Saijo

Provision of study materials or patients: Hideo Kunitoh, Harubumi Kato, Masahiro Tsuboi, Hisao Asamura, Yukito Ichonose, Nobuyuki

Katakami, Kanji Nagai, Tetsuya Mitsudomi, Akihide Matsumura, Ken Nakagawa, Hirohito Tada

Collection and assembly of data: Masahiro Tsuboi, Taro Shibata

Data analysis and interpretation: Taro Shibata

Manuscript writing: Hideo Kunitoh, Taro Shibata

Final approval of manuscript: Hideo Kunitoh, Harubumi Kato, Masahiro Tsuboi, Taro Shibata, Hisao Asamura, Yukito Ichonose, Nobuyuki Katakami, Kanji Nagai, Tetsuya Mitsudomi, Akihide Matsumura, Ken Nakagawa, Hirohito Tada, Nagahiro Saijo

1. Pancoast HK: Importance of careful roentgen-ray investigation of apical chest tumors. JAMA 83:1407-1411, 1924

2. Pancoast H: Superior pulmonary sulcus tumor: Tumor characterized by pain, Horner's syndrome, destruction of bone and atrophy of hand muscles. JAMA 99:1391-1396, 1932

3. Arcasoy SM, Jett JR: Superior pulmonary sulcus tumors and Pancoast's syndrome. N Engl J Med 337:1370-1376, 1997

4. Shaw RR, Paulson DL, Kee JL Jr: Treatment of superior sulcus tumor by irradiation followed by resection. Ann Surg 154:29-40, 1961

5. Paulson DL: The survival rate in superior sulcus tumors treated by presurgical irradiation. JAMA 196:342, 1966

6. Hilaris BS, Luomanen RK, Beattie EJ Jr: Integrated irradiation and surgery in the treatment of apical lung cancer. Cancer 27:1369-1373, 1971

7. Paulson DL: Carcinomas in the superior pulmonary sulcus. J Thorac Cardiovasc Surg 70:1095-1104, 1975

8. Devine JW, Mendenhall WM, Million RR, et al: Carcinoma of the superior pulmonary sulcus treated with surgery and/or radiation therapy. Cancer 57:941-943, 1986

9. Anderson TM, Moy PM, Holmes EC: Factors affecting survival in superior sulcus tumors. J Clin Oncol 4:1598-1603, 1986

10. Hilaris BS, Martini N, Wong GY, et al: Treatment of superior sulcus tumor (Pancoast tumor). Surg Clin North Am 67:965-977, 1987

11. Shahian DM, Neptune WB, Ellis FH Jr: Pancoast tumors: Improved survival with preoperative and postoperative radiotherapy. Ann Thorac Surg 43:32-38, 1987

12. Neal CR, Amdur RJ, Mendenhall WM, et al: Pancoast tumor: Radiation therapy alone versus preoperative radiation therapy and surgery. Int J Radiat Oncol Biol Phys 21:651-660, 1991

13. Maggi G, Casadio C, Pischedda F, et al: Combined radiosurgical treatment of Pancoast tumor. Ann Thorac Surg 57:198-202, 1994

14. Komaki R, Roth JA, Walsh GL, et al: Outcome predictors for 143 patients with superior sulcus tumors treated by multidisciplinary approach at the University of Texas M. D. Anderson Cancer Center. Int J Radiat Oncol Biol Phys 48:347-354, 2000

15. Martinod E, D'Audiffret A, Thomas P, et al: Management of superior sulcus tumors: Experience with 139 cases treated by surgical resection. Ann Thorac Surg 73:1534-1539, 2002

16. Muscolino G, Valente M, Andreani S: Pancoast tumours: Clinical assessment and long-term results of combined radiosurgical treatment. Thorax 52:284-286, 1997

17. Ginsberg RJ, Martini N, Zaman M, et al: Influence of surgical resection and brachytherapy in the management of superior sulcus tumor. Ann Thorac Surg 57:1440-1445, 1994

18. Rusch VW, Parekh KR, Leon L, et al: Factors determining outcome after surgical resection of T3 and T4 lung cancers of the superior sulcus. J Thorac Cardiovasc Surg 119:1147-1153, 2000

19. Furuse K, Fukuoka M, Kawahara M, et al: Phase III study of concurrent versus sequential thoracic radiotherapy in combination with mitomycin, vindesine, and cisplatin in unresectable stage III non-small-cell lung cancer. J Clin Oncol 17:2692-2699, 1999

20. Curran WJ Jr: Treatment of locally advanced non-small cell lung cancer: What we have and have not learned over the past decade. Semin Oncol 32:S2-S5, 2005

21. Zatloukal P, Petruzelka L, Zemanova M, et al: Concurrent versus sequential chemoradiotherapy with cisplatin and vinorelbine in locally advanced non-small cell lung cancer: A randomized study. Lung Cancer 46:87-98, 2004

22. Sause W, Kolesar P, Taylor SI, et al: Final results of phase III trial in regionally advanced unresectable non-small cell lung cancer: Radiation Therapy Oncology Group, Eastern Cooperative Oncology Group, and Southwest Oncology Group. Chest 117:358-364, 2000

23. Dillman RO, Seagren SL, Propert KJ, et al: A randomized trial of induction chemotherapy plus high-dose radiation versus radiation alone in stage III non-small-cell lung cancer. N Engl J Med 323:940-945, 1990

24. Schaake-Koning C, van den Bogaert W, Dalesio O, et al: Effects of concomitant cisplatin and radiotherapy on inoperable non-small-cell lung cancer. N Engl J Med 326:524-530, 1992

25. Rusch VW, Giroux DJ, Kraut MJ, et al: Induction chemoradiation and surgical resection for non-small cell lung carcinomas of the superior sulcus: Initial results of Southwest Oncology Group Trial 9416 (Intergroup Trial 0160). J Thorac Cardiovasc Surg 121:472-483, 2001

26. Tobinai K, Kohno A, Shimada Y, et al: Toxicity grading criteria of the Japan Clinical Oncology Group. Jpn J Clin Oncol 23:250-257, 1993

27. Miller AB, Hoogstraten B, Staquet M, et al: Reporting results of cancer treatment. Cancer 47:207-214, 1981

28. Wright CD, Menard MT, Wain JC, et al: Induction chemoradiation compared with induction radiation for lung cancer involving the superior sulcus. Ann Thorac Surg 73:1541-1544, 2002

29. Rusch VW, Giroux D, Kraut MJ, et al: Induction chemoradiation and surgical resection for superior sulcus non-small-cell lung carcinomas: Long-term results of Southwest Oncology Group trial 9416 (Intergroup trial 0160). J Clin Oncol 25:313-318, 2007

30. Albain KS, Rusch VW, Crowley JJ, et al: Concurrent cisplatin/etoposide plus chest radiotherapy followed by surgery for stages IIIA (N2) and IIIB non-small-cell lung cancer: Mature results of Southwest Oncology Group phase II study 8805. J Clin Oncol 13:1880-1892, 1995

31. Albain KS, Swann RS, Rusch VR, et al: Phase III study of concurrent chemotherapy and radiotherapy (CT/RT) vs CT/RT followed by surgical resection for stage IIIA(pN2) non-small cell lung cancer (NSCLC): Outcomes update of North American Intergroup 0139 (RTOG 9309). Proc Am Soc Clin Oncol 23:624S, 2005 (abstr 7014)

32. Kunitoh H, Saijo N, Tsuboi M, et al: A pilot trial of preoperative MVP-combined chemoradiotherapy: Mitomycin C (MMC), vindesine (VDS), and cisplatin (CDDP), concurrently given with thoracic radiotherapy (TRT) in N2 non-small cell lung cancer (NSCLC). Proc Am Soc Clin Oncol 19:530a, 2000 (abstr 2085)

33. Stuschke M, Eberhardt W, Pottgen C, et al: Prophylactic cranial irradiation in locally advanced non-small-cell lung cancer after multimodality treatment: Long-term follow-up and investigations of late neuropsychologic effects. J Clin Oncol 17:2700-2709, 1999

Acknowledgment

Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

Proteomic analysis of stage I primary lung adenocarcinoma aimed at individualisation of postoperative therapy

J Maeda[1], T Hirano[*,1], A Ogiwara[2,3], S Akimoto[2,3], T Kawakami[2], Y Fukui[4], T Oka[4], Y Gong[1], R Guo[1], H Inada[1], K Nawa[1], M Kojika[1], Y Suga[1], T Ohira[1], K Mukai[5] and H Kato[1]

[1]Department of Surgery, Tokyo Medical University, 6-7-1 Nishi-shinjuku, Shinjuku-ku, Tokyo 160-0023, Japan; [2]Clinical Proteome Center, Tokyo Medical University, 2-6-1 Nishi-shinjuku, Shinjuku-ku, Tokyo 163-0217, Japan; [3]Medical ProteoScope Co. Ltd, 2-6-1 Nishi-shinjuku, Shinjuku-ku, Tokyo 163-0217, Japan; [4]Taiho Pharmaceutical Co. Ltd, Tokyo, Japan; [5]Department of Pathology, Tokyo Medical University, 6-7-1 Nishi-shinjuku, Shinjuku-ku, Tokyo 160-0023, Japan

Although postoperative adjuvant chemotherapy (PAC) with uracil–tegafur significantly improves the prognosis of patients with stage I lung adenocarcinoma, subset analysis has revealed that only 11.5% of patients with stage IB derive actual benefit from such therapy. Therefore, it is extremely important to identify patients for whom adjuvant chemotherapy will be beneficial. We performed comprehensive protein analysis of 24 surgically resected specimens of stage I adenocarcinoma using liquid chromatography-tandem mass spectrometry (LC-MS/MS), followed by bioinformatical investigations to identify protein molecules. Furthermore, we carried out immunohistochemical studies of 90 adenocarcinoma specimens to validate the results of LC-MS/MS. We detected two kinds of protein molecules (myosin IIA and vimentin) by LC-MS/MS. We confirmed their immunohistochemical expression and distribution, and evaluated the relationship between the expression of these proteins and prognosis after adjuvant chemotherapy. Patients with no expression of either myosin IIA or vimentin showed a significantly better outcome regardless of PAC using uracil–tegafur. However, we were unable to select responders to uracil–tegafur using these proteins. Cases of adenocarcinoma lacking expression of either myosin IIA or vimentin show a good outcome without PAC, and therefore do not require such treatment.
British Journal of Cancer (2008) 98, 596–603. doi:10.1038/sj.bjc.6604197 www.bjcancer.com
Published online 22 January 2008
© 2008 Cancer Research UK

Keywords: myosin IIA; vimentin; postoperative adjuvant chemotherapy; responder to uracil–tegafur; stage I lung adenocarcinoma

Death due to lung cancer is still increasing in most industrialised countries, including Japan, despite improvement of various diagnostic and therapeutic modalities. Even though the opportunities to detect lung cancer at an early stage are increasing, approximately 60 000 patients with lung cancer die every year in Japan, usually due to distant metastasis. Distant metastasis, including intrapulmonary metastasis, frequently occurs in patients with advanced-stage non-small cell lung cancer (NSCLC) who undergo only surgical resection, because in such cases micrometastases probably exist at the time of surgery. The concept of postoperative adjuvant chemotherapy (PAC) for control of micrometastasis does not conflict with the improved prognosis of NSCLC patients. However, the efficacy of PAC in patients after complete resection of NSCLC was a matter of controversy in the 1990s. Even as recently as 2003, the efficacy of PAC could not be demonstrated (Scagliotti et al, 2003). In 2004, however, some studies demonstrate a beneficial effect of PAC (Arriagada et al, 2004; Strauss et al, 2004; Winton et al, 2005). We have also reported that PAC with oral uracil–tegafur (DPD Inhibitory Fluoropyrimidine, Taiho Pharmaceutical Co. Ltd, Tokyo, Japan) provided better survival than surgical treatment alone in patients

with stage I adenocarcinoma of the lung (Kato et al, 2004). The combination of uracil and tegafur (also referred to as UFT) at a molar ratio of 4:1 is an oral anticancer agent with good absorption in the small intestine (Fujii et al, 1979). Tegafur is a prodrug that is gradually converted into fluorouracil in the liver by the cytochrome P-450 enzyme system. Uracil enhances the serum concentration of fluorouracil by competitive inhibition of dihydropyrimidine dehydrogenase, the enzyme responsible for fluorouracil catabolism (Ikenaka et al, 1979). Oral uracil–tegafur generates a higher maximal plasma level of fluorouracil than protracted intravenous infusion of fluorouracil at a dose that is equimolar to the amount of tegafur in uracil–tegafur (Ho et al, 1998).

Even though PAC with uracil–tegafur has significantly improved the prognosis of patients with stage I primary lung adenocarcinoma, even subset analysis of stage IB has revealed that 11.5% of patients actually derive some benefit from the treatment (Kato et al, 2004). Nonresponders to uracil–tegafur, including relapse-free patients without any adjuvant therapy, gain no benefit from PAC. In this context, it is important to establish biomarkers for prediction of responders to uracil–tegafur, and/or for favourable prognosis without the use of PAC.

Clarification of the entire human genome is one of the most significant events in the history of bioscience, and has accelerated the comprehensive analysis of human genes and their protein

products. Many biologists recognise the importance of protein analysis, because proteins play central role various cellular functions. However, in cancer research, there has been a tendency for most researchers to avoid investigation of cancer-related proteins, because their structures are more complicated than those of the genes. Nevertheless, techniques for the comprehensive analysis of proteins have improved greatly in recent years. The concept of comprehensive protein analysis has been established, and the new research field of proteomics has been developed. One of the main purposes of clinical proteomics in the field of oncology is the development of new therapeutic strategies for cancer, centred on individualised therapy. In this study, we attempted to identify biomarkers for the selection of responders to uracil–tegafur and nonresponders, including relapse-free patients without any requirement for PAC, using clinical proteomics methodology.

MATERIALS AND METHODS

Institutional review board approval for this investigation

The institutional review board approved the use of proteomics analysis to explore biomarkers for selection of responders to oral uracil–tegafur (294/323/480/702).

Surgical samples of stage I lung adenocarcinoma for mass spectrometry

After obtaining written informed consent, lung cancer tissues were obtained from patients with pathologically confirmed stage I adenocarcinoma resected at Tokyo Medical University Hospital between 1995 and 2001. Tissues were kept frozen at −80°C until use. We collected 11 lung adenocarcinoma specimens from 11 patients who subsequently underwent PAC using uracil–tegafur for more than 2 years. In 5 of these 11 patients, recurrent lesions were detected within 2 years after surgery (U1R1), and the remaining 6 were confirmed to be disease-free for 5 years after surgery (U1R0). Furthermore, 13 specimens of lung cancer were collected from patients receiving no adjuvant therapy after surgery. In 6 of these 13 patients, recurrence was recognised within 2 years after surgery (U0R1), but no recurrent lesions were detected during 5 years after surgery in the other 7 (U0R0).

Protein extraction

The surgically resected materials were suspended and homogenised in PBS supplemented with a protease inhibitor cocktail (Roche Diagnostics Inc., Basel, Switzerland) at 4°C. The cell lysate was then fractionated by ultracentrifugation (52 000 g, 4°C, 20 min). The resulting pellet containing plasma membranes from the cells, was solubilised in PBS containing 5% SDS with continuous ultrasonication. The resulting solution was taken as the insoluble fraction, whereas the supernatant from the ultracentrifugation, containing mainly cytosolic proteins, was taken as the soluble fraction. The total protein concentrations of both fractions were measured (Lowry et al, 1951) using bovine serum albumin as a standard.

Protein condensation with SDS-PAGE

We added 150 pmol egg white lysozyme (Sigma-Aldrich Inc., St Louis, MO, USA) to an aliquot containing 75 μg protein from each fraction, then dried it under vacuum. The mixture was then solubilised in sample buffer (Laemmli, 1970) with gentle stirring at 37°C for 1 h. A two-third volume of the solution containing 50 μg sample protein and 100 pmol lysozyme was subjected to SDS-PAGE on 12.5% polyacrylamide gel 1-mm-thick. SDS-PAGE was carried out at a constant current of 20 A until the bromophenol blue marker passed the boundary between the stacking and

separation gels. In this 'halfway' running, most proteins remained stacked in a small area of approximately 2 mm in height between the gel boundary and the blue marker. After electrophoresis, this small gel area was excised from the gel slab, and the proteins were fixed in the gel slice with an excess volume of aqueous solution containing 40% methanol and 10% acetic acid.

In-gel tryptic digestion of protein

The gel slice was subjected to an in-gel tryptic digestion process (Shevchenko et al, 1996), with minor modifications. Briefly, after S-carboxyamidomethylation of Cys residues with iodoacetamide, the gel slice was incubated in a small volume of 50 mM ammonium bicarbonate buffer solution containing 1 μg of trypsin (Promega Co., Madison, WI, USA). The resulting peptides were extracted from the gel matrix, and dried under vacuum.

Liquid chromatography-tandem mass spectrometry

The peptide mixture (1 μg) was analysed using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system in a fully automated manner (Kawakami et al, 2005). Briefly, reversed-phase peptide separation was performed on a C18 capillary LC column (Michrom BioResources Inc., Auburn, CA, USA) at a flow rate of 1 μl/min. For gasification of the protonated peptides, the LC effluent was directly interfaced with an electrospray ionisation (ESI) source in a positive ion mode modified on a Finnigan LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) (Schwartz et al, 2002). The ESI used a Teflon-coated spray needle (20 μm id, AMR Inc., Tokyo, Japan). The ESI-MS/MS operation and continuous data acquisition of full MS scan and subsequent three MS/MS scans were carried out on an Xcalibur system controller (Thermo Fisher Scientific).

Semiquantitative analysis

All full MS data were investigated using an i-OPAL semiquantitative LC-MS data analysis system (i-OPAL algorithm: Patent no. WO 2004/090526 AI). First, the signal intensity of the full MS scan was normalised so that the total signal intensity of each sample became the same value. Several standard signals derived either from the injected egg white lysozyme or from sample intrinsic common proteins were selected as i-OPAL alignment markers. The i-OPAL alignment programme was used to align the nonlinearly fluctuating LC retention time axis of all LC-MS data to finally generate a single combined LC-MS data set for the soluble and the insoluble fractions, respectively. Analysis of variance (ANOVA) was applied for each peak signal in the final combined LC-MS data set to select candidate marker signals whose intensity differed significantly in a particular patient group. ANOVA was carried out using a Spotfire DecisionSite package.

Database searches

All MS/MS data were investigated using the Mascot search engine (Matrix Science Ltd., London, UK, http://www. matrixscience.com) against the Homo sapiens (human) subset of the Swiss-Prot and the RefSeq protein sequence databases. The database searches were performed allowing for fixed modification of cysteine residues (S-carbamidomethylation, +57.0 Da) and variable modification of methionine residues (oxidation, +16.0 Da), peptide mass tolerance ±2.0 Da and fragment m/z tolerance ±0.8.

Surgical specimens of stage I lung adenocarcinoma for immunohistochemical staining

Sample set A for confirmation of LC-MS semiquantitative results To confirm the semiquantitative results of LC-MS, 23

Table I Clinical features of lung adenocarcinoma cases subjected to LC-MS/MS

Characteristic	(%)
Age (year)	
Median	65.0
Range	32–78
Gender	
Male	19 cases (79.2%)
Female	5 cases (20.8%)
Pathological stage	
IA	10 cases (41.7%)
IB	14 cases (58.3%)
Presence of recurrence	
(+)	11 cases (45.8%)
(−)	13 cases (54.2%)
PAC	
(+)	11 cases (45.8%)
(−)	13 cases (54.2%)

PAC = postoperative adjuvant chemotherapy with oral uracil–tegafur.

Table 2 Clinical features of lung adenocarcinoma cases as revealed by immunohistochemical staining

Characteristic	(%)
Age (year)	
Median	64.8
Range	45–82
Gender	
Male	54 cases (60.0%)
Female	36 cases (40.0%)
Pathological stage	
IA	33 cases (36.7%)
IB	57 cases (63.3%)
Existence of recurrence	
(+)	41 cases (45.6%)
(−)	49 cases (54.4%)
PAC with urasil-tegafur	
(+)	51 cases (56.7%)
(−)	39 cases (43.3%)

PAC = postoperative adjuvant chemotherapy.

formalin-fixed, paraffin-embedded specimens derived from the same cases as those used for LC-MS analysis were collected for immunohistochemical investigation. As one formalin-fixed specimen had already been exhausted for the previous investigations, the remaining 23 specimens were investigated.

Sample set B for validation To validate the expression of the protein molecules on lung adenocarcinoma cells, 90 formalin-fixed, paraffin-embedded specimens from patients with lung adenocarcinoma, resected at Tokyo Medical University Hospital between 1995 and 2001, were used. All the patients had undergone curative resection of lung cancer, and after surgery, a pathologically definitive diagnosis of stage I adenocarcinoma had been obtained. We evaluated recurrence after surgery using chest roentgenography and serum tumour markers (CEA, CA19-9 and SLX) every 3 months and computed tomography of the head and body, and bone scintigraphy, every 6 months. When it was difficult to evaluate roentgenographically whether the lesion was recurrent or not, either cytological or pathological examinations were performed to obtain a definitive diagnosis (Table 1).

Of the 90 patients, 51 underwent PAC using uracil–tegafur. These 51 cases included 24 recurrences (U1R1) within 5 years and 27 cases without recurrence (U1R0) within 5 years after surgical treatment. The remaining 39 patients did not receive any adjuvant chemotherapy. These 39 patients included 17 with recurrence (U0R1) and 22 without recurrence (U0R0). The clinicopathological backgrounds of the 90 patients with lung adenocarcinoma are summarised in Table 2.

Immunohistochemical staining of surgically resected specimens of stage I lung adenocarcinoma

Four-micrometer-thick tissue sections were prepared from formalin-fixed, paraffin-embedded surgical specimens and collected on glass slides. The sections were stained immunohistochemically by the ABC method using either anti-myosin IIA mouse monoclonal antibody (clone ab24762, abcam, Cambridge, CB4 0FW, UK) (diluted 1:500) or anti-vimentin antibody (Dako Cytomation, Denmark A/S) (diluted 1:100) as the first antibody. After deparaffinisation, specimens were treated with 0.01% trypsin and an autoclave antigen retrieval system (Barbareschi et al, 1994). Sequentially, after inhibition of endogenous peroxidase activity with 0.5% hydrogen peroxide and incubation with 2% normal

swine serum, the first antibody was applied. Biotinylated anti-mouse immunoglobulin (Vector Laboratories Inc., Burlingam, CA, USA) was applied as the second antibody (diluted 1:200), followed by application with avidin–biotin peroxidase complex (Vector Laboratories Inc.) (diluted 1:100). The specimens were reacted with 0.06% 3,3'-diaminobenzidine tetrahydrochloride and 0.03% hydrogen peroxide in Tris-buffered saline to visualise the positive areas. Meyer's haematoxylin was used for counterstaining.

Evaluation of immunohistochemically stained preparations

Cells showing cytoplasmic staining were evaluated as positive. For myosin IIA immunohistochemical staining, we evaluated a case as positive when more than 50% of the cells were stained. Also, for vimentin immunostaining, cases in which more than 25% of the cells were stained were evaluated as positive. For both kinds of staining, normal alveolar epithelium served as an internal negative control.

Statistical analysis

Statistical analysis was carried out using the SPSS program. Statistical significance of the relationship between recurrence and immunohistochemical reactivity was evaluated using χ^2 test. Disease-free survival curves were calculated from the day of surgery using the Kaplan–Meier method, and the significance of differences in survival rates between the patient groups was calculated by the log-rank test. In all statistical analyses, a P-value of < 0.05 was taken to indicate a statistically significant difference.

RESULTS

LC-MS data analysis

After i-OPAL alignment and peak detection, we obtained 13 136 signal peaks from the soluble fraction and 14 984 peaks from the insoluble fraction. Using Spotfire, we restricted the candidate signal peaks on the basis of the following conditions:

(1) A Mascot search result with a score equal to or more than 50.
(2) An ANOVA P-value equal to or less than 1×10^{-5} (for the soluble fraction) or 1×10^{-6} (for the insoluble fraction).
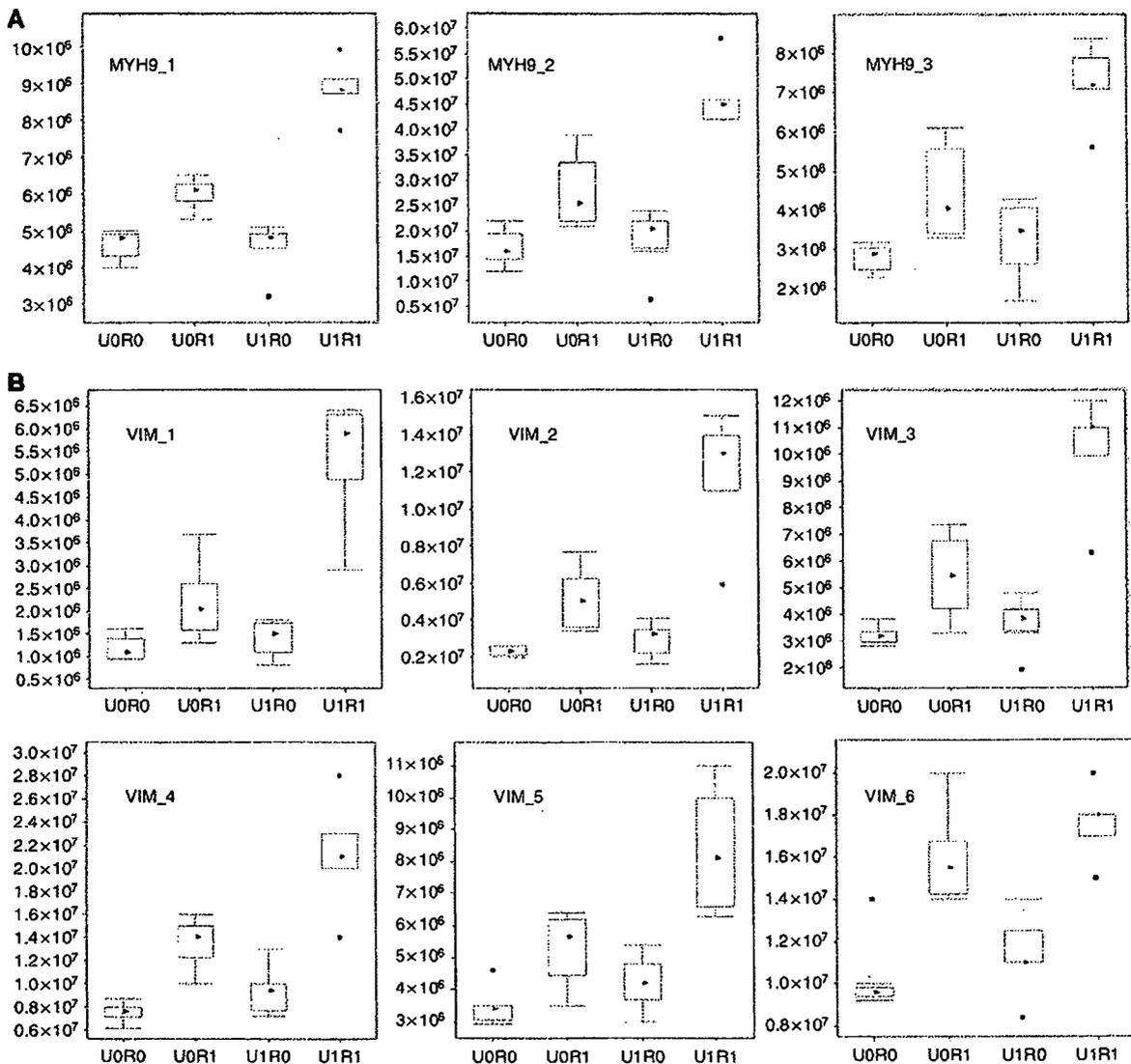
Figure 1 Comparison of the intensity of peptide signals originating from the same protein molecule in each group detected by LC-MS. The vertical axis indicates normalised signal intensity measured by LC-MS. In each box plot, the upper and lower sides of the box represent the upper and the lower quartile values (Q3/Q1), and the upper and lower horizontal bars outside the box indicate the upper and the lower adjacent values (UAV/LAV). Note that UAV is the largest observation value that is less than or equal to Q3 + 1.5 × (Q3−Q1), and LAV is the smallest observation greater than or equal to Q1 − 1.5 × (Q3−Q1). Black triangle marks represent the median values, and black square marks represent outliers. U0R0: patients without PAC showing no recurrence within 5 years after surgery. U0R1: patients without PAC in showing recurrence within 5 years after surgery. U1R0: patients who received PAC with uracil−tegafur and showed no recurrence within 5 years after surgery. U1R1: patients who received PAC with uracil−tegafur and showed recurrence within 5 years after surgery. (**A**) These three peptide signals were shown by MS/MS to have originated from myosin IIA. There was a significant difference between the U1R1 and the other groups ($P<9.7 \times 10^{-7}$). (**B**) These six peptide signals were shown by MS/MS to have originated from vimentin. There was also a significant difference between the U1R1 and the other groups ($P<8.3 \times 10^{-6}$).

As the peptide compositions of the soluble and the insoluble fractions differed, we applied different criteria to obtain approximately the same number of candidate signals. As a result, we were able to restrict the number of candidate signals to 23 and 28 for the soluble and insoluble fractions, respectively. From the restricted candidate signals, we selected several myosin IIA and vimentin signals as final candidate biomarker signals, because these two candidate biomarkers were identified by more than one distinct peptide sequence, and almost all of these signals had similar patterns of intensity (Figure 1A and B; Table 3).

Table 3 lists the amino-acid sequences from the selected candidate biomarker signals described above. These sequences were identified from MS/MS data using Mascot software.

Figure 1A shows the distribution of the signal intensity of several peptide ions derived from myosin IIA, and Figure 1B shows the signal intensity distribution of vimentin-derived peptide ions. For most signals, the intensity for group U1R1 patients showed patterns that differed significantly (i.e., were markedly higher) from those of the other patient groups.

Immunohistochemical staining of myosin IIA and vimentin

Representative staining of myosin IIA and vimentin is shown in Figure 2, and a summary of the immunohistochemical data is presented in Table 4. Cytoplasmic staining was observed in cases

Table 3 Amino-acid sequences from the selected peptide ion signals

Name	Fraction	Sequence
Myosin, heavy polypeptide 9, non-muscle		
MYH9_1	Insoluble	IRELESQISELQEDLESER
MYH9_2	Insoluble	KANLQIDQINTDLNLER
MYH9_3	Insoluble	HEMPPHIYAITDTAYR
Vimentin		
VIM_1	Insoluble	ETNLDSLPLVDTHSK
VIM_2	Insoluble	NLQEAEEWYK
VIM_3	Insoluble	LGDLYEEEMR
VIM_4	Insoluble	LLQDSVDFSLADAINTEFK
VIM_5	Soluble	SGDAAIVDMVPGKPMCVESFSDYPPLGR
VIM_6	Soluble	ILTVEDHYYEGGIGEAVSSAVVGEPGITVTHLAVNR

positive for myosin IIA and vimentin. We evaluated cases in which more than 50% of the cells showed immunohistochemical reactivity for myosin IIA, considered as overexpressing (positive). We also evaluated overexpressing (positive) cases in which more than 25% of the cells showed immunohistochemical reactivity for vimentin. On the basis of these criteria, we evaluated sample sets A and B.

Immunohistochemical evaluation of sample set A (Table 4A): all patients with cancers lacking expression of both myosin IIA and vimentin showed relapse-free survival at 5 years. On the other hand, all patients with cancers showing positive expression of both myosin IIA and vimentin suffered disease recurrence.

Immunohistochemical evaluation of sample set B (Table 4B): among 90 cases, 75 (83.3%) showed overexpression of myosin IIA, and 48 (53.3%) showed overexpression of vimentin. There was no
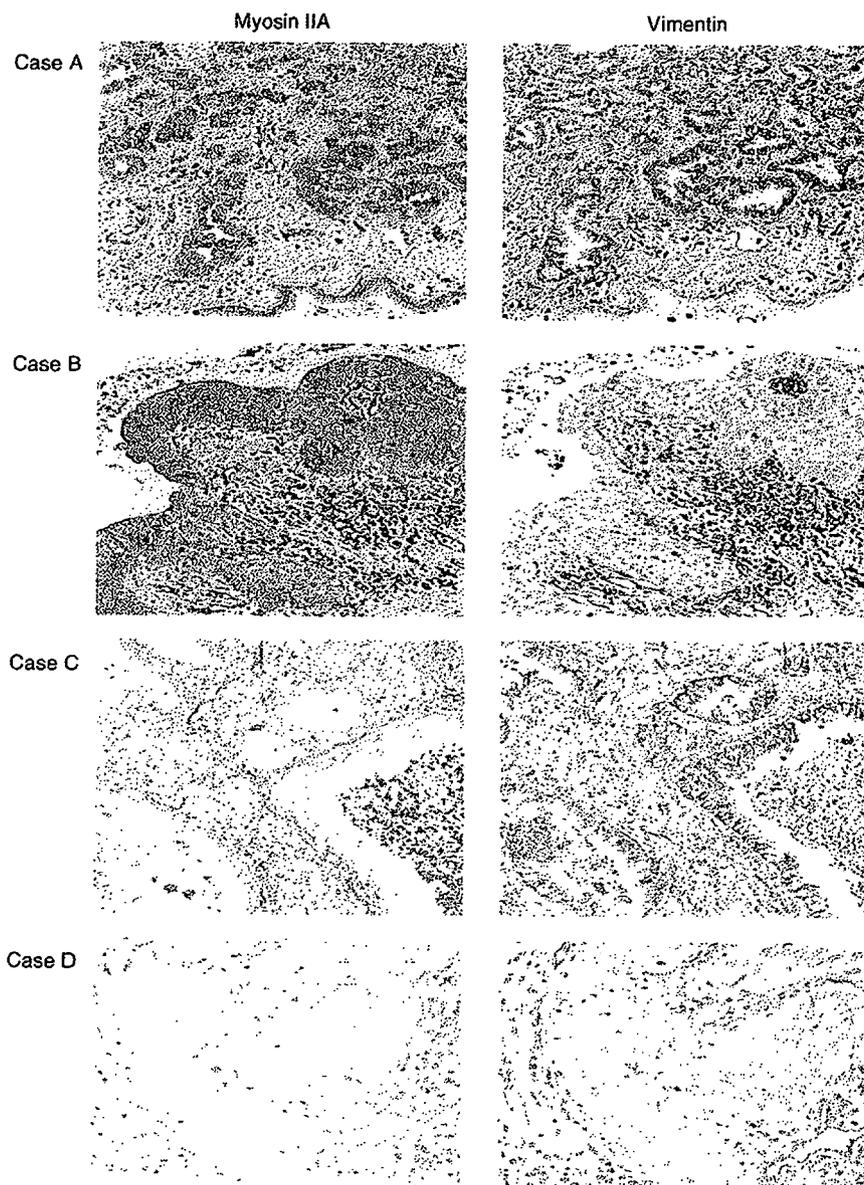
Figure 2 Immunohistochemical reactivity of representative cases using anti-myosin IIA antibody, ab24762 (abcam, Cambridge, UK) and anti-vimentin antibody (Dako Cytomation, Denmark A/S). Case A showed positive cytoplasmic staining for both myosin IIA and vimentin. Case B showed positive cytoplasmic staining for myosin IIA and negative cytoplasmic staining for vimentin. Case C showed negative cytoplasmic staining for myosin IIA and positive cytoplasmic staining for vimentin. Case D showed negative cytoplasmic staining for both myosin IIA and vimentin.

Table 4 Relationship between PAC, recurrence and immunohistochemical reactivity for myosin IIA and vimentin

	M(−)V(−)	M(−)V(+) or M(+)V(−)	M(+)V(+)	ND
(A) Sample set A (n = 24) derived from the same cases as those subjected to LC-MS/ MS				
U0R0	5	2	0	0
U1R0	2	3	0	1
U0R1	0	2	4	0
U1R1	0	0	5	0
(B) Sample set B (n = 90) for validation by immunohistochemical analysis				
U0R0	6	11	5	0
U1R0	3	12	12	0
U0R1	0	6	11	0
U1R1	0	10	14	0

M = expression of myosin IIA; ND = not done; U0R0 = patients without PAC showing no recurrence within 5 years after surgery; U0R1 = patients without PAC showing recurrence within 5 years after surgery; U1R0 = patients who received PAC with uracil-tegafur and showed no recurrence within 5 years after surgery; U1R1 = patients who received PAC with uracil-tegafur and showed recurrence within 5 years after surgery; V = expression of vimentin. *Statistically significant difference between U0R0 and U1R1 was detected (P = 0.008).

relationship between the immunohistochemical reactivities of myosin IIA and vimentin. All nine patients whose cancers lacked immunohistochemical reactivity for both myosin IIA and vimentin showed relapse-free survival at 5 years. Among cases that were immunohistochemically negative for both myosin IIA and vimentin, we recognised a statistically significant difference between U1R1 and U0R0 ($P = 0.008$), but there were no significant differences between U1R1 and the other groups.

Disease-free survival and coexpression of myosin IIA and vimentin in sample set B

The non-relapse survival curves of cases with/without PAC are shown in Figure 3A and B. Irrespective of whether patients had undergone PAC or not, the non-relapse survival rate of cases lacking expression of both myosin IIA and vimentin was 100%. Among patients who had not undergone PAC, there was a statistically significant difference between cases lacking expression of both myosin IIA and vimentin and cases that were positive for both ($P = 0.011$) (Figure 3A). Among the patients who received PAC, there was no statistically significant difference in this respect (Figure 3B). When the cases showing positive expression of both myosin IIA and vimentin were evaluated, we recognised a 5-year-survival rate benefit of approximately 19% in patients who had undergone PAC with uracil-tegafur, but there was no statistically significant difference in this respect between patients who had and who had not received PAC.

When we evaluated the non-relapse survival curves of all the studied cases, there were statistically significant differences between cases negative for myosin IIA and vimentin expression and cases that were positive for both ($P = 0.006$), and between cases positive for either myosin IIA or vimentin and cases that were negative for both ($P = 0.029$; Figure 4).

DISCUSSION

Lung cancer is the leading cause of cancer death in Japan, and its incidence is still increasing. Even if surgical resection involving either lobectomy or pneumonectomy accompanied by lymph node dissection is performed at a relatively early stage, distant metastasis often occurs within a few years. More than 20% of patients with stage I NSCLC suffer recurrence caused by distant
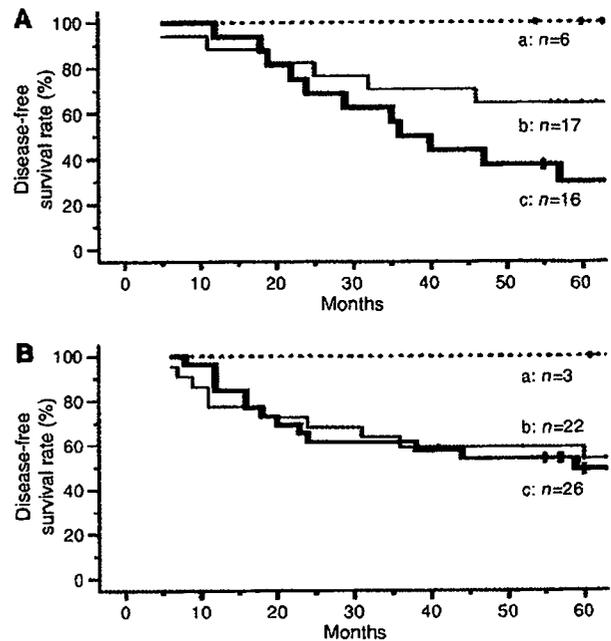
Figure 3 Kaplan–Meier curves for disease-free survival after complete resection in patients with stage I lung adenocarcinoma who received PAC with uracil-tegafur (**A**), or did not receive any PAC (**B**). a: Cases lacking both myosin IIA and vimentin expression (non-relapse survival rate at 5 years: 100% in panels **A** and **B**). b: Cases negative for myosin IIA expression and positive for vimentin expression, or positive for myosin IIA and negative for vimentin expression (non-relapse survival rate at 5 years: 64.7% in panel **A** and 53.7% in panel **B**). c: Cases positive for both myosin IIA and vimentin expression (non-relapse survival rate at 5 years: 30.0% in panel **A** and 49.0% in panel **B**). In patients who did not receive adjuvant chemotherapy, there was a statistically significant difference in disease-free survival between those who were negative and those who were positive for both proteins (a–c: $P = 0.011$). No significant difference in this respect was recognised in patients who received PAC with uracil-tegafur.
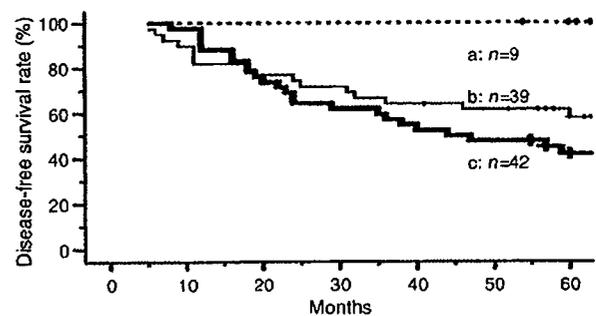
Figure 4 Kaplan–Meier curves for disease-free survival after complete resection in patients with stage I lung adenocarcinoma. a: Cases lacking both myosin IIA and vimentin expression (non-relapse survival rate at 5 years: 100%). b: Cases negative for myosin IIA and positive for vimentin, or positive for myosin IIA and negative for vimentin (non-relapse survival rate at 5 years: 58.0%). c: Cases positive for both myosin IIA and vimentin (non-relapse survival rate at 5 years: 42.0%). Group a: showed significantly higher survival than group b, and significantly higher survival than group c (a–b: $P = 0.029$; a–c: $P = 0.006$).

metastasis. Distant metastasis is the most frequent mode of recurrence in patients who undergo surgical resection of lung cancer, and it is believed that in such patients, micrometastasis is

invariably present at the time of initial treatment. If an efficient PAC regimen could be devised for total control of micrometastasis, then the prognosis of patients with lung cancer would be markedly improved. A meta-analysis conducted in the 1990s showed that PAC using platinum-based agents had no effect on the survival of patients with NSCLC, even though previous studies had suggested a 5% increase in survival at 5 years (Non-Small Cell Lung Cancer Collaborative Group, 1995). At the 2004 ASCO meeting, the results of two randomised adjuvant trials showing the efficacy of platinum-based chemotherapy – the CALGB-9633 trial (carboplatin and paclitaxcel) (Strauss et al, 2004) and the JBR 10 trial (cisplatin and vinorelbine) – were reported. Furthermore, a recent large-scale randomised clinical trial involving meta-analysis concluded that patients assigned to cisplatin-based PAC had a significantly higher survival rate than those assigned to postoperative observation (44.5 vs 40.4% at 5 years; $P < 0.03$) (Arriagada et al, 2004). Also our previous study showed that PAC with uracil–tegafur conferred a survival benefit for patients with resected stage I adenocarcinoma of the lung (Kato et al, 2004). Also, meta-analysis of PAC with tegafur–uracil supported this result (Hamada et al, 2005). However, even though a significant difference was found in this study, the 5-year-survival rate benefit of this therapy was 11.5% for stage IB adenocarcinoma (Kato et al, 2004). At present, although leading lung cancer experts appear to have reached a consensus concerning the effectiveness of PAC, none of the present PAC regimens are of benefit to more than 15% of patients with NSCLC. In this context, it is very important to predict the response to PAC and to select potential responders before carrying out PAC. Therefore, we attempted to identify biomarkers of either responders or nonresponders including relapse-free patients without PAC using uracil–tegafur, for selection of patients who would benefit from PAC using proteomic analysis of surgically resected specimens of stage I lung adenocarcinoma.

As proteins play a role in both physiological and pathological functions, it is now recognised that investigation of proteins is essential to obtain an accurate grasp of cellular physiology. Recent advances in proteomic techniques, including two-dimensional polyacrylamide gel electrophoresis and MS, have brought hope that the pathogenesis of any type of malignant neoplasm will be ultimately clarified. We believe that the present concepts of proteomic analysis will prove to be extremely valuable in the field of clinical oncology, and will lead to the development of new therapeutic strategies. Liquid chromatography-tandem mass spectrometry enables simultaneous evaluation of a large number of polypeptides, and furthermore, MS/MS has made it possible to identify protein molecules by obtaining information about their amino-acid sequences. We attempted to identify proteins associated with the effectiveness of postoperative uracil–tegafur chemotherapy and the favourable prognosis of stage I adenocarcinoma, and detected two kinds of protein molecules (myosin IIA and vimentin) showing significantly high expression in the group that suffered recurrence despite administration of uracil–tegafur, in comparison with the other groups. Our semiquantitative results of LC-MS were confirmed by immunohistochemistry for myosin IIA and vimentin (Table 4A).

Nonmuscle myosin IIA is a major component of the actomyosin cytoskeleton and is generally considered to contribute to contraction of the cell posterior during migration (Ridley et al, 2003). However, there is still a profound lack of understanding of the exact mechanical roles of myosin IIA during cell migration. A recent clinical study of patients with NSCLC found a significant positive correlation between the expression levels of myosin light chain kinase (which activates myosin II) and the likelihood of disease recurrence and metastasis (Minamiya et al, 2005), indicating that myosin IIA activation could be a factor contributing to metastasis. A key role for myosin IIA in cancer cell metastasis has been further suggested, indirectly, by a number of

published studies focusing on the small calcium-binding protein, metastasin-1. This protein is upregulated in many metastasis cell lines, and when overexpressed enhances metastatic behaviour (Davies et al, 1993). A major cellular target of metastasin-1 seems to be myosin IIA (Garrett et al, 2006). Although studies of metastasin-1 suggest critical roles for myosin IIA in metastasis, it remains completely unknown how myosin IIA contributes to metastasis, and which isoforms are important for this process.

Vimentin is the most ubiquitous intermediate filament protein and the first to be expressed during cell differentiation. All primitive cell types express vimentin, but in most nonmesenchymal cells, it is replaced by other intermediate filament proteins during differentiation. Vimentin is expressed in a wide variety of mesenchymal cell types (fibroblasts, endothelial cells, etc), and also in a number of other cell types derived from mesoderm, mesothelium and ovarian granulose cells. Epithelial–mesenchymal transition is a key mechanism operating in the normal development of multicellular organisms. During this process, epithelial cells progressively acquire a reversible or irreversible mesenchymal phenotype that is essential for organogenesis (Thiery, 2002). Morphogenetic epithelial–mesenchymal transition is aberrantly recapitulated during tumorigenesis in a variety of epithelial cancers, including those of the thyroid, liver, kidney, prostate, breast and lung (Arias, 2001; Thiery, 2002). The common signature of this process involves disruption of normal epithelial integrity, with loss of morphological features including polarised epithelia, and partial or total gain of mesenchymal markers with progressive acquisition of a motile and invasive phenotype (Islam et al, 1996). In addition to a disrupted epithelial morphology, dysregulation of adhesion and junctional molecules and aberrant expression of N-cadherin, epithelial–mesenchymal transition involves de novo expression of other mesenchymal markers, such as fibronectin and vimentin in epithelial cells. Aberrant expression of vimentin in tumours and transformed cell lines has been correlated with increased motility, invasive behaviour and poor prognosis (Gilles et al, 1996; Hendrix et al, 1997). Recently, it was reported that the presence of vimentin-positive tumour cells mainly in fibrotic areas is consistent with other studies that have shown a correlation between tumour fibrosis and epithelial–mesenchymal transition (Blanco et al, 2004).

These two molecules identified by proteomic analysis might reflect the cellular functions of metastasis and the mechanism of recurrence of malignant neoplasms. We attempted to validate the results of LC-MS/MS using immunohistochemistry of an additional sample set (sample set B: 90 surgically resected lung cancer specimens) with monoclonal antibodies against the two proteins. The results showed that cases lacking expression of the two proteins had a good prognosis, irrespective of whether the patients had undergone PAC. Therefore, these two proteins appear to be potentially useful biomarkers for the selection of patients who do not require PAC. In the cases positive for both of these proteins, the 5-year-survival benefit was approximately 19% in patients with adenocarcinoma who underwent PAC with uracil–tegafur. However, there was no significant difference between patients who did, and did not, undergo PAC with uracil–tegafur. Therefore, in this investigation, we failed to select patients who might benefit from this adjuvant chemotherapy. A larger-scale investigation is therefore needed to establish suitable biomarkers for the selection of patients who might benefit from PAC with uracil–tegafur, because a few per cent of patients with stage I adenocarcinoma do obtain such a benefit.

Individualised chemotherapy for lung cancer patients is currently attracting attention, because the efficacy of systemic chemotherapy using any single agent is less than 30%. Therefore, it is extremely important to select patients who might benefit from chemotherapy. Until an ideal chemotherapy agent is established, we propose that rather than focusing only on improving the efficacy of chemotherapy regimens, we should also make efforts to identify patients who will show a good response to regimens that