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 (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.

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

	EGFR mutation positive ($n = 32$)		*EGFR* mutation negative ($n = 85$)		
Characteristics	**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.

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

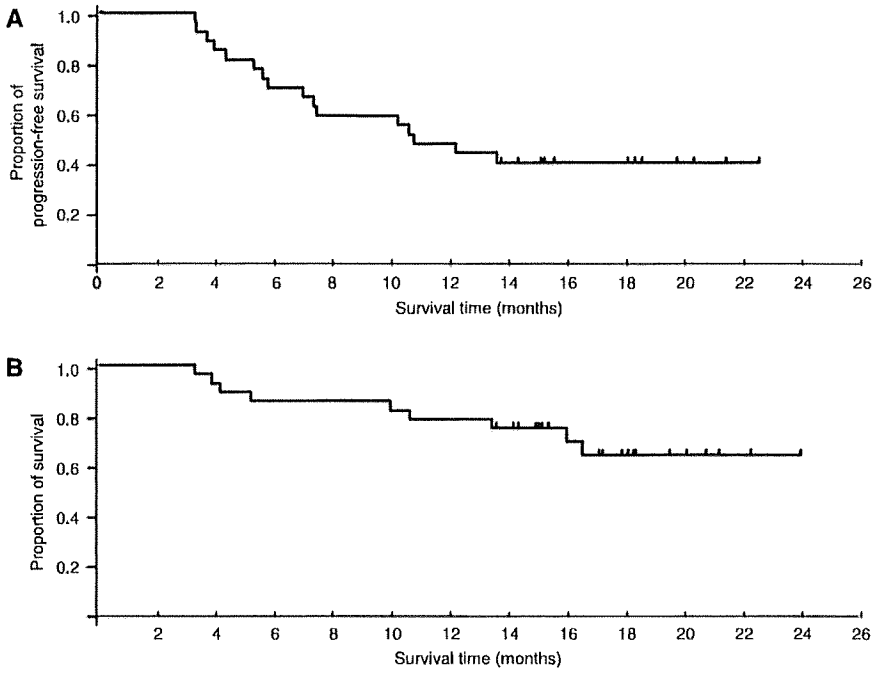

Figure 1 (**A**) Progression-free survival (PFS) and (**B**) overall survival (OS) of all eligible patients ($n = 28$). The median PFS was 11.5 months. The median OS has not yet been reached. The 1-year survival rate was 79%.

Table 5 Common adverse events ($n = 28$)

	No. of patients (%)			
Adverse events	**Grade 1**	**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	0.48
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.

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A Phase II Trial of Gefitinib Monotherapy in Chemotherapy-Naïve Patients of 75 Years or Older with Advanced Non-small Cell Lung Cancer

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Background: Gefitinib has shown modest activity in patients with recurrent non-small cell lung cancer (NSCLC) after platinum-based chemotherapy. However, the activity of gefitinib as first-line chemotherapy remains unclear, especially unknown in elderly patients. A multicenter phase II trial was conducted to evaluate the efficacy and tolerability of gefitinib for elderly patients with chemotherapy-naïve NSCLC.

Methods: Elderly chemotherapy-naïve patients with advanced NSCLC, ECOG PS of 0–2, and adequate organ functions received 250 mg/day of gefitinib. The primary objective of this study was to determine the objective response rate (RR). Secondary endpoints were tolerability, disease-related symptom using lung cancer subscale (LCS) in FACT-L, progression free survival (PFS) and overall survival (OS). We investigated mutation status of the epidermal growth factor receptor (EGFR) gene in cases with available tumor samples.

Results: Fifty patients were enrolled, of whom 49 were eligible. Median age (range) was 80 (75–90) years. Thirty-two patients were female (65%) and 40 patients had adenocarcinoma (82%). The objective RR was 25% (CI 95%, 13–39). Median survival time was 10 months (CI 95%, 7–20) and 1-year survival rate was 50%. The most frequent adverse events were skin disorders (76%). Fifteen patients (30%) experienced toxicities ≥grade 3. There were four patients with possible interstitial lung disease including two treatment-related deaths. Symptom improvement rate using LCS was 49% at 4 weeks of gefitinib therapy. Tumor samples from 17 patients were analyzed for *EGFR* mutation status. *EGFR* mutations were detected in tumor tissues from 7 patients, of which 5 had partial responses (71%).

Conclusions: Gefitinib monotherapy is effective and relatively well tolerated in chemotherapy-naïve elderly patients with advanced NSCLC. Gefitinib has potential as a first-line therapeutic option in elderly patients with advanced NSCLC.

Key Words: Gefitinib, Non-small cell lung cancer, Elderly

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The incidence of lung cancer, the major cause of cancer-related mortality worldwide, increases with age.[1] More than 30% of lung cancer patients are diagnosed at an age of ≥75 years,[2] and this proportion is likely to increase over the coming years. Approximately 80 to 85% of lung cancer subtypes are of non-small cell histology. In patients with advanced non-small cell lung cancer (NSCLC) chemotherapy improves survival, disease-related symptoms, and quality of life (QoL) compared with best supportive care[3] and combination chemotherapy involving newer agents is now considered the standard first-line treatment for most patients with advanced NSCLC.[4,5] However, combination chemotherapy causes increased hematologic and neuropsychiatric toxicity in older patients,[6,7] and ≥90% of elderly patients experience a grade ≥3 toxicity when treated with a platinum-based doublet.[8] Therefore, single-agent chemotherapy is considered as a standard treatment for elderly patients with advanced NSCLC.[9,10] An effective, less toxic therapy might help extend potentially beneficial treatment to a greater proportion of older patients who would otherwise be considered unsuitable for chemotherapy. Recently, molecular-targeted agents have been introduced for the treatment of NSCLC. Gefitinib, an orally active epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI), is a leading agent in the field of EGFR-targeted

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therapy.[11] In two international, randomized phase II trials (Iressa Dose Evaluation in Advanced Lung cancer [IDEAL]-1 and IDEAL-2) in patients with advanced or metastatic NSCLC after platinum-based chemotherapy, treatment with gefitinib 250 mg monotherapy resulted in response rates (RRs) of 12 to 18%, good tolerability and symptom improvement.[12,13] The subset analyses in both trials revealed no significant difference in terms of the RR and adverse events (AEs) reported with gefitinib between younger and older patients. Despite the high incidence of NSCLC and its high mortality rate in elderly patients,[1] the likelihood of receiving systemic chemotherapy seems to decrease with increasing age in particular, the age of ≥75 years due to the progressive decline in organ function, and age-related comorbidities. Therefore, we conducted a phase II study of gefitinib monotherapy in chemotherapy-naive patients ≥75 years of age with advanced NSCLC.

PATIENTS AND METHODS

Patient Population

Patients with histologically or cytologically confirmed NSCLC that was inoperable as a result of substantial comorbidity, impairment of respiratory function or anatomic contraindication were eligible. Patients were to be aged 75 years or older, and to have an Eastern Co-operative Oncology Group Performance Status 0 to 2, measurable disease, Pao_2 ≥60 mmHg and adequate organ function. Exclusion criteria were: prior chemotherapy or thoracic radiotherapy; interstitial pneumonia or pulmonary fibrosis; as determined by chest computed tomography (CT); paralytic ileus or vomiting; symptomatic brain metastases; active infection; active concomitant malignancy; pregnancy or breast-feeding; and severe allergy to study drugs. All patients gave written informed consent before enrolment. This protocol was approved by the Institutional Review Boards of the participating centers.

Treatment Plan

Baseline assessment included a medical history and physical examination, standard laboratory studies, ECG, CT of the chest and abdomen, head CT or magnetic resonance imaging, and disease-related symptoms using lung cancer subscale (LCS) in The Functional Assessment of Cancer Therapy–Lung.[14] Patients were treated with gefitinib 250 mg daily until disease progression, severe or intolerable toxicity, or withdrawal of consent.

Patients were evaluated for objective response every month using Response Evaluation Criteria in Solid Tumors guidelines.[15] The objective response was evaluated centrally by an independent review board. All AEs reported during gefitinib treatment were recorded and severity was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 2.0.[16] Treatment interruptions up to 14 days were allowed for toxicities ≥grade 3, and patients were restarted if toxicities recovered to ≤grade 2 within 14 days.

Disease-Related Symptom Analysis

Disease-related symptoms were assessed by LCS before therapy and at 1, 2, and 4 weeks. Symptom improvement was defined as a 2-point or greater increase in score on the summed LCS.[17]

Mutation Analysis of the EGFR Gene

Tumor specimens (paraffin embedded) were collected from previous diagnostic or surgical procedures. DNA was extracted from tumor tissues derived either by macrodissection or by laser-capture microdissection performed to enrich tumor cells, using the QIAamp Micro kits (QIAGEN KK, Tokyo, Japan). The amplification-refractory mutation system (ARMS) with designed "scorpion" primers were applied for the allele-specific detection of *EGFR* mutations.[18] Only the following mutations described in previous studies were classified as mutations in the present study: G719X in exon 18, deletion of E746 to A750 or of neighboring residues in exon 19, and L858R and L861Q in exon 21. All mutations were confirmed by analysis of at least two independent amplification products.

Statistical Analysis

This study was a multicenter, single-arm, noncomparative phase II clinical trial. The primary end point was objective RR. Secondary endpoints were tolerability, disease-related symptoms using LCS in Functional Assessment of Cancer Therapy Lung, progression free survival (PFS) and overall survival (OS). With the target activity level of 30% and the lowest RR of interest set at 15%, 48 patients were required with $\alpha = 0.048$ and $\beta = 0.18$ assuming binomial distribution of RR. Allowing for a loss, a total of 50 patients were planned to enroll. All eligible patients were included in the analysis of response. The 95% confidence interval (CI) of the RR was calculated by the exact method, assuming a binomial distribution of data. PFS was defined as the time from registration until objective tumor progression or death. Patients whose disease had not progressed at the time of discontinuation of the study treatment continued to be assessed until progression was documented. If a patient died without documentation of disease progression, the patient was considered to have had tumor progression at the time of death, unless there was sufficient documented evidence to conclude otherwise. PFS and OS were estimated using the Kaplan-Meier method.[19] To examine the association of tumor response to gefinitib and OS with clinical factors including *EGFR* mutations, two-sided Fisher's exact test and Log-rank test, respectively, were used. To examine the time tendency of disease related symptoms, Generalized Estimation Equation model was used.[20] The model includes the repeated measured LCS as a continuous dependent variable and time in week as a continuous explanatory variable. A two-sided $p < 0.05$ was considered statistically significant.

RESULTS

Patients and Treatment Administration

Fifty patients were enrolled between April 2004 and May 2006 at 22 centers across Kyushu in Japan. Of these, 49 patients were deemed eligible (one stage IB patient with no indications for surgery or curative radiotherapy due to severe emphysema). One patient was ineligible because of stage IIIA with an indication for curative radiotherapy. Patient characteristics are listed in Table 1. Median age (range) was

TABLE 1. Patient Characteristics

Characteristics	No. of Patients	%
Patients enrolled	50	
Patients eligible	49	
Age		
Median	80	
Range	75–90	
Sex		
Male	17	35
Female	32	65
Performance status		
0	13	27
1	24	49
2	12	24
Stage at enrolment		
IB[a]	1	2
IIIB	8	16
IV	40	82
Histology		
Adenocarcinoma	40	82
Squamous cell carcinoma	6	12
Large cell carcinoma	0	0
Other	3	6
Smoking status		
Never	30	61
Former	16	33
Current	3	6

[a] No indications for surgery or curative radiotherapy due to severe emphysema

TABLE 2. Common Adverse Events (n = 50)

	All		Grade 3–5[a]	
Toxicity	No.	%	No.	%
Dermal[b]	38	76	10	20
Anaemia	33	66	3	6
Anorexia	25	50	6	12
Fatigue	21	42	5	10
Hepatic	20	40	11	22
Diarrhoea	14	28	2	4
Nausea	11	22	1	2
Neutropenia	11	22	0	0
Renal	11	22	0	0
Mucositis	7	14	1	2
ILD	4	8	3	6
Vomiting	3	6	0	0

[a] Two patients experienced grade 5 events related to gefitinib-induced ILD
[b] Dermal toxicities including rash, dry skin, pruritus, acne and nail changes
ILD, interstitial lung disease

80 (75–90) years. Thirty-two patients were female (65%) and 12 patients (24%) had a performance status (PS) of 2. The most common histologic NSCLC subtype was adenocarcinoma (82%). Most patients (82%) had stage IV disease or recurrence after surgical resection and 30 patients were never smokers (61%). Of the 49 patients, 46 patients discontinued gefitinib treatment by reason of progression of disease (n = 31), treatment-related AEs (n = 13), patient request (n = 1), and another disease (n = 1). The median treatment period was 2.8 months (range: 0.1–16 months). Three patients are still receiving treatment with gefitinib.

Adverse Events

Table 2 lists the AEs by grade. The most frequent AEs were skin disorders (76%) including rash, dry skin, pruritus, acne, and nail changes. In general, toxicities were mild (grade 1–2) and easily managed. Fifteen patients (30%) experienced toxicities ≥grade 3. Thirteen were withdrawn from the study because of treatment-related AEs—four patients with interstitial lung disease (ILD), four with rash, one with asthenia, one with diarrhea, one with mucositis, one with hypoxemia, and one with hepatic toxicity. There were four patients with possible ILD including two treatment-related deaths.

Response

There were 12 partial responses (PRs) among the 49 patients, yielding an overall RR of 25% (95% CI, 13–39%). Seventeen patients (35%) achieved stable diseases (SDs) and the overall disease control rate (PR + SD) was 59% (95% CI, 44–73%). Four patients were removed from the study before being evaluated for response; two patients had ILD, one had a stroke, and one had deteriorated PS. (Table 3).

PFS and Survival

All 49 eligible patients were assessed for PFS and survival. At the time of analysis, there were 31 deaths, 17 patients confirmed alive, and one patient lost to follow-up. Median survival time was 10 months (95% CI, 7–20; Figure 1*A*). Median PFS was 4 months (95% CI, 3–8; Figure 1*B*). The 1- and 2-year survival rates were 50% and 23%, respectively.

Disease-Related Symptoms

With respect to disease-related symptoms, the mean LCS scores were 21, 22, 22, and 23 points before the therapy and at 1, 2, and 4 weeks, respectively. The time tendency of change in LCS scores was significant (p = 0.017) (Table 4). There was no significant association between response and symptom improvement (Table 5).

TABLE 3. Best Overall Objective Response (n = 49)

Type of Response	No. of Patients	% of Patients	
Complete response	0	0	
Partial response	12	25	
Stable disease	17	35	
Progressive disease	16	33	
Not evaluable[a]	4	8	
Overall response (CI 95%)	12	25	(13–39)

[a] Four patients were removed from study before being evaluated for response; two patients had interstitial lung disease (ILD), one had stroke and one had deteriorated performance status (PS)
CI 95%, 95% confidence interval.

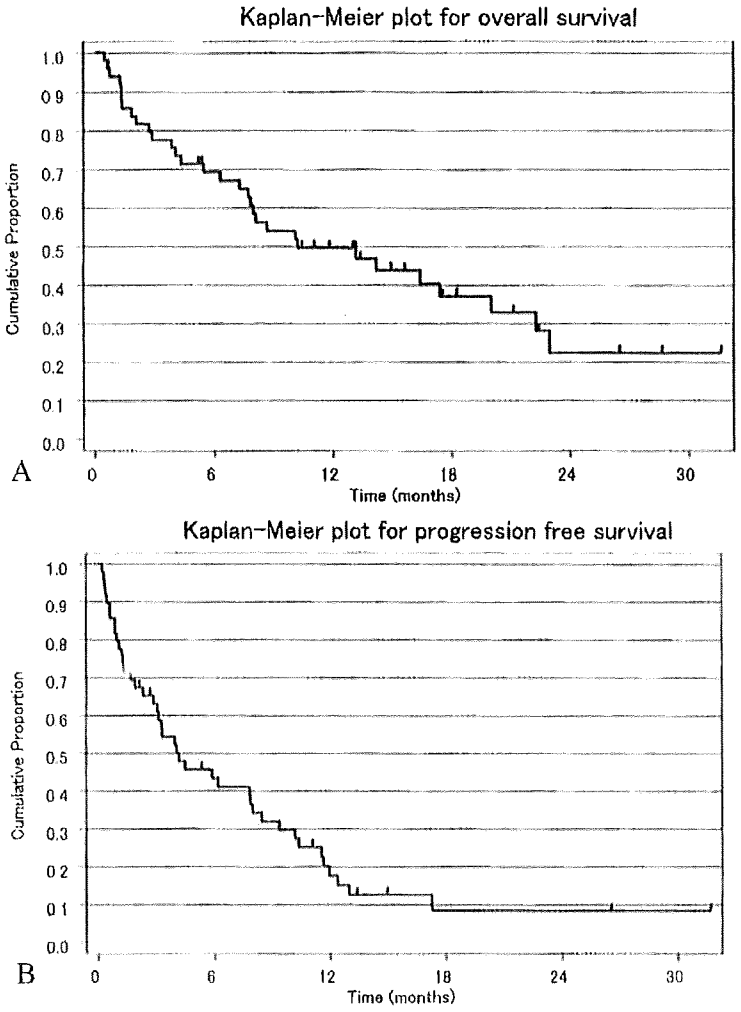

FIGURE 1. *A*, overall survival (OS) and (*B*) progression-free survival (PFS) of all eligible patients (n = 49). Median OS was 10 months. Median PFS was 4 months.

Mutation Analysis of the EGFR Gene

Tumor samples from 17 patients were analyzed for *EGFR* mutations by Scorpion-ARMS. *EGFR* mutations, consisting of in-frame deletions in exon 19 (n = 2) and point mutations in exon 21 (n = 5) were detected in 7 tumor tissues (41%). All patients harboring *EGFR* mutations achieved either PRs or SDs. Five patients with *EGFR* mutations had PRs and the RR was 71%. Of the 10 patients without *EGFR* mutations (59%), 2 patients had PRs, 3 had SDs and 5 had progressive diseases. The presence of *EGFR* mutations was associated with prolonged survival in this trial (Table 5).

TABLE 4. Disease-Related Symptoms Evaluated by Lung Cancer Subscale

		Before Therapy n = 42	After 1 wk n = 41	After 2 wk n = 39	After 4 wk n = 35
Mean LCS score		21	22	22	23
Change of LCS Score from Baseline			**No. of Patients (%)**		
≥ 2	Improved	15 (37)	14 (36)	17 (49)	
≤ 2	Worsened	5 (12)	10 (26)	8 (23)	
Otherwise	No change	21 (51)	15 (39)	10 (29)	

The time trend in repeated measured LCS was statistically significant (p = 0.017) according to Generalized Estimation Equation model analysis.
LCS, lung cancer subscale

Second-Line Chemotherapy

Thirteen patients received second-line chemotherapy (gefitinib rechallenge, n = 5; docetaxel monotherapy, n = 4; gemcitabine plus tegafur-uracil, n = 2; gemcitabine plus vinorelbine, n = 1; or gemcitabine monotherapy, n = 1). Altogether, two patients partially responded to second-line gemcitabine plus tegafur-uracil, or gemcitabine monotherapy, with no responses among patients receiving other regimens. Overall RR to second-line chemotherapy was 15%.

DISCUSSION

Given the increasing number of elderly individuals with advanced NSCLC, it is important for clinicians to be ready to manage these challenging patients in the coming decades. Prospective randomized trials in elderly (70 years or older)

TABLE 5. Association of Tumor Response to Gefitinib and Median Overall Survival with Clinicopathological Factors

Characteristics (no. of patients)	ORR		MS	
	%	P_1	mo	P_2
Gender				
Male (17)	0	<0.01	8	0.12
Female (32)	38		17	
Histology				
Adeno ca. (40)	28	0.42	13	0.06
Nonadeno ca. (9)	11		4	
Smoking				
Never (30)	37	0.02	17	0.10
Current/Former (19)	5		8	
LCS score				
Improved/No change (27)[a]	33	0.39	17	0.43
Worsened (8)[a]	13		6	
EGFR mutation				
Positive (7)	71	0.06	>27[b]	0.01
Negative (10)	20		7	

[a] This is at 4 wk of therapy.
[b] MS has not yet been reached.
ORR, objective response rate; MS, median survival; P_1, two-sided P for difference in ORR; P_2, two-sided P for difference in overall survival; LCS, lung cancer subscale.

advanced NSCLC patients are now available. The Elderly Lung Cancer Vinorelbine Italian Study Group reported significantly superior survival and QoL with single-agent vinorelbine over best supportive care.[9] The conclusive results were reported in other Multicenter Italian Lung Cancer in the Elderly Study, which enrolled more than 700 patients and reported no significant survival difference between single-agent vinorelbine, single-agent gemcitabine, or a regimen with both agents combined.[10] These studies together provide evidence that single-agent chemotherapy is considered as a standard treatment for advanced NSCLC elderly patients.

The present phase II study was designed to evaluate the efficacy and tolerability of single agent treatment with gefitinib in previously untreated NSCLC patients ≥75 years of age. The observed RR of 25% (95% CI, 13–39%), median PFS of 4 months, median survival of 10 months and 1-year survival rate of 50% are promising in elderly advanced NSCLC. One potential limitation in interpreting efficacy data from this trial is a possible selection bias on the part of treating physicians. Although this study planned to recruit unselected patients, a higher percentage of women (65%), never smokers (61%), and patients with adenocarcinoma (82%) were enrolled. This was a result of the growing evidence that these clinical characteristics are more often associated with benefit from EGFR-TKIs.[12,13,21] A randomized phase II study (median age <70) comparing combination chemotherapy with another EGFR-TKI, erlotinib for chemotherapy-naive advanced NSCLC patients with a PS of 2 (who accounted for 24% of our study enrollment) showed a superior response rate, PFS and OS in combination chemotherapy relative to erlotinib. However, subgroup analyses revealed a longer PFS in erlotinib relative to combination chemotherapy for women, never smokers, and patients with adenocarcinoma.[22] Indeed, our subgroup analyses demonstrated that all responders in the present study were women, and never smokers had a higher response rate than smokers (37 versus 5%). Imbalance of responders by gender may be attributed to the fact that most women enrolled in this study were never smokers. On the other hand, histologic subtype as another predicting factor did not show any significant differences in response rate, possibly due to the small sample size.

The recent discovery of somatic mutations in the tyrosine kinase domain of EGFR and of the association of such mutations with a high response rate to EGFR-TKIs has had a profound impact on the treatment of advanced NSCLC.[23–25] In the present study, we used Scorpion-ARMS which is more sensitive than direct sequencing for detection of the known *EGFR* mutations that reflect responsiveness to EGFR-TKIs.[18] Our analyses demonstrated that *EGFR* mutations were detected in 7 of 17 patients (41%), and those 5 patients achieved PRs and 2 patients had SDs. It is noteworthy that the presence of *EGFR* mutations was associated with longer survival (median >27 months), although it remains unclear whether *EGFR* mutations are predictive of EGFR-TKIs treatment benefit or merely prognostic of prolonged survival. Disease-related symptom improvement may be more important factors than tumor response and survival in the treatment of elderly patients with advanced NSCLC. In the present study, LCS revealed a significant symptom improvement from the start of gefitinib therapy to 4 weeks. Symptom improvement rate was 49% at 4 weeks of gefitinib therapy, which compares favorably with the improvement rate reported for the overall population in IDEAL-2 (39%).[17]

The toxicity of gefitinib in this study compares favorably with other studies performed in patients with NSCLC older than age 70 years. Fifteen patients (30%) experienced toxicities ≥grade 3. The most frequent AEs (≥G3) were skin disorders and hepatic dysfunction. There were four patients with possible ILD including two treatment-related deaths.

Gefitinib-induced ILD in the Japanese patients, of which multivariate analysis identified male sex, a history of smoking, and coincidence of interstitial pneumonia as significant risk factors, revealed a higher incidence of 3.2%, ranging from 0.4% in female never-smokers to 6.6% in male smokers, than in other ethnicities.[21] Despite strict exclusion of interstitial pneumonia by chest CT, there were 4 patients with possible ILD (8%) in the present study, including 2 treatment-related deaths (4%). One patient with treatment-related death was a male smoker with the higher risk of gefitinib-induced ILD, however, another was a female never-smoker with the lower risk. With strict exclusion of interstitial pneumonia by chest CT, it might be difficult to completely prevent development of gefitinib-induced ILD as it was previously reported that 5 of 34 patients without interstitial shadow by chest CT experienced ILD.[26] Further scientific investigations are required to elucidate gefitinib-induced ILD.

A recent phase III study for elderly patients (≥70) with advanced NSCLC (WJTOG9904) reported that docetaxel monotherapy significantly improved RR, PFS, and overall disease-related symptoms compared with vinorelbine mono-

therapy.[27] The data suggest that docetaxel monotherapy should be considered as an option in the standard treatment in this patient population. More recently, a large second line trial comparing gefitinib with docetaxel (INTEREST trial $n = 1440$) demonstrated the noninferiority of gefitinib to docetaxel in terms of OS, with a more favorable toxicity profile and QoL score in gefitinib arm.[28] These findings support the notion that EGFR-TKIs may be an ideal agent to investigate in the first line setting in elderly patients with advanced NSCLC. It has been recently reported that another EGFR-TKIs, erlotinib, is active and relatively well tolerated in chemotherapy-naive elderly patients (≥70) with advanced NSCLC.[29]

In conclusion, we showed the effective outcome of gefitinib monotherapy in chemotherapy-naive patients ≥75 years of age with advanced NSCLC. Despite our intentions not to discriminate, a higher percentage of women (65%), never smokers (61%), and patients with adenocarcinoma (82%) were enrolled because of a possible selection bias on the part of treating physicians. Although our present study suggests that gefitinib is a viable option for such selected patients, our data may not be applicable to elderly patients in general. Further studies are required to determine which patients will ultimately benefit from this therapy.

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CD5 expression is potentially predictive of poor outcome among biomarkers in patients with diffuse large B-cell lymphoma receiving rituximab plus CHOP therapy

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Background: Several biomarkers indicating poor prognosis have been reassessed in patients receiving rituximab combination chemotherapy for diffuse large B-cell lymphoma (DLBCL). However, few studies have investigated outcome in relation to a combination of these biomarkers. In addition, no large-scale studies have reassessed the outcome of patients with CD5-positive DLBCL treated with rituximab.

Patients and methods: We conducted a retrospective study and investigated the predictive value of three biomarkers—BCL2, germinal center (GC) phenotype and CD5—in 121 DLBCL patients treated with rituximab plus cyclophosphamide, doxorubicin, vincristine and prednisone.

Results: CD5-positive patients showed significantly poorer event-free survival (EFS) and overall survival (OS) than CD5-negative patients (2-year EFS, 18% versus 73%, $P < 0.001$; 2-year OS, 45% versus 91%, $P = 0.001$). However, no significant difference in outcome according to BCL2 or GC phenotype was observed. Multivariate analysis revealed that CD5 expression was a significant prognostic factor for EFS [hazard ratio 14.2, 95% confidence interval (CI) 4.7–43.2] and OS (hazard ratio 20.3, 95% CI 3.6–114.4).

Conclusions: CD5 expression was the only significant prognostic factor among the biomarkers examined in this study. Further studies with larger numbers are warranted to confirm the prognostic significance of CD5 expression for patients with DLBCL receiving rituximab-containing chemotherapy.

Key words: biomarker, CD5, diffuse large B-cell lymphoma, rituximab

introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin's lymphoma (NHL) [1]. It shows an aggressive clinical course and comprises a heterogeneous group of lymphomas in terms of morphology, immunophenotype, molecular abnormality and clinical behavior. Although the cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) regimen has been the mainstay of treatment for aggressive lymphomas for several decades [2], a significantly improved outcome has been obtained in both young and elderly patients by combining the CHOP regimen with rituximab (an anti-CD20 chimeric antibody) [3–5].

In the era when CHOP was used alone, the International Prognostic Index (IPI) was the primary clinical tool employed for prediction of outcome in patients with aggressive NHL [6]. Although the IPI is considered to be the most important prognostic factor for DLBCL, the five risk factors used for assessing it do not provide any information about biologic features. To date, several biomarkers have been shown to predict the outcome and responsiveness of DLBCL to therapy. Overexpression of BCL2 family proteins has also been shown to indicate resistance to chemotherapy both *in vitro* and *in vivo* [7, 8]. BCL6 family proteins are reportedly associated with a better prognosis, and patients with BCL6-positive DLBCL have a relatively favorable outcome when treated with the CHOP regimen [9]. On the other hand, it has been reported that CD5-positive DLBCL has a very poor prognosis and high stage with more extranodal sites in comparison with CD5-negative DLBCL [10, 11]. Moreover, on the basis of the data obtained using complementary DNA (cDNA) microarray, DLBCL has been divided into two distinct subtypes that reflect the different stages of B-cell differentiation, i.e. germinal center

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B-cell-like (GCB) and activated B-cell like (ABC) [12]. The ABC subtype is associated with a poorer prognosis than the GCB subtype. It has been reported that the immunostaining patterns of CD10, BCL6 and MUM1 are an alternative means of identifying germinal center (GC) or non-GC DLBCL including the ABC subtypes and that non-GC DLBCL shows poor responsiveness to anthracycline-based regimens [13].

Recently, it has been recognized that addition of rituximab to anthracycline-based regimens may alter the previously identified prognostic factors, in view of the markedly improved outcome of patients with DLBCL. The study from British Colombia demonstrated that the IPI remained predictive, but reclassified patients into three prognostic groups after reassessing the five prognostic factors [14]. Moreover, several studies have investigated whether these biomarkers predict responsiveness to rituximab combination chemotherapy and outcome. The prognosis of BCL2- or BCL6-overexpressing DLBCL and GC phenotype has been reassessed in patients receiving rituximab combination chemotherapy [15–17]. On the other hand, no large-scale studies of CD5 expression in the rituximab era have been reported.

Although several studies analyzing the prognostic significance of individual biomarkers have been carried out since the introduction of rituximab, none have investigated outcome by considering these biomarkers together. The aims of the present study were to reassess the predictive values of these biomarkers at a single institution and to investigate which factor among BCL2 expression, GC phenotype and CD5 expression has the greatest influence on the outcome of DLBCL patients.

patients and methods

patient characteristics

In the present study, we reviewed the medical records of patients with CD20-positive DLBCL who received CHOP with or without rituximab as a first-line therapy at the Cancer Institute Hospital from April 2004 to May 2007 and were followed until January 2008. The study protocol and sampling were approved by the Institutional Review Board of the Cancer Institute Hospital. Informed consent for retrospective analysis and additional immunophenotypic analysis and gene rearrangement studies was obtained.

Patients were analyzed if they were older than 18 years and had a performance status (PS) of zero to three according to the criteria of the European Cooperative Oncology Group. Patients were excluded if they had clinically relevant cardiac diseases or positivity for antibodies against human immunodeficiency virus-1 or -2. Patients with primary mediastinal large B-cell lymphoma, primary central nervous system lymphoma and primary testicular lymphoma were also not included in this study.

The disease stage was evaluated according to the Ann Arbor staging system. All patients had undergone staging investigations, including physical examinations, blood and serum analysis, bone marrow aspiration and biopsy and computed tomography of the neck, chest, abdomen and pelvis. Magnetic resonance imaging was used for evaluation of involved organs in the head and neck. The following clinical and laboratory data were available at the time of diagnosis: age, sex, serum lactate dehydrogenase level, PS, presence of B symptoms, clinical stage and number of extranodal sites. This information allowed IPI scores to be determined in the included patients. Patients were categorized into either a low-risk group (IPI score, 0–2) or a high-risk group (IPI score, 3–5).

treatment

All patients received rituximab plus CHOP (RCHOP) chemotherapy. For patients with stage IB–IV, rituximab was administered at the standard dose of 375 mg/m^2 once weekly for 8 weeks and CHOP chemotherapy was given concurrently triweekly, as described previously [18]. CHOP chemotherapy was given for a total of six cycles. For patients with stage IA, CHOP chemotherapy was repeated for three cycles and rituximab was continued in the same way as for patients with stages IB–IV, with subsequent radiotherapy.

pathological studies

Biopsy samples collected at the time of diagnosis were fixed in formalin, embedded in paraffin, sliced and stained with hematoxylin and eosin for morphological analysis. Immunohistochemical analysis was carried out using the dextran-polymer method (EnVision+; Dako, Glostup, Denmark) using mAbs against CD10 (56C6, Novocastra, Newcastle-upon-Tyne, UK), BCL6 (PG-B6p, Dako), MUM1 (MUM1p, Dako), BCL2 (124, Dako), CD5 (4C7, Novocastra) and cyclin D1 (P2D11F11, Novocastra) at our institution. For all the antibodies, heat-induced antigen retrieval pretreatment using Target Retrieval Solution, pH. 9 (Dako) was carried out. BCL6, MUM1 and BCL2 were designated as positive when the proportion of stained lymphoma cells was 30% or higher. CD5 and CD10 were considered to be immunohistochemically positive when at least a small population of the neoplastic cells was positive. To classify the samples into immunohistochemically defined GC or non-GC phenotypes, we used an algorithm previously described by Hans et al. [13].

For examination of CD5 expression, we reviewed the results of flow cytometry analysis. Cases were defined as CD5 positive if CD5 expression was detected by flow cytometry, irrespective of the result of CD5 immunohistochemistry. Excluded were those positive for cyclin D1 or those with a history of chronic lymphocytic leukemia/small lymphocytic lymphoma. Patients with a small-cell component implying transformation from low-grade/indolent B-cell lymphoma were also excluded. All the histopathology samples were reviewed by an expert hematopathologist (KT), and flow cytometric analyses were reviewed by two of the authors independently (DE and KT).

statistical analysis

The main outcomes of this study were event-free survival (EFS) and overall survival (OS). EFS was calculated from the date of diagnosis to the date of documented disease progression, relapse or death from any cause or to the date on which the study was stopped. OS was calculated from the date of diagnosis until death from any cause or the last follow-up. If the stopping date was not reached, the data were censored at the date of the last follow-up evaluation. Survival curves were estimated by the Kaplan–Meier method, and overall differences were compared by the log-rank test. Cox multivariate analysis was carried out to estimate the prognostic impacts of the biomarkers and IPI risk factors on EFS and OS. Comparisons of basic characteristics between the CD5-positive and -negative groups were tested by Fisher's exact test and Student's *t*-test. Data were analyzed using SPSS software version 11.0 for Windows (SPSS, Chicago, IL).

results

patient characteristics

During the study period, 180 patients were included, and data for all three biomarkers and flow cytometric analysis were available for 121 patients. The characteristics of these patients are listed in Table 1. CD5 was expressed in 11 of 121 patients with DLBCL (9%). None of the CD5-positive patients

Table 1. Patient characteristics

Clinical parameter	Frequency (%)
Sex	
Male	65 (54)
Female	56 (46)
Age	
Median, range	66, 23–88
≤60	37 (31)
>60	84 (69)
Stage	
1–2	85 (70)
3–4	36 (30)
Performance status	
0–1	104 (86)
2–4	17 (14)
Lactate dehydrogenase	
Normal	65 (54)
High	56 (46)
No. of extranodal sites	
0–1	95 (79)
2–4	26 (21)
International Prognostic Index score	
0–2	89 (74)
3–5	32 (26)
BCL2	
Positive	79 (65)
Negative	42 (35)
GC phenotype	
GC type	73 (60)
Non-GC type	48 (40)
CD5	
Positive	11 (9)
Negative	110 (91)

GC, germinal center.

had a history of other lymphoproliferative disorders, and all were found to have *de novo* CD5-positive DLBCL. Of these 11 patients, seven were positive by both flow cytometry and immunohistochemistry and four were positive only by flow cytometry. In all the seven cases defined as CD5 positive by both methods, the lymphoma cells expressed less CD5 than normal T cells in the background. Expression of BCL2 was detected in 79 of 121 cases (65%). CD10 was expressed in 45 cases (37%), BCL6 in 89 (66%) and MUM1 in 55 (45%). Overall, 48 of 121 cases (40%) were categorized into the non-GC group. No significant difference in basic characteristics was found between the 121 and 59 patients for whom all biomarkers were and were not available, respectively. No patients had central nervous system or testicular lesions.

survival analysis

The 2-year OS was 85% and EFS was 79% with a median follow-up of 28 months. We compared the survival curves in accordance with the expression of the three biomarkers. The Kaplan–Meier method revealed that the EFS rates at 2 years were 76% for BCL2-positive patients and 91% for BCL2-negative patients. The corresponding OS rates were 77% and 97%, respectively. Although both survival rates were inferior in BCL2-positive patients, the differences did not reach statistical significance ($P = 0.080$ and $P = 0.060$, respectively, log-rank test). Similarly, the EFS and OS rates at 2 years were 70% and 77%, respectively, for non-GC patients and 90% and 91%, respectively, for GC patients, there being no significant differences in these parameters between the two groups ($P = 0.080$ and $P = 0.120$, respectively). The IPI score at the baseline did not differ significantly according to BCL2 or GC phenotype. On the other hand, the differences in the EFS and OS rates between CD5-positive and CD5-negative patients were significant (EFS, 18% versus 73%, $P < 0.001$; OS, 45% versus 91%, $P = 0.001$) (Figure 1A and B).

For comparison with the biomarkers, we compared the survival curves according to the IPI. The EFS rates at 2 years were 52% for high and high-intermediate IPI and 91% for low and low-intermediate IPI. The OS rates were 64% and 92% for the high and low IPI groups, respectively. The differences in the EFS and OS rates were significant ($P = 0.001$ and $P = 0.010$, respectively) (Figure 1C and D).

The clinical and biological features in relation to CD5 expression are summarized in Table 2. Among the 11 patients who were CD5 positive, the primary site was extranodal in five (bone in two and intestine, thyroid and nasal cavity in one case each). No patient had bone marrow involvement. Significantly more CD5-positive than -negative patients had a poor PS ($P = 0.01$). Although no other significant differences were detected in the distributions of the other patient characteristics, CD5-positive patients were more frequently BCL2 positive ($P = 0.095$).

To further investigate the prognostic impact of CD5 expression, Cox multivariate analysis was carried out adjusted for the IPI categorization. As shown in Table 3, CD5 expression had significant prognostic value for both EFS [hazard ratio 14.2, 95% confidence interval (CI) 4.7–43.2; $P < 0.001$] and OS (hazard ratio 20.3, 95% CI 3.6–114.4; $P = 0.001$). The prognostic significance of CD5 remained even after adjustments by BCL2 expression or GC/non-GC categorization.

discussion

This analysis of biomarkers in 121 DLBCL patients receiving RCHOP highlighted the potentially poor outcome of patients with CD5-positive DLBCL. Multivariate analysis including the IPI revealed that CD5 expression and IPI were independent factors associated with poor prognosis. On the other hand, significant differences in survival were not detected in relation to BCL2 and immunohistochemically defined GC phenotype.

De novo CD5-positive DLBCL, a distinct subgroup that accounts for 5%–10% of all DLBCL, has been reported to be associated with elderly onset, female predominance, frequent involvement of extranodal sites and inferior survival [10, 11]. The largest study of CD5-positive DLBCL demonstrated a 5-year survival rate of 34% for CD5-positive DLBCL treated with an anthracycline-based regimen [11]. The Nordic Lymphoma Study Group also demonstrated that CD5 expression was associated with significantly inferior OS and failure-free survival [19]. In contrast, other authors showed that

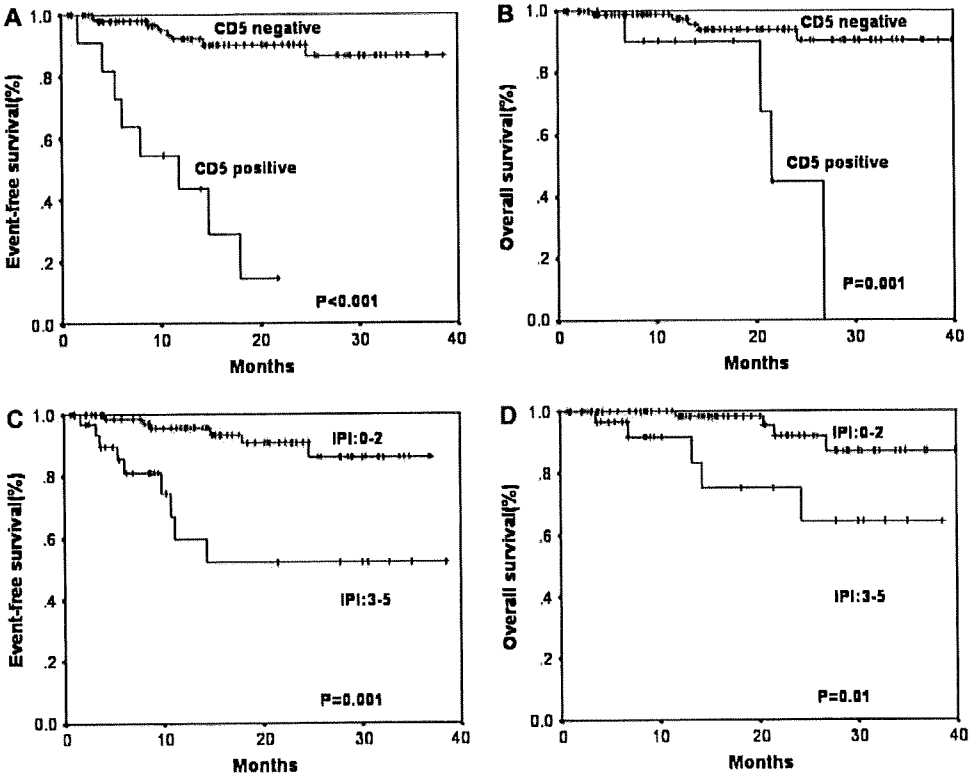

Figure 1. Event-free survival (EFS) and overall survival (OS) curves for diffuse large B-cell lymphoma patients treated with rituximab plus cyclophosphamide, doxorubicin, vincristine and prednisone according to CD5 expression and clinical factors. EFS (A) and OS (B) curves according to positive (n = 11) versus negative (n = 110) CD5 expression. EFS (C) and OS (D) curves according to the IPI (0–2, n = 89 versus 3–5, n = 32).

Table 2. Patient characteristics in relation to CD5 expression

Characteristic	CD5 positive (n = 11), n (%)	CD5 negative (n = 110), n (%)	P
Sex: male	6 (55)	59 (53)	1.0
Age: median, range	68, 33–76	66, 23–88	0.27
IPI score 3–5	4 (36)	28 (25)	0.47
Stages III–IV	4 (36)	32 (30)	0.74
Elevated LDH level	7 (64)	49 (44)	0.54
More than one extranodal site	5 (45)	22 (20)	0.38
PS >1	5 (45)	12 (11)	0.013
BCL2 positive	10 (91)	69 (62)	0.095
Non-GC type	6 (55)	42 (38)	0.54

IPI, International Prognostic Index; LDH, lactate dehydrogenase; PS, performance status; GC, germinal center.

CD5-positive DLBCL did not show distinctive clinical features or inferior survival [20]. In the present study, patients who received immunochemotherapy showed significantly poor OS and EFS, whereas the factors comprising the IPI were similar between the patients who were positive for CD5 and those who were negative. We consider that this poor prognosis of CD5-positive DLBCL in the rituximab era is noteworthy and that a large-scale study is warranted.

CD5 is a 67-kDa transmembrane glycoprotein that is expressed by most normal T cells and less brightly by a subset of B cells known as B1 cells [21]. Reflecting this difference in expression-level neoplastic CD5-positive B cells also usually express less CD5. Therefore, even if successfully stained by immunohistochemistry, these cells are usually stained less strongly than normal background T cells with anti-CD5 antibody. In the authors' experience, until the introduction of antigen retrieval techniques and effective antibodies like mAb 4C7, it was very difficult to detect CD5-positive B cells immunohistochemically on formalin-fixed paraffin-embedded sections [22]. However, even since the introduction of these techniques, CD5 immunohistochemistry using paraffin sections still remains less sensitive than flow cytometry and frozen section immunohistochemistry [23]. In fact, in the present study, only seven cases of DLBCL were positive for CD5 by immunohistochemistry out of 11 cases that were CD5 positive by flow cytometry. In an attempt to overcome this lower sensitivity of CD5 immunohistochemistry for CD5-positive DLBCL, de Jong et al. examined the usefulness of recently developed immunohistochemical enhancement techniques (Powervision; Immunovision Technologies, Duiven, The Netherlands and ChemMate; Dako). However, although they acquired higher sensitivity, there was also a loss of

Table 3. Cox multivariate analysis for EFS and OS

Variable	Unfavorable	HR	95% CI	P
EFS				
CD5	Positive	14.2	4.7–43.2	<0.001
IPI	3–5	7.6	2.5–22.8	<0.001
OS				
CD5	Positive	20.3	3.6–114.4	0.001
IPI	3–5	10.5	1.9–56.8	0.006

HR, hazard ratio; CI, confidence interval; EFS, event-free survival; IPI, International Prognostic Index; OS, overall survival.

reproducibility due to the unacceptable level of background staining [24]. Taken together, CD5 is usually expressed weakly by a subset of normal and neoplastic B cells, and CD5 paraffin immunohistochemistry is less sensitive for these B cells. For these reasons, we consider that flow cytometric analysis or frozen section immunohistochemistry needs to be carried out for detection of CD5 in DLBCL. Differences in the method of CD5 detection might lead to differences among studies in the apparent impact of CD5 on prognosis.

In addition to these differences in clinical aspects, there are several lines of evidence for genetic differences between CD5-positive and -negative DLBCL. Microarray studies have suggested that integrin beta-1 in tumor cells and CD36 in vascular endothelium are expressed more frequently in CD5-positive than in CD5-negative DLBCL [25]. Comparative genomic hybridization studies have revealed that CD5-positive DLBCL has a different pattern of chromosomal gain and loss compared with CD5-negative DLBCL [20, 26]. Loss of 9q21 (*p16 INK4a*), which is strongly associated with lymphoma progression, has been observed more frequently in CD5-positive DLBCL [27].

Previous studies also showed that IPI values remained in patients with DLBCL receiving immunochemotherapy [14–17]. In some studies, IPI category was divided into two risk groups—low or low intermediate and high or high intermediate—and this remained a predictive tool in DLBCL patients receiving immunochemotherapy [15–17]. Other authors have reassessed the IPI risk factors of DLBCL patients treated with RCHOP and divided them into three distinct prognostic groups referred to as R-IPI groups. Significant differences were also demonstrated in the same cohort upon division into two risk groups [14]. In the present study, IPI values were used to delineate two risk groups, and prognostic values were retained, although we did not evaluate each of the IPI risk factors. A consensus will be required for accurate handling of these risk factors in the rituximab era.

BCL2 overexpression was associated with poorer survival in the prerituximab era [7, 8]. In contrast, several studies conducted in the rituximab era demonstrated that addition of rituximab to chemotherapy eliminated the prognostic significance of BCL2 overexpression in DLBCL [15]. However, these studies did not reveal any data on the association between CD5 expression and BCL2 overexpression. Moreover, in previous studies of CD5-positive DLBCL, no association between CD5 expression and BCL2 overexpression in patients with DLBCL was demonstrated [10, 11]. The present study demonstrated that 10 of 11 CD5-positive patients had BCL2 overexpression. The OS and EFS of BCL2-positive, CD5-negative patients were significantly superior to those of patients positive for both BCL2 and CD5 (data not shown), suggesting that the poorer survival trend of patients with BCL2 overexpression in the present series may have been influenced by CD5 expression. A large-scale analysis of BCL2 expression in CD5-positive DLBCL will be needed to clarify the association between expressions of BCL2 and CD5.

There have been several studies of the relationship between CD5 expression and GC/ABC phenotype [27–29]. An analysis of genomic imbalance showed that most cases of CD5-positive DLBCL were included in the ABC type [27], and another study of somatic mutations of the immunoglobulin heavy chain variable region suggested that the cells from which CD5-positive DLBCL arise are predominantly of post-GC origin [28, 29]. These conclusions were based on molecular-based analyses and not by immunohistochemistry. Our study found no association between CD5 expression and GC phenotype. This may be because GC phenotype in the present study was defined by an immunophenotypic algorithm, which reproduced ~80% of the GC phenotype defined by cDNA microarray [13]. A new algorithm using five types of immunostaining—GCET1, MUM1, CD10, BCL6 and FOXP1—has been introduced recently and provided an improved GC/ABC subclassification [30]. Application of this approach to our series might lead to a consistent result.

In conclusion, we have investigated the outcome of DLBCL patients receiving rituximab combination chemotherapy by considering several biomarkers together and demonstrated that CD5 expression is a potentially useful indicator of poor prognosis. To accurately confirm whether CD5 expression influences the outcome of patients receiving RCHOP, further large-scale and prospective studies of CD5-positive patients will be required.

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Synergistic antitumor effect of S-1 and the epidermal growth factor receptor inhibitor gefitinib in non-small cell lung cancer cell lines: role of gefitinib-induced down-regulation of thymidylate synthase

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Abstract

Somatic mutations in the epidermal growth factor receptor (*EGFR*) gene are associated with the therapeutic response to EGFR tyrosine kinase inhibitors (TKI) in patients with advanced non-small cell lung cancer (NSCLC). The response rate to these drugs remains low, however, in NSCLC patients with wild-type *EGFR* alleles. Combination therapies with EGFR-TKIs and cytotoxic agents are considered a therapeutic option for patients with NSCLC expressing wild-type *EGFR*. We investigated the antiproliferative effect of the combination of the oral fluorouracil S-1 and the EGFR-TKI gefitinib in NSCLC cells of differing *EGFR* status. The combination of 5-fluorouracil and gefitinib showed a synergistic antiproliferative effect *in vitro* in all NSCLC cell lines tested. Combination chemotherapy with S-1 and gefitinib *in vivo* also had a synergistic antitumor effect on NSCLC xenografts regardless of the absence or presence of *EGFR* mutations. Gefitinib inhibited the expression of the transcription factor E2F-1, resulting in the down-regulation of thymidylate synthase at the mRNA and protein levels. These observations suggest that gefitinib-induced down-regulation of thymidylate synthase is responsible, at least in part, for the synergistic antitumor effect of combined treatment with S-1 and gefitinib and provide a basis for clinical evaluation of combination chemotherapy with S-1 and EGFR-TKIs in patients with solid tumors. [Mol Cancer Ther 2008;7(3):599–606]

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Introduction

Targeted therapy in the treatment of cancer has made substantial progress over the last few years. The ErbB family of receptor tyrosine kinases includes the epidermal growth factor receptor (EGFR; ErbB1), ErbB2 (HER2/*neu*), ErbB3, and ErbB4 and is important for normal development as a result of its roles in cell proliferation and differentiation (1–3). Aberrant expression of EGFR has been detected in a wide range of human epithelial malignancies, including non-small cell lung cancer (NSCLC), and is correlated with poor prognosis and reduced survival time (4, 5). Agents that specifically target EGFR are therefore under development as anticancer drugs. Indeed, two inhibitors of the tyrosine kinase activity of EGFR (EGFR-TKI), gefitinib and erlotinib, both of which compete with ATP for binding to the catalytic pocket of the receptor, have been extensively studied in individuals with NSCLC (6–9). Somatic mutations in the region of *EGFR* that encodes the tyrosine kinase domain have been associated with tumor responsiveness to EGFR-TKIs in a subset of NSCLC patients (10–17). In contrast, achievement of a clinical benefit of these drugs in NSCLC patients who express wild-type *EGFR* has been problematic.

S-1 (Taiho Pharmaceutical) is an oral anticancer agent composed of tegafur, 5-chloro-2,4-dihydroxypyridine (CDHP), and potassium oxonate in a molar ratio of 1:0.4:1 (18). Tegafur is a prodrug that generates 5-fluorouracil (5-FU) in blood largely as a result of its metabolism by cytochrome P450 in the liver. CDHP increases the plasma concentration of 5-FU through competitive inhibition of dihydropyrimidine dehydrogenase (DPD), which catalyzes 5-FU catabolism (19). Oxonate reduces the gastrointestinal toxicity of 5-FU (20). A response rate of 22% and a median survival time of 10.2 months were obtained in a clinical trial of S-1 in patients with advanced NSCLC not subjected previously to chemotherapy (21). Few severe gastrointestinal or hematologic adverse events were reported. Moreover, a phase II trial of S-1 plus cisplatin in NSCLC patients revealed a 47% response rate and an acceptable safety profile (22).

Based on this background, we examined the anticancer effect of the combination of S-1 and gefitinib in NSCLC cell lines of differing *EGFR* status. We found that the combination of S-1 (or 5-FU) and gefitinib exhibited a marked and synergistic antiproliferative effect both *in vivo*