80.4 %. 2 patients had CR, 17 patients had PR, and 18 patients had SD.

Figure 1 shows the OS of the 46 enrolled patients. The median OS was 17.4 months (95 % CI, 14.1 to 21.6), with 1-year survival rate of 67.4 % (95 % CI, 52.0 to 80.5). The median PFS was 7.5 months (Fig. 2).

Toxicity Major grade 3 and 4 toxicities are summarized in Table 2. Myelosuppression was the most common toxicity of grade 3/4, with neutropenia occurring in 13 (28.3 %) patients, anemia in 8 (17.4 %) patients, and thrombocytopenia in 6 (13.0 %) patients. Severe nonhematologic toxicities were uncommon in this study. There was no treatment-related death.

Discussion

Diagnosis of metastatic CUP has long been considered synonymous with poor prognosis. Combination regimens containing a taxane and a platinum agent have been most extensively studied [3–7]. In practical settings, empiric chemotherapy is generally administered to patients with CUP. However, empiric chemotherapy, for example, paclitaxel and carboplatin, is not expected to be efficacious in patients with several carcinomas. Moreover, this regimen is believed to be poorly effective when the primary site is colorectal, renal, pancreatic, prostatic, or hepatic. In this respect, S-1 is considered to have broad spectrum of clinical activity in solid tumors. In the present study, we demonstrated that CDDP plus S-1 combination chemotherapy achieved a response rate of 41.3 %, median MST of 17.4 months, and 1-year survival rate of 67 %.

Table 2 Treatment-Related Toxicity (N=46)

Toxicity	No. of patients (%)	
	Grade 3	Grade 4
Hematologic		
Leukopenia	4 (8.7)	1 (2.2)
Neutropenia	8 (17.4)	5 (10.9)
Anemia	5 (10.9)	3 (6.5)
Thrombocytopenia	4 (8.7)	2 (4.3)
Nonhematologic		
Neutoropenic fever	1 (2.2)	0
Anorexia	4 (8.7)	0
Nausea/vomiting	2 (4.3)	0
Fatigue	1 (2.2)	–
Diarrhea	1 (2.2)	0

However, careful attention should be paid to the interpretation of our promising results. Importantly, patient selection may have influenced the study outcomes. Poor prognostic factors—including male gender, PS score greater than 1, high number of metastatic sites, presence of liver metastases, elevated serum alkaline phosphatase, elevated lactate dehydrogenase (LDH), and low serum albumin—have been identified in CUP patients. In our study, the majority (89.1 %) of the patients had good PS scores of 0–1, and 30 % of the patients was squamous cell carcinoma, whereas a small percentage (10.9 %) of patients had liver metastases, 39.1 % had high LDH levels, and 39.1 % had nodal disease only. These favorable prognostic features of enrolled patients may contribute to good clinical outcome observed in this study.

Recently, various targeted agents have been included in the treatment of several common solid tumors [18, 19]. As development of molecular target agents advances, prognostics may be improved by classifying cases with CUP into subgroups according to the expression of specific biomarkers.

On the other hand, molecular profiling assays, which may assist in the identification of the tissue of origin, have attracted increasing attention. Indeed, the development molecular technologies that allow tumor gene expression profiling provides an opportunity for improved diagnosis of patients with CUP [20, 21]. Clinical trials are currently being conducted to examine the efficacy of therapy directed based on the molecular profiling assay diagnosis of patients with CUP.

In our study, the treatment-related toxicity of the S-1/CDDP regimen was mild in the majority of the patients. However, we should note that the recommend dose of S-1 may be different between Japanese and Western patients. In the FLAGS trial (Multicenter Phase III Comparison of Cisplatin/S-1 with Cisplatin/Infusional Fluorouracil in Advanced Gastric or Gastroesophageal Adenocarcinoma Study), patients in the CDDP plus S-1 arm received S-1 at 50 mg/m^2/day [22] . This dose has been considered the maximum-tolerated dose in combination with CDDP in Western patients. On the other hand, we and other Japanese trial [23, 24] have shown that Japanese patients tolerate well S-1 at 80 mg/m^2/day. This difference in tolerance to S-1 has been suggested to be likely related to polymorphic differences in the key *CYP2A6* gene [25]. Thus, ethnic differences should be taken into account before introduction of S-1 plus CDDP combination therapy in patients with CUP.

In conclusion, this nonrandomized phase II trial demonstrated the efficacy and safety of using S-1 in combination with CDDP as the first-line treatment for patients with CUP.

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Disclosure of potential conflicts of interest All authors declare no conflicts of interest.

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RESEARCH ARTICLE Open Access

Prognostic impact of serum CYFRA 21–1 in patients with advanced lung adenocarcinoma: a retrospective study

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Abstract

Background: Serum CYFRA 21–1 is one of the most important serum markers in the diagnosis of non-small cell lung cancer (NSCLC), especially squamous-cell carcinoma. However, it remains unknown whether pretreatment serum CYFRA 21–1 values (PCV) may also have prognostic implications in patients with advanced lung adenocarcinoma.

Methods: We retrospectively reviewed the data of 284 patients (pts) who were diagnosed as having advanced lung adenocarcinoma and had received initial therapy.

Results: Of the study subjects, 121 pts (43%) had activating epidermal growth factor receptor (EGFR) mutations (Mt+), while the remaining 163 pts (57%) had wild-type EGFR (Mt-). Univariate analysis identified gender (male/ female), ECOG performance status (PS) (0-1/ ≥2), PCV (<2.2 ng/ml/ ≥2.2 ng/ml), EGFR mutation status (Mt+/ Mt-), pretreatment serum CEA values (<5.0 ng/ml/ ≥5.0 ng/ml), smoking history (yes/ no) and EGFR-TKI treatment (yes/ no) as prognostic factors ($p = .008$, $p < .0001$, $p < .0001$, $p < .0001$, $p = .036$, $p = .0012$, $p < .0001$ respectively). Cox's multivariate regression analysis identified PCV < 2.2ng/ml as the only factor significantly associated with prolonged survival ($p < .0001$, hazard ratio: 0.43, 95% CI 0.31-0.59), after adjustments for PS ($p < .0001$), EGFR mutation status ($p = .0069$), date of start of initial therapy ($p = .07$), gender ($p = .75$), serum CEA level ($p = .63$), smoking history ($p = .39$) and EGFR-TKI treatment ($p = .20$). Furthermore, pts with Mt+ and PCV of <2.2 ng/ml had a more favorable prognosis than those with Mt+ and PCV of ≥2.2 ng/ml (MST: 67.0 vs. 21.0 months, $p < .0001$), and patients with Mt- and PCV of <2.2 ng/ml had a more favorable prognosis than those with Mt- and PCV of ≥2.2 ng/ml (MST: 24.1 vs. 10.2 months, $p < .0001$).

Conclusion: PCV may be a potential independent prognostic factor in both Mt+ and Mt- patients with advanced lung adenocarcinoma.

Keywords: Lung adenocarcinoma, Prognostic factor, CYFRA 21–1, CEA, EGFR mutation, Tumor heterogeneity, EGFR-TKI, Chemotherapy

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Background

Lung cancer is the leading cause of cancer death, and at present, there exists no cure of stage IV non-small cell lung cancer (NSCLC) [1]. Adenocarcinoma and squamous cell carcinoma are the most common histological subtypes of lung cancer and account for about 70% of all lung cancers [2]. The folate antagonist pemetrexed has been shown to exhibit efficacy against non-squamous cell lung cancers [3], and is currently used in combination with cisplatin as a standard treatment regimen for patients with non-squamous cell lung carcinoma. Chemotherapy with the angiogenesis inhibitor bevacizumab administered in combination with platinum agents has also been shown to exhibit favorable efficacy against non-squamous cell lung carcinoma [4,5]. Somatic gain-of-function mutations in exons encoding the EGFR tyrosine kinase domain have been identified in NSCLC [6,7]. Several previous studies have reported prolongation of the survival time in patients with EGFR-mutation-positive lung carcinomas treated with EGFR-tyrosine kinase inhibitors (TKIs) [8-11], therefore, EGFR-TKIs are widely used in medical practice. EGFR mutations occur more frequently in lung cancer patients who are Asians, females and non-smokers with the histological subtype of adenocarcinoma [12-14]. On the other hand, while there have also been scattered reports of EGFR mutations among cases of lung squamous-cell carcinoma [15-17], a recent report showed that there were no EGFR mutation-positive cases among lung cancer patients with pure squamous cell carcinoma [18,19].

CYFRA 21–1 is a fragment of cytokeratin (CK) 19. CKs, which are now called keratins, are the principal structural elements of the cytoskeleton (keratin filaments) of epithelial cells, including bronchial epithelial cells, and have been classified into 20 subtypes based on differences in the molecular mass and isoelectric point as determined by 2-dimensional electrophoresis [20,21]. CK types 1–8 are categorized as type I CKs, and CKs 9–20 as type II CKs. Microfilaments are heteropolymers formed from type I and type II keratins, and constitute the cytoskeleton [22]. CK19 is a soluble type I CK (acidic type), and has the lowest molecular mass (40 kDa) among the CKs. It is expressed in the unstratified or pseudostratified epithelium lining the bronchial tree [23], and been reported to be overexpressed in many lung cancer tissue specimens [24]. The CK expression patterns in tissues are well-maintained even during the process of transformation of the tissue from normal to tumor tissue [25]. Accelerated CK19 degradation occurs in neoplastically transformed epithelial cells as a result of increased protease activity of caspase 3, a regulator of the apoptosis cascade, and fragments are released into the blood. This results in an increase of the blood CYFRA 21–1 values, because CK19 fragments are recognized by two monoclonal antibodies [26].

Measurement of serum CYFRA 21–1 level is a useful auxiliary test in the diagnosis of NSCLC, and particularly high specificity of this test has been reported for the diagnosis of squamous cell carcinoma of the lung [27,28]. On the other hand, a meta-analysis also revealed that serum CYFRA 21–1 may be a useful prognostic factor in NSCLC patients [29]; analysis of the histological background in the aforementioned meta-analysis showed that non-adenocarcinoma accounted for the majority of cases of NSCLC (65%). There has also been a report suggesting that serum CYFRA 21–1 levels might serve as a prognostic factor in patients with recurrent NSCLC receiving 3rd-line or later gefitinib therapy [30]. Some studies have suggested the possible prognostic value of pretreatment serum CYFRA 21–1 values (PCV) in patients with surgically treated lung adenocarcinoma [31] and advanced NSCLC [32-34]. However, none of the studies suggesting serum CYFRA 21–1 as a prognostic factor in patients with untreated advanced lung adenocarcinoma has included the EGFR mutation status as a variable. Therefore, in the present study, we investigated the impact of serum CYFRA 21–1 on the prognosis of untreated advanced lung adenocarcinoma patients.

Methods

Patients

Of patients diagnosed as having primary lung carcinoma between January 2003 and June 2010 at the Shizuoka Cancer Center, EGFR mutation analysis was performed on 424 patients from April 2008 to June 2010. Of these, 284 lung adenocarcinoma patients had received initial therapy, and we retrospectively reviewed the data of the 163 patients who were found to harbor wild-type EGFR and 121 patients who were found to harbor activating EGFR mutations (Figure 1). The following inclusion criteria were set for this study; patients with pathologically proven adenocarcinoma who had received initial therapy (including chemotherapy or chemoradiotherapy) and survived for more than one month; Eastern Cooperative Oncology Group performance status (ECOG PS) of 3 or less. The histological and cytological diagnoses were performed according to the WHO classification criteria [35]. The study was conducted with the approval of the Shizuoka cancer center Institutional Review Board #1 (HHS IRB registration number; IRB00006744).

We outsourced some of the clinical laboratory tests, such as measurement of the tumor markers and EGFR mutation analysis. Serum CYFRA 21–1 and serum CEA concentrations were measured at the baseline, before the initial therapy. The serum CYFRA 21–1 concentration was measured using a Lumipulse Presto® kit (FUJIREBIO Inc, Tokyo, Japan), based on a CLEIA (chemiluminescent enzyme immunoassay) method, while the serum CEA concentrations were measured using an ARCHITECT® kit

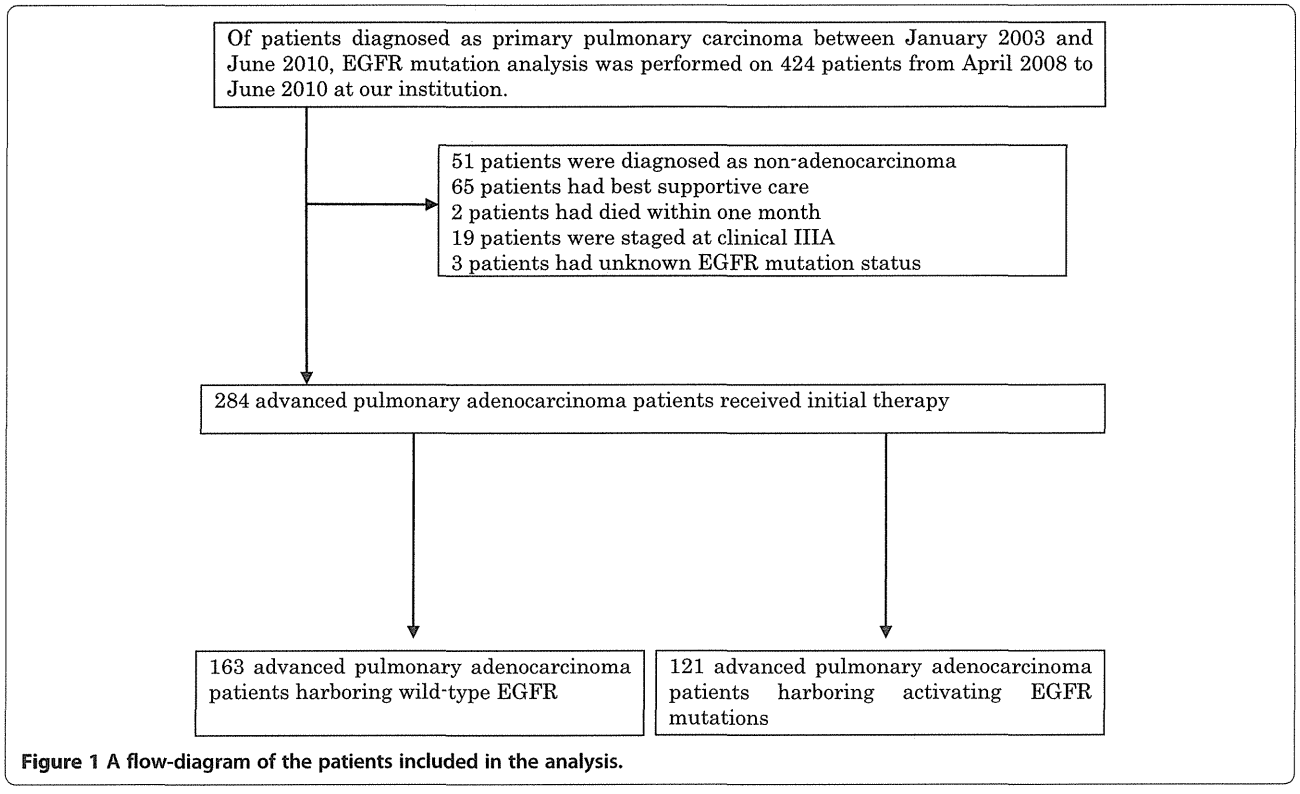

Figure 1 A flow-diagram of the patients included in the analysis.

(Abbott Japan, Tokyo, Japan). EGFR mutation analysis was performed by fragment analysis using polymerase chain reaction (PCR) and the cycleave real-time quantitative PCR technique (SRL Inc, Tokyo, Japan).

The reported upper limit of normal for the diagnosis of NSCLC and upper limit of the percentiles for healthy individuals of serum CYFRA 21–1 as measured by EIA are 3.5 ng/ml and 2.8 ng/ml, respectively [36]. In contrast, the reported upper limit of the percentiles for healthy individuals of serum CYFRA 21–1 measured by the CLEIA method is 1.6 ng/ml [37], a lower value as compared to that set for measurement by the EIA method. Therefore, for our study, we set the cutoff value for CYFRA 21–1 at 2.2 ng/ml, based on the mean value for healthy subjects + 3SD [37], a lower value as compared to that set for measurement by the EIA method. The cutoff value for serum CEA was set at 5.0 ng/ml, which is the upper limit of normal.

A standard evaluation of the patients, including assessment of the medical history, physical examination and routine laboratory tests, was performed before each treatment. All patients were staged based on the International Association for the Study of Lung Cancer (IASLC) TNM (tumor-node-metastasis) classification, 7th edition [38].

Statistical methods

There were no missing data in our study. Survival was estimated using the Kaplan-Meier method. Overall survival was measured from the date of the first course of the initial therapy to the date of death or that of the last follow-up examination. A log-rank test was performed to evaluate the significance of differences in the overall survival among the groups. P values < 0.05 were considered to be indicative of statistical significance. A multivariate analysis using the Cox proportional hazards model was used to establish the association between the clinical variables and survival. All statistical analyses were carried out using SPSS, version 11.0 for Windows (SPSS Inc., Chicago, IL, USA). To reduce the potential bias arising from some patients dying too early to receive initial therapy, the two patients who died within a month (30 days) of the start of initial therapy were excluded from the analysis.

Results

The cohort consisted of 284 patients who were diagnosed as having stage IIIB or IV lung adenocarcinoma and had received initial therapy.

The clinical characteristics of the patients are summarized in Table 1. The median patient age prior to the start of initial therapy was 65 years (range, 23 to 87 years). The patients were predominantly younger than 70 years of age (81%), the ECOG PS was 0–2 in 93% of patients, and 91% of the patients had stage IV disease. While the lung adenocarcinoma patients with EGFR mutations were predominantly female (64%) and non-smokers

Table 1 Patient characteristics

Characteristic	Mt + (n= 121)		Mt – (n= 163)		All (n= 284)	
	No.	%	No.	%	No.	%
Age, years						
Median (range)	66 (32–87)		65 (23–83)		65 (23–87)	
< 70	97	80	134	82	231	81
≥ 70						
	24	20	29	18	53	19
Gender						
Male	43	36	125	77	168	59
Female	78	64	38	23	116	41
ECOG PS						
0-1	103	85	135	83	238	84
> 2	18	15	28	17	46	16
Smoking status						
Yes	50	41	124	76	174	61
No	71	59	39	24	110	39
Stage						
IIIB	6	5	19	12	25	9
IV	115	95	144	88	259	91
EGFR mutation						
Exon 19 deletion	59	49			59	21
Exon 21 L858R	57	47			57	20
Exon 18 G719X	5	4			5	2
Wild type			163	100	163	57
PCV						
Median (range)	1.6 (0.1-110.0)		2.3 (0.1-80.0)		2.0 (0.1-110.0)	
< 2.2 ng/ml	72	60	78	48	150	53
≥ 2.2 ng/ml	49	40	85	52	134	47
CEA						
Median (range)	8 (0.7-11942)		7 (0.5-14985)		7.4 (0.5-14985)	
< 5.0 ng/ml	45	37	63	39	108	38
≥ 5.0 ng/ml	76	63	100	61	176	62

EGFR: epidermal growth factor receptor, Mt+: mutant EGFR, Mt-: wild-type EGFR, PCV: pretreatment CYFRA 21–1 value.

(71%), those with wild-type EGFR were predominantly male (77%) and smokers (76%).

Details about the first-line chemotherapy were available for 284 patients including both patient groups with wild-type (Mt-) and mutant EGFR (Mt+) groups (Table 2). About 40% of the EGFR mutation-positive patients received EGFR-TKIs as the initial treatment.

Carboplatin-paclitaxel, the treatment of choice across both groups, was administered to half of the platinum doublet cohort in the Mt- patient group. Meanwhile, docetaxel was administered to half of the monotherapy cohort in the same patient group. However, cisplatin-pemetrexed was the most common regimen of second choice across both the Mt+ and Mt- groups.

The EGFR-TKI used for each treatment line in the Mt+ group is shown in Table 3. Forty-one (58%) patients received gefitinib, while 16 (22%) received erlotinib as first- or second-line treatment in the Mt+ group with PCV (<2.2 ng/ml). Thirty-seven (73%) patients received gefitinib, and 10 (20%) patients received erlotinib as first- or second-line treatment in the Mt+ group with PCV (≥2.2 ng/ml). Of the 121 patients in the Mt+ group, 27 did not receive gefitinib at any treatment-line stage of treatment; among these 27 patients, 19 received erlotinib (6 as first-line, 10 as second-line, 1 as third-line and 2 as further-line treatment). In the Mt+ group, a total of 113 patients (93%) received EGFR-TKIs, while 8 patients did not receive EGFR-TKIs at any stage of treatment.

Table 2 Summary of initial treatment delivered among 284 patients

EGFR mutation	Mt – (n= 163)				Mt + (n= 121)			
	IIIB		IV		IIIB		IV	
	(n= 19)		(n= 144)		(n= 6)		(n= 115)	
	No.	%	No.	%	No.	%	No.	%
Treatment								
Platinum doublet	4	3	114	70	2	2	54	45
Monotherapy	0		30	18	0		11	9
EGFR-TKI	0		0		0		50	41
Chemoradiotherapy	15	9	0		4	3	0	
Specific regimens								
Cisplatin-pemetrexed	1		24	15	1		9	7
Carboplatin-paclitaxel	3		52	32	0		27	22
Carboplatin-paclitaxel+ bev	0		2		0		2	
Other platinum doublets	0		36	22	1		12	10
Gefitinib	0		0		0		41	34
Erlotinib	0		0		0		7	6
Docetaxel	0		16	10	0		3	
Vinorelbine	0		5		0		2	
Others	0		24	15	0		6	

Mt+: mutant EGFR, Mt-: wild-type EGFR, bev: bevacizumab.

Furthermore, of the 160 patients in the Mt- group, 30 patients received EGFR-TKIs (11 as second-line, 7 as third-line, 6 as fourth-line, 3 as fifth-line, 1 as sixth-line, 1 as seventh-line, and 1 as eighth-line treatment). Fifty-three patients (18%) were still alive at the time of the analysis. The median follow-up period for determining the survival was 39.3 (range; 11.8-84.9) months after the start of initial therapy. The clinical variables identified by univariate analysis to be associated with significantly better survival (Table 4) included female gender (MST 32.4 months versus 20.1 months in males: p = .0086), no smoking history (33.4 months versus 20.1 months in smokers, p = .0012), ECOG PS (0–1) (29.5 months versus 7.9 months in those with a PS of 2–3, p < .0001), presence of EGFR mutation (39.2 months versus 17.8 months in patients without EGFR mutations, p < .0001), PCV < 2.2 ng/ml (38.6 months versus 15.0 months in those with PCV ≥ 2.2 ng/ml, p < .0001), serum CEA < 5.0 ng/ml (32.6 months versus 21.0 months in those with serum CEA ≥ 5.0 ng/ml, p = .036), start date of initial therapy before April 1, 2008 (34.1 months versus 19.3 months in the group that received the initial therapy after April 1, 2008, p = .003) and EGFR-TKI treatment (33.7 months versus 15.3 months in the group not treated with EGFR-TKIs, p < .0001). Multivariate analysis identified EGFR mutation positivity (HR 0.53; 95% CI: 0.34-0.84, p = .0069) and PCV < 2.2 ng/ml (HR 0.43; 95% CI: 0.31-0.59, p < .0001) as independent favorable prognostic factors. Another factor that was found to be an independent prognostic indicator of overall survival was the PS (Table 4). The overall survival rates of patients with advanced lung adenocarcinoma with/ without EGFR mutation are shown in Figure 2. Among the Mt+ patients, the prognosis was more favorable in the group with PCV < 2.2 ng/ml (n = 70) than in the group with PCV > 2.2 ng/ml (n = 48) (median survival time [MST]: 67.0 vs. 21.0 months, p < 0.0001). Among the patients with Mt- also, the prognosis was more favorable in the group with PCV < 2.2 ng/ml (n = 78) than in the group with PCV ≥ 2.2 ng/ml (n = 86) (MST: 24.1 vs. 10.2 months, p < 0.0001).

Table 3 Summary of EGFR-TKI delivered among EGFR mutation positive patients

	EGFR mutation positive							
	Low PCV				High PCV			
	(< 2.2 ng/ml) (n= 72)				(≥ 2.2 ng/ml) (n= 49)			
	Gefitinib		Erlotinib		Gefitinib		Erlotinib	
	No.	%	No.	%	No.	%	No.	%
First-line	20	28	5	7	23	47	2	4
Second-line	21	29	11	15	14	29	8	16
Third-line	9	12	6	8	3	6	4	8
Further-line	2	3	14	20	2	4	4	8
Unadministered	20	28	36	50	7	14	31	63

PCV: pretreatment CYFRA 21–1 value.

Discussion

In the present study, we demonstrated PCV and EGFR mutation status as independent prognostic factors in untreated advanced lung adenocarcinoma patients. We also showed that PCV < 2.2 ng/ml was a predictor of a favorable outcome in both advanced lung adenocarcinoma patients with wild-type and mutant EGFR.

Serum CYFRA 21–1 has been reported as a prognostic factor in patients with a variety of cancer types, including resectable NSCLC [39,40], biliary tract cancer [41], urothelial cancer [42], head and neck cancer [43], esophageal cancer [44], and cervical cancer [45].

A meta-analysis of CYFRA 21–1 as a prognostic indicator in advanced NSCLC patients showed that the PCV may be a reliable prognostic factor [29], however, since non-adenocarcinoma accounted for 65% of the cases and squamous cell carcinoma for 50%, the role of serum CYFRA 21–1 as a prognostic indicator in the lung adenocarcinoma population remained unclear. Moreover, in a study of PCV as a prognostic indicator in advanced NSCLC patients in whom gefitinib was used as 3rd-line or later therapy, adenocarcinoma accounted for fewer than a half of the cases (47%) [30]. The EGFR mutation status was not included as a variable in the analysis, and the test population was small, consisting of only 50 patients.

Table 4 Variables associated with overall survival among 284 patients

Co-variable	No.	Univariate analysis		Multivariate analysis			
		MST (months)	P	Variate	OR	95% CI	P
Age							
< 70	231	22.8					
> 70	53	24.3	0.625				
Gender							
Male	168	20.1					
Female	116	32.4	0.0086	Female	1.06	0.75-1.58	0.75
Smoking status							
Yes	174	20.1					
No	110	33.4	0.0012	No smoking status	0.84	0.52-1.24	0.39
ECOG PS							
0-1	238	29.5					
>2	46	7.9	<.0001	PS 0-1	0.34	0.24-0.50	<.0001
Stage							
IIIB	25	30.2					
IV	259	22.5	0.269				
EGFR mutation							
Mt (+)	121	39.2					
Mt (–)	163	17.8	<.0001	Mutant EGFR	0.53	0.34-0.84	0.0069
PCV							
< 2.2 ng/ml	150	38.6					
≥ 2.2 ng/ml	134	15.0	<.0001	< 2.2 ng/ml	0.43	0.31-0.59	<.0001
CEA							
< 5.0 ng/ml	108	32.6					
≥ 5.0 ng/ml	176	21.0	0.036	< 5.0 ng/ml	0.93	0.67-1.26	0.63
Start dates of IT							
Before 1/ 4/ 2008	79	34.1		After 1/ 4/ 2008			
After 1/ 4/ 2008	205	19.3	0.0030		0.73	0.50-1.15	0.07
EGFR-TKI treatment							
Yes	143	33.7					
No	141	15.3	<.0001	Yes	0.76	0.50-1.15	0.20

IT: initial therapy, PCV: pretreatment CYFRA 21–1 value, Mt(+): mutant EGFR, M(–): wild-type EGFR.

Several factors may have contributed to identification of serum CYFRA 21–1 as a prognostic indicator in the advanced lung adenocarcinoma population in the present study. First, there could be a relationship between the serum levels of CYFRA 21–1 and the microfilament formation trend in the tumor cells [22]. CKs are the principal structural elements of intracellular microfilaments. Microfilaments have been shown to be heteropolymers formed from type I and type II keratins which form the cytoskeleton. Moreover, while the CKs (CKs 1, 2, 10/11), on which the degree of keratinization within tumors depends, are strongly expressed in well-differentiated squamous cell carcinomas, they are not detected in the serum. The possibility that they are preferentially removed by macrophages because of their poor solubility has been suggested as the reason for the failure to detect them in the serum [46]. By contrast, soluble CK19 is degraded by tumor lysis and tumor necrosis and released into the blood. Therefore, serum levels of CK19 may indicate the degree of cytoskeleton formation by microfilaments within the tumor cells. Second, there may also be a relationship between serum CYFRA 21–1 levels and the degree of tumor differentiation towards squamous epithelium. CKs with a relatively high molecular mass tend to be associated with differentiation into squamous cell carcinoma, while CKs with a relatively low molecular mass tend to be associated with differentiation into adenocarcinoma [47]. In a study in

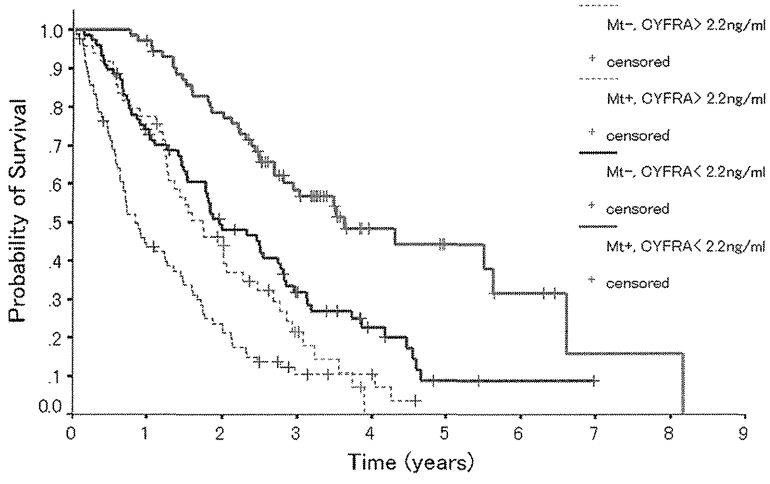

Figure 2 Kaplan-Meier curves for overall survival in four groups, EGFR mutation status- stratified by PCV. M+: mutant EGFR, M-: wild-type EGFR, PCV: pretreatment CYFRA 21–1 value.

which monoclonal antibodies were used, the number of cells containing CK19 increased with decreasing degree of differentiation into squamous cell carcinoma, and the presence of intracellular CK19 was consistently demonstrated in pure lung adenocarcinomas [25]. On the other hand, a negative correlation between intracellular CK19 expression and serum CYFRA 21–1 levels has also been shown [24]. Increase in the serum level of CYFRA 21–1 may also be the result of a greater degree of degradation and release of intracellular CK19 into the serum with an increasing tendency towards differentiation into squamous cell carcinoma.

Because identical EGFR mutations have been seen in both the adenocarcinoma component and squamous cell carcinoma component in resected cases of adenosquamous carcinoma [48], it has been suggested that the two components may arise from a single clone [48,49]. Resected cases of adenosquamous carcinoma have been reported to account for 3% of all cases of NSCLC [50], and adenosquamous carcinoma patients have also been reported to have a poor prognosis [51]. The prognosis of patients in whom the tumor tissue consists of a mixture of mutant EGFR cells and wild-type EGFR cells has been reported to be inferior to that of patients with tumors consisting of only mutant EGFR cells, and intratumor heterogeneity has also been investigated [52]. On the other hand, there is a report suggesting that no intratumor heterogeneity of EGFR expression is found in mutant EGFR lung adenocarcinomas, and also that no disparity is found between the EGFR mutation status of the primary tumor and lymph node metastasis [53].

There are several limitations of the present study. The first is that it was a retrospective study conducted at a single institution, and the possibility of a selection bias is undeniable. The prognosis of patients who received initial therapy before April 1, 2008 was significantly superior to that of those who received their initial therapy after 2008. Because we started to perform EGFR mutation analysis in routine clinical practice from April 1, 2008, there is the possibility of a selection bias towards patients who received the initial therapy before April 1, 2008. This is one of the major limitations of our retrospective study. Some studies have reported that EGFR mutations may be a positive prognostic factor for survival in advanced NSCLC patients, regardless of EGFR-TKI therapy [54,55]. Also in the BR.21 trial, the median survival time was reported to be longer in patients with mutant EGFR as compared to that in patients with wild-type EGFR [56]. Although mutant EGFR patients not treated with EGFR-TKIs were found to be a confounding factor, we performed adjustment for the confounding factor using a Cox proportional hazards model. According to the univariate analysis, the date of start of the initial therapy (before April 1, 2008) was a favorable prognostic factor. However, PCV < 2.2 ng/ml, EGFR mutation positivity and PS 0–1 were found to be independent favorable prognostic factors after adjustment for the date of start of the initial therapy. In this study, while the MST (39.2 months) in the mutant EGFR group was not favorable as compared to previous reports [57], the mutant EGFR group with PCV < 2.2 ng/ml had a more favorable prognosis than that of the mutant EGFR group with PCV ≥ 2.2 ng/ml. The proportion of patients who received erlotinib was less in the group with PCV ≥ 2.2 ng/ml than in the group with PCV < 2.2 ng/ml, which could have influenced the more favorable prognosis in the group with PCV < 2.2 ng/ml than in the group with PCV ≥ 2.2 ng/ml. All of the patients with advanced lung adenocarcinoma in whom the diagnosis was made after April 1, 2008 were tested for EGFR mutations at

the time of the diagnosis, whereas in the patients with other histological types of lung cancer, the testing was performed at the discretion of the attending physician. Second, the follow-up period was inadequate, especially in the mutant EGFR group with PCV < 2.2 ng/ml, and the censored cases were conspicuous. There was also a problem with the stage distribution (there were relatively few stage IIIB cases). Distant metastasis occurred in all of the stage IIIB cases in which local treatment had been performed, and all of the patients with disease recurrence were tested for EGFR mutations. Moreover, significant survival differences in stage IIIB/ IV were not found in the univariate analysis. Furthermore, the treatment regimens used in the stage IV cases were not standardized, with each of the attending physicians administering any of the various standard treatments used in routine clinical practice recommended by the guidelines of the Japan Lung Cancer Society.

In advanced lung adenocarcinoma, which may be considered as a generalized systemic disease, it may be particularly difficult to determine the characteristics of an entire heterogeneous tumor by tissue diagnosis alone based on examining just one part of the tumor. Based on the results of the present study, we propose that mutant EGFR patients with serum PCV < 2.2 ng/ml have a better prognosis than the mutant EGFR patients with higher PCV.

Conclusions

The potential applications of PCV measurements might include identification of candidates in whom it might have some prognostic value. Furthermore, PCV might be regarded as a routine demographic variable having prognostic value in patients with advanced lung adenocarcinoma.

Abbreviations

NSCLC: Non-small cell lung cancer; PCV: Pretreatment serum CYFRA 21–1 levels; pts: patients; EGFR: Epidermal growth factor receptor; Mt+: Mutant EGFR; Mt-: Wild-type; TKI: Tyrosine kinase inhibitor; CK: Cytokeratin; ECOG PS: Eastern Cooperative Oncology Group performance status; CLEIA: Chemiluminescence enzyme immunoassay; PCR: Polymerase chain reaction; IASLC: International Association of the Study of Lung Cancer; TNM: Tumor-node-metastasis.

Competing interests

The authors have no competing interests to declare.

Authors' contributions

AO contributed to the drafting of this manuscript and data collection, and KM contributed to the study design and statistical analysis. TT, HA, TS, TT, HK, TN, HM, TN, ME, NY contributed to analysis of the data and interpretation of the findings. All authors have read and approved of the submission of the final manuscript.

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Detection of epidermal growth factor receptor T790M mutation in plasma DNA from patients refractory to epidermal growth factor receptor tyrosine kinase inhibitor

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A secondary epidermal growth factor receptor (EGFR) mutation, the substitution of threonine 790 with methionine (T790M), leads to acquired resistance to reversible EGFR-tyrosine kinase inhibitors (EGFR-TKIs). A non-invasive method for detecting T790M mutation would be desirable to direct patient treatment strategy. Plasma DNA samples were obtained after discontinuation of gefitinib or erlotinib in 75 patients with non-small cell lung cancer (NSCLC). T790M mutation was amplified using the SABER (single allele base extension reaction) technique and analyzed using the Sequenom MassARRAY platform. We examined the T790M mutation status in plasma samples obtained after treatment with an EGFR-TKI. The SABER assay sensitivity using mixed oligonucleotides was determined to be 0.3%. The T790M mutation was detected in 21 of the 75 plasma samples (28%). The presence of the T790M mutation was confirmed by subcloning into sequencing vectors and sequencing in 14 of the 21 samples (66.6%). In this cohort of 75 patients, the median progression-free survival (PFS) of the patients with the T790M mutation ($n = 21$) was not statistically different from that of the patients without the mutation ($n = 54$, $P = 0.94$). When patients under 65 years of age who had a partial response were grouped according to their plasma T790M mutation status, the PFS of the T790M-positive patients ($n = 11$) was significantly shorter than that of the T790M-negative patients ($n = 29$, $P = 0.03$). The SABER method is a feasible means of determining the plasma T790M mutation status and could potentially be used to monitor EGFR-TKI therapy. (*Cancer Sci* 2013; 104: 1198–1204)

Despite responses to epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, in the majority of lung cancer patients carrying an EGFR mutation, most of these patients eventually become resistant to EGFR-TKIs.[1] The most common known mechanisms of acquired resistance are MET amplification, increased levels of hepatocyte growth factor, and secondary mutations in EGFR.[2–5] The substitution of threonine 790 with methionine (T790M) leads to acquired resistance to reversible EGFR-TKIs. The frequency of this secondary mutation is 30–50% among patients who are resistant to EGFR-TKIs. Understanding the mechanisms of resistance to EGFR-TKIs is important because second and third generations EGFR-TKIs, including irreversible EGFR inhibitors, are presently under clinical development with the goal of conquering resistance mechanisms to EGFR-TKIs.

Here, we report the development of a highly sensitive single allele base extension reaction (SABER) method capable of detecting low levels of T790M mutation. We used this method to assess the T790M mutation status of plasma samples from non-small cell lung cancer (NSCLC) patients to clarify (i) the frequency of tumor samples carrying the T790M mutation after EGFR-TKI treatment; and (ii) the association between the T790M mutation status and the clinical outcome. The detection of the T790M mutation in plasma samples could enable the clinical response to EGFR-TKIs to be monitored.

Materials and Methods

Patients. A total of 75 NSCLC patients with progressive disease (PD) after undergoing EGFR-TKI treatment (gefitinib or erlotinib) at the Thoracic Oncology Center, Cancer Institute Hospital, Japanese Foundation for Cancer Research between 2006 and 2011 were included in this study. Progressive disease was defined as the appearance of a new lesion or a 20% increase in the size of a primary tumor. The period between the detection of PD and the collection of the plasma sample used to determine the T790M mutation status varied (median 44 days; range, 0–803 days). Epidermal growth factor receptor mutation status in tumor samples obtained before treatment with an EGFR-TKI were identified using direct sequencing. Table 1 shows the clinical characteristics of the patients. Plasma samples obtained after discontinuation of EGFR-TKI were used to examine the T790M mutation status. All the patients provided informed written consent, and the study was approved by the Institutional Review Board at the Cancer Institute Hospital and the Kinki University Faculty of Medicine.

Sample processing. Plasma DNA was purified using a QIAamp Circulating Nucleic Acid Kit (Qiagen, Valencia, CA, USA). The extracted DNA was stored at −80°C until analysis.

Assay design. The assay was designed using MassARRAY Assay Design 4.0 software (Sequenom, San Diego, CA, USA) with a slight modification to enable use with the SABER method; the assay was intentionally designed so that it would only include terminators for the mutated nucleotide, and not the terminator for the wild-type nucleotide. A schematic diagram of the assay is shown in Figure 1. An amplification control assay was incorporated into each reaction using a conserved sequence in the amplified EGFR transcript so that

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Table 1. Clinical characteristics of patients with progressive disease (PD) after epidermal growth factor-tyrosine kinase inhibitor (EGFR-TKI) treatment (n = 75)

EGFR-TKI-treated Patients	All patients (n = 75) No. (%)	Post-treatment T790M mutation positive patients (n = 21)	Post-treatment T790M mutation negative patients (n = 54)	P†
Age, years(mean, 61.6 ± 8.5 years)				
≤65	47 (62.7)	11	36	0.294
>65	28 (37.3)	10	18	
Sex				
Male	21 (28.0)	7	14	0.573
Female	54 (72.0)	14	40	
Smoking				
No	44 (58.7)	12	32	1.000
Yes	31 (41.3)	9	22	
Histology				
Ad	71 (94.7)	19	52	0.311
Large/Sq	4 (5.3)	2	2	
EGFR mutation (pre-treatment, tumor)				
Activating mutation	60 (80.0)	18	42	0.535
Wild type/unknown	15 (20.0)	3	12	
Response to EGFR-TKI				
PR	60 (80.0)	20	40	0.053
SD/PD/NE	15 (20.0)	1	14	

†Fisher's exact test. Ad, adenocarcinoma; Large, large cell carcinoma; NE, not evaluable; PD, progressive disease; PR, partial response; SD, stable disease; Sq, squamous cell carcinoma.

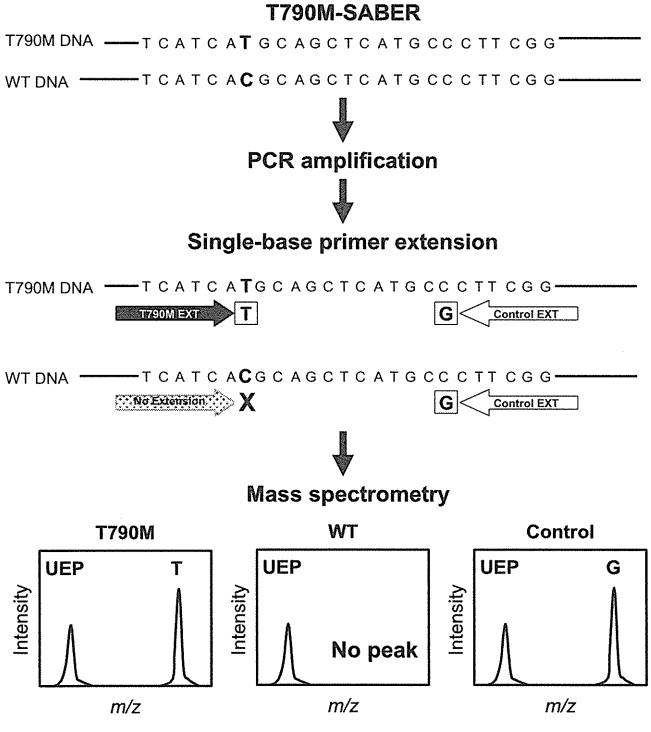

Fig. 1. Schematic diagram of the T790M-SABER method, based on MassARRAY assays. The DNA samples were first amplified using polymerase chain reaction (PCR). The PCR products were then subjected to a single base extension reaction. The T790M-SABER reaction only included terminators for the mutated nucleotide, and not the terminators for the wild-type nucleotide. An internal amplification control was designed to prevent false-negative results caused by PCR inhibitors. The primer extension products were analyzed using matrix-assisted laser desorption ionization-time of flight mass spectroscopy (MALDI-TOF MS). UEP, unextended primer.

Table 2. Primers used in the T790M SABER (single allele base extension reaction) method

	Sequences
PCR	
Forward	5′- ACGTTGGATGATCTGCCTCACCTCCACCGT -3′
Reverse	5′- ACGTTGGATGTGTTCCCGGACATAGTCCAG -3′
Extension	
T790M	5′- CACCGTGCAGCTCATCA -3′
Internal control	5′- GTCCAGGAGGCAGCCGAAG -3′

PCR, polymerase chain reaction.

amplification would always occur in the presence of input template DNA. The PCR and extension primer sequences are listed in Table 2.

SABER method. The SABER method, where the primer extension was restricted to the mutant-specific allele, was performed using Sequenom iPlex Pro biochemistry with resultant products detected with the MassARRAY platform. The PCR reactions were performed in 5-μL volumes containing 1.5 μL of eluted serum DNA, 200 nM of each primer, 50 μM of dNTPs (Sequenom), 0.2 U of PCR Taq DNA polymerase (Sequenom), 2.0 mM of $MgCl_2$, and 1 × PCR buffer (Sequenom). The thermal cycling for the PCR was performed as follows: 2 min at 94°C, followed by 45 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. The program was terminated after a final incubation at 72°C for 5 min. After the completion of the PCR, 2 μL (0.5 U) of shrimp alkaline phosphatase (Sequenom) was added to each reaction and the resulting mixture was incubated for 40 min at 37°C before enzyme inactivation by incubating the sample for 5 min at 85°C. The single-base primer extension reaction (SABER) was then performed in a final volume of 9 μL, containing 1 μmol of each extension primer, a mixture of three iPLEX mass-modified terminators (Sequenom), and 2.56 U of

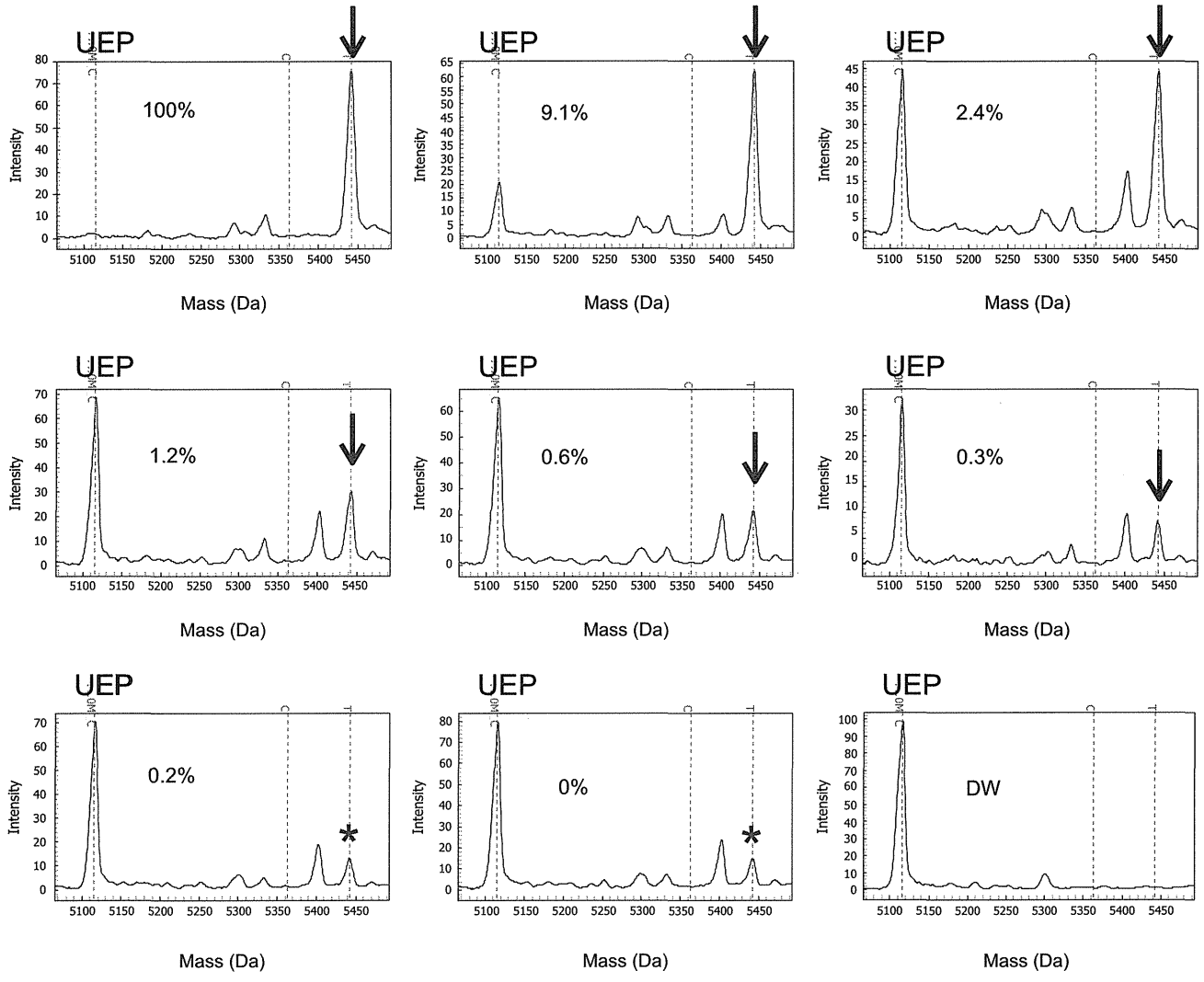

Fig. 2. Sensitivity of T790M detection. The percentages indicate the calculated proportion of T790M DNA in a mixture with wild-type DNA. An arrow at 5442 Da indicates the detection of the T790M mutation. An asterisk indicates a non-specific background peak. Three independent experiments were performed in duplicate, with identical results. DW, distilled water; UEP, unextended primer.

ThermoSequenase (Sequenom). The thermal cycling program for the reaction included an initial denaturation for 30 s at 94°C followed by five cycles of 5 s at 52°C and 5 s at 80°C. Forty additional annealing and extension cycles (5 s at 94°C, 5 s at 52°C, and 5 s at 80°C) were then performed. The final extension was performed at 72°C for 3 min, and the samples were then cooled to 4°C. The reaction products were desalted by dilution with 41 μL of distilled water, the addition of 15 mg of ion-exchange resin (Sequenom), and subsequent separation of the resin by centrifugation. The products were spotted on a SpectroChip II (Sequenom), processed, and analyzed using a Compact Mass Spectrometer and MassARRAY Workstation (version 3.3) software (Sequenom). The data analysis was performed using MassARRAY Typer software, version 4.0 (Sequenom).

Sequencing analysis. The PCR products were subcloned into a pTA2 vector (Toyobo, Osaka, Japan) and sequenced using an automated sequencer (ABI Prism 3100 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) and M13 universal primers (Applied Biosystems).

Scorpion ARMS analysis. Scorpion ARMS analysis used the DxS EGFR Mutation Test Kit for research use only (Qiagen) and was carried out according to the manufacturer's instructions.

Statistical analyses. The Fisher exact test was used to assess the relationship between the T790M mutation status and different clinical characteristics, including patient sex and the primary mutation status. The objective tumor response (partial response [PR], stable disease [SD], or progressive disease [PD]) was evaluated according to the Response Evaluation Criteria in Solid Tumors guidelines. Progression-free survival (PFS) was defined as the period from the start of treatment until the date when disease progression was observed. The survival curves were derived using the Kaplan–Meier method and were compared using the log-rank test. All the statistical analyses were performed using JMP software (version 10; SAS Institute, Cary, NC, USA). A P-value < 0.05 was considered statistically significant.

Results

Assay sensitivity. The assay sensitivity was determined using T790M DNA oligonucleotide (5.0×10^{-16}, 2.5×10^{-16}, 1.3×10^{-16}, 6.3×10^{-17}, 3.1×10^{-17}, 1.6×10^{-17}, and

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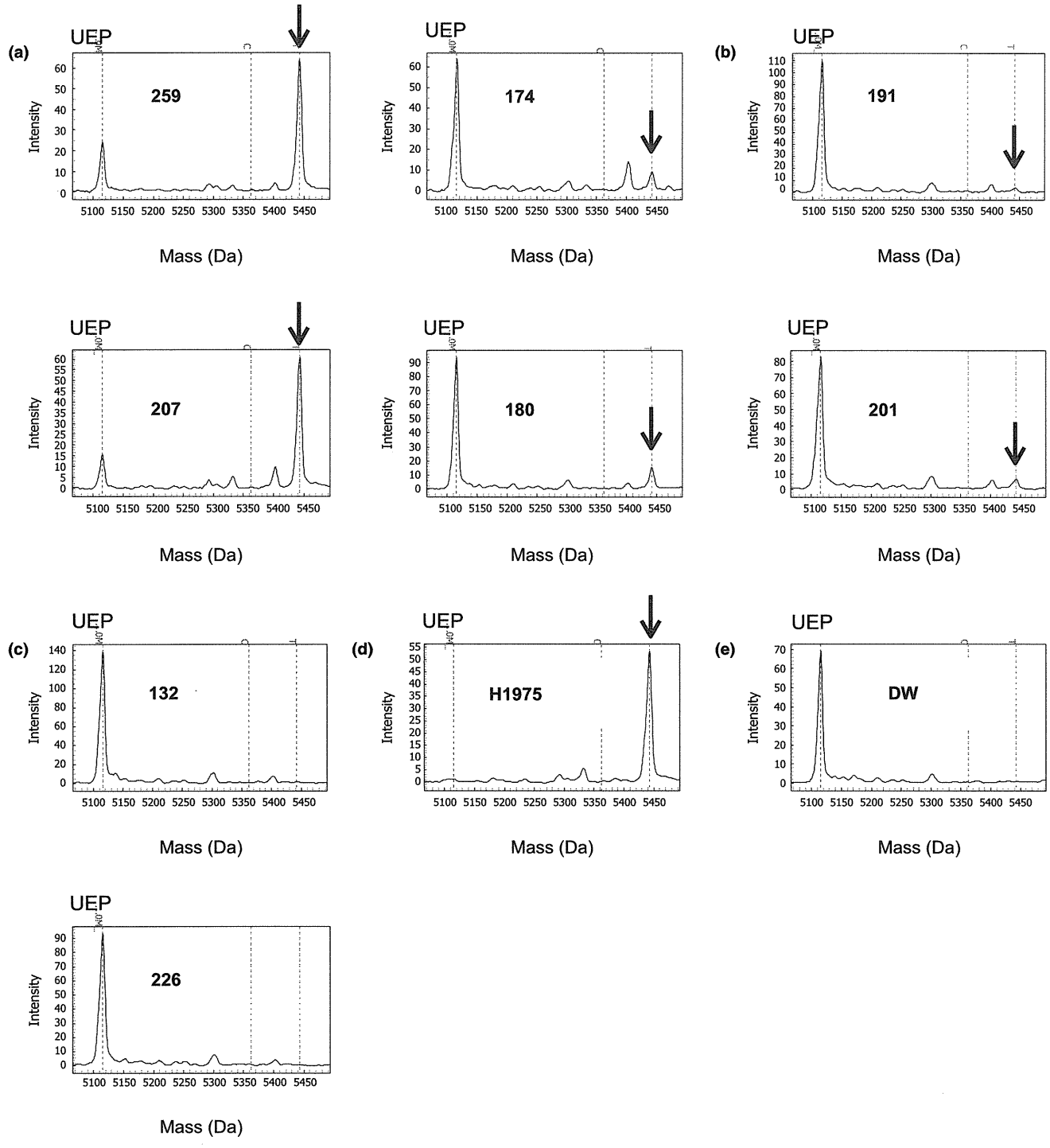

Fig. 3. Mass spectroscopy (MS) analysis of the T790M mutation in plasma DNA. An arrow at 5442 Da indicates the detection of the T790M mutation. (a) Example of a T790M-SABER/subcloning double-positive sample. (b) Example of a T790M-positive case that was only detected using the T790M SABER method. (c) Example of a T790M-negative case, as detected using the T790M SABER method. (d) Positive control for the T790M-SABER method using T790M-positive human non-small cell lung cancer (NSCLC) H1975 cells. (e) Negative control for the T790M-SABER method using distilled water. The experiment was repeated twice with identical results. DW, distilled water; UEP, unextended primer.

7.8×10^{-18} mol/reaction (i.e. 4.8%, 2.4%, 1.2%, 0.6%, 0.3%, 0.2%, and 0.1%, respectively) mixed with a fixed amount (1×10^{-14} mol/reaction) of wild-type DNA oligonucleotide. Figure 2 shows the MALDI-TOF MS spectra. Concentrations as low as 3.1×10^{-17} mol of T790M DNA mixed with 1×10^{-14} mol of wild-type DNA could be detected, indicating a detection sensitivity of approximately 0.3%, which is in agreement with previous studies using SABER.[6]

Detection of T790M in plasma DNA. We examined the T790M mutation status in plasma samples obtained after dis-

Table 3. Clinical characteristics of T790M mutation positive patients with acquired resistance to epidermal growth factor-tyrosine kinase inhibitor (EGFR-TKI)

No.	Age	Sex	Histology	Pre-treatment EGFR mutation status (tumor)†		Response to EGFR-TKI	From PD to plasma collection (days)	Post-treatment T790M mutation status (plasma)‡	
				Direct sequencing	SABER			SABER	Clonal analysis T790M clones/Total clones
147	69	F	Ad	unknown	–	PR	139	+	0/105
162	76	F	Ad	L858R	–	PR	190	+	0/105
174	60	F	Ad	del E746-A751	n.t.	PR	272	+	2/20
180	67	F	Ad	del E746-A751	n.t.	PR	34	+	3/25
185	75	M	Ad	G719X	+	PR	17	+	1/55
191	56	F	Ad	L858R	–	PR	17	+	0/105
192	68	M	Ad	del E746-A751	–	PR	65	+	0/105
199	72	M	Ad	L858R	–	PR	0	+	0/100
201	73	F	Ad	del E746-A751	–	PR	390	+	0/105
207	64	F	Ad	L858R	–	PR	44	+	4/35
214	61	F	Ad	L858R	–	PR	60	+	2/65
217	71	F	Ad	unknown	n.t.	PR	128	+	3/20
235	69	F	Ad	unknown	n.t.	PR	222	+	1/35
243	59	M	Ad	L858R	–	PR	14	+	1/25
246	54	F	Ad	del E746-A751	–	PR	182	+	1/20
248	42	F	Large	del E746-A751	n.t.	PR	23	+	1/30
258	44	M	Ad	del E746-A751	–	PR	299	+	1/100
259	60	F	Ad	L858R	–	PR	237	+	2/25
279	71	F	Ad	L858R	n.t.	SD	379	+	1/30
306	43	M	Ad	del E746-A751	n.t.	PR	34	+	1/57
308	65	M	Sq	del E746-A751	n.t.	PR	42	+	0/105

†The EGFR mutation status of each tumor sample was analyzed using direct sequencing and SABER (single allele base extension reaction) method. ‡The EGFR mutation status of each plasma sample was analyzed using the SABER method and was confirmed using clonal analysis. +, T790M positive; –, T790M negative; Ad, adenocarcinoma; Large, large cell carcinoma; n.t., not tested; PD, progressive disease; PR, partial response; SD, stable disease; Sq, squamous cell carcinoma.

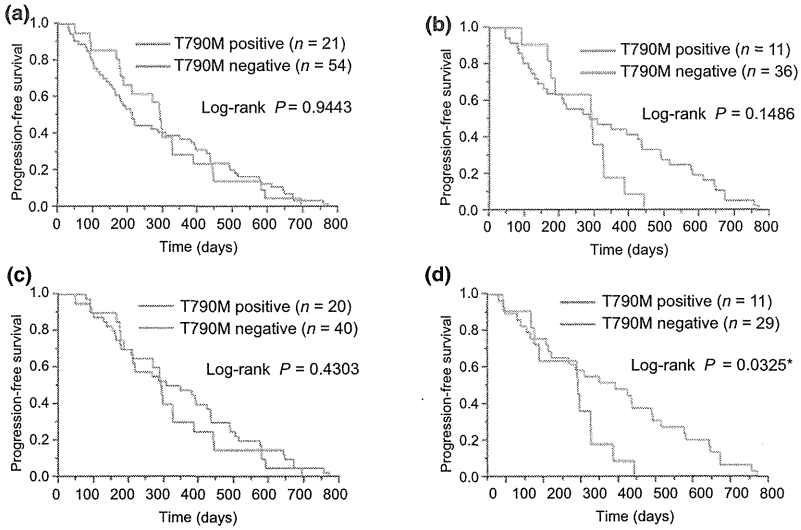

Fig. 4. Progression-free survival according to T790M mutation status as measured in plasma DNA. The Kaplan–Meier progression-free survival curves are shown for all the T790M-positive and T790M-negative patients (n = 75) in (a), for patients aged 65 years or younger (n = 47) in (b), for patients with a partial response to epidermal growth factor-tyrosine kinase inhibitors (EGFR-TKIs) treatment (n = 60) in (c), and for patients aged 65 years or younger who had a partial response to EGFR-TKI treatment (n = 40) in (d).

continuation of EGFR-TKI. Using the SABER method, the internal control was successfully amplified and detected in all the samples (data not shown). The T790M mutation was detected in 21 of the 75 samples (28%). The key results of the SABER method are shown in Figure 3. The clinical characteristics of the T790M-positive patients are shown in Table 3. The presence of the T790M mutation was confirmed by subcloning into sequencing vectors and sequencing. When up to 105 colonies were selected and sequenced (theoretical median limit of detection of 0.95%), the T790M mutation was confirmed in 14 of the 21 (66.6%) PCR products. The T790M mutation in plasma was also identified by the Scorpion ARMS (Table S1).

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T790M was detected in 6/75 cases (8%). Of these cases, 5/6 were also positive by the SABER method. Compared to the positive rate (28%, 21/75) when using the SABER method, the rate detected by the Scorpion ARMS was relatively low.

Detection of T790M in pretreatment tumor specimens. We analyzed the T790M mutation status in the tumor samples using the SABER method. All of the tumor samples were obtained before the EGFR-TKI treatment. No tumor sample was obtained by the re-biopsy after EGFR-TKI treatment. T790M was detected in two tumor samples (Table S1). One case (no. 185) was double positive (tissue +, plasma +). Another case (no. 167) was T790M positive in tumor samples only. The positive rate (7%, 2/28) was relatively low as compared with that of plasma samples (46%, 13/28). These data suggest that the T790M mutation detected in the plasma is acquired by EGFR-TKI treatment and that detection of T790M in the plasma is feasible to detect EGFR-TKI refractory cases.

Correlation between plasma T790M mutation status and clinical outcome. There was no significant difference in clinical characteristics between patients with or without T790M mutation (Table 1). T790M positive detection tends to be observed more frequently in PR patients compared with non-PR patients although the correlation is not highly significant ($P = 0.053$, Table 1).

In our cohort of 75 patients, the median PFS of the patients with the T790M mutation ($n = 21$) was not statistically different from that of the patients without the mutation ($n = 54$) ($P = 0.9443$), being 289 days and 210 days, respectively (Fig. 4a). When patients aged 65 years or younger were subdivided into two groups according to their plasma T790M status, the median PFS of the T790M-positive patients ($n = 11$) tended to be shorter than that of the T790M-negative patients ($n = 36$, $P = 0.1486$; Fig. 4b). We also compared the PFS of patients according to their response to the EGFR-TKIs and found no statistical differences between the responders (PR) and the non-responders ($P = 0.4303$; Fig. 4c). When patients aged 65 years or younger who had a PR were grouped according to their plasma T790M mutation status, the PFS of the T790M-positive patients ($n = 11$) was significantly shorter than that of the T790M-negative patients ($n = 29$, $P = 0.0325$; Fig. 4d) being 289 days and 391 days, respectively.

Discussion

Our findings show that the T790M mutation can be detected in plasma samples obtained after discontinuation of EGFR-TKI and that the SABER method is a feasible means of determining the plasma T790M mutation status. We detected the T790M mutation in 21 out of 75 plasma samples that were obtained from patients after discontinuation of EGFR-TKI (28%). This frequency seems to be lower than the positive rate (~50%) in tumor tissue samples reportedly.[7] However, the positive rate when using SABER is relatively higher than when using Scorpion-Arms (Table S1) for the same samples and our previous report.[8] We can speculate that the sensitivity of the SABER assay for circulating samples (plasma or serum) is much improved. In this study, plasma samples were collected following therapy with the EGFR-TKI; therefore, the time between the detection of PD and that of sample collection was varied. To conclude whether this sensitivity is enough or not, it will be necessary to conduct a prospective comparison study using the paired samples of plasma and re-biopsy samples as the next step. Previously, we attempted to determine the EGFR mutation status in serum samples using the highly sensitive Scorpion-Arms method.[8,9] In that cohort, a high false-negative rate was observed, and more sensitive methods of detecting EGFR mutations in serum samples are desirable. Another group reported the results of a serum EGFR mutation analysis using the MBP-QP method,[10] which yielded a detection sensitivity equivalent to that of the SABER method used in the present report. Automated and high throughput analysis is an advantage of SABER method.

Direct sequencing of the subcloned PCR products confirmed the presence of the T790M mutation, suggesting that this SABER method is highly specific. However, the clonal analysis did not detect the T790M mutation in seven of the 21 (33.3%) samples that were found to be T790M-positive using SABER. In this cohort, we selected and sequenced up to 105 colonies (theoretical median limit of detection of 0.95%). Therefore, the number of tested colonies might not have been large enough to detect the low frequency of mutant clones with normal sampling error distribution.

In our study, the median PFS of the T790M-positive patients was significantly shorter than that of the T790M-negative patients with a clinical PR among younger (≤65 years) patients. It is likely that the prognosis of patients with an activating mutation who acquire a resistance mutation is less favorable. However, Oxnard *et al.*[11] showed that EGFR-TKI resistant patients with T790M identified in re-biopsy specimens had a relatively favorable prognosis compared with patients without the T790M mutation. Their data seems to be inconsistent with our result. Different materials and detection methods may be the cause of this discrepancy between two studies.

The early detection of T790M mutation may be beneficial to such patients.

The intrinsic existence of T790M clones in pretreatment tumors has been previously suggested. Indeed, the T790M mutation has been detected in a few pretreatment tumor samples.[12,13] The T790M-SABER method is highly sensitive and is capable of detecting this mutation in plasma samples; consequently, the detection of the T790M mutation before or during EGFR-TKI treatment may be possible. Consequently, the T790M-SABER method is a promising tool for the detection of T790M mutation in a diagnostic setting.

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Disclosure Statement

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. A comparison of T790M detection by SABER and Scorpion-ARMS methods.

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Pemetrexed and Cisplatin for Advanced Non-squamous Non-small Cell Lung Cancer in Japanese Patients: Phase II Study

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Abstract. *Background: Although pemetrexed/cisplatin (P-C) is a standard treatment for advanced non-squamous non-small cell lung cancer (Nsq-NSCLC), neither its efficacy nor the effects of potential differences between driver mutations, such as the anaplastic lymphoma kinase (ALK) translocation and epidermal growth factor receptor (EGFR) mutations, have been thoroughly examined. Patients and Methods: A single-arm phase II study of P-C was conducted in Japanese patients with chemo-naïve advanced Nsq-NSCLC. Patients received four cycles of pemetrexed (500 mg/m^2) combined with cisplatin (75 mg/m^2) on day 1 every three weeks. The primary end-point was the response rate (RR) and the secondary end-points were toxicity, progression-free survival (PFS), and overall survival (OS). Results: A total of 50 patients were analyzed (males, 68%; adenocarcinoma, 80%). The RR was 44.0%. The median PFS and OS were 4.3 months and 22.2 months, respectively. Toxicities were mild, and no new toxicity profiles were identified. Among the 39 out of 50 samples, six (15.4%) presented ALK translocation and nine (23.1%) presented EGFR mutations; of the remaining patients, 24 (61.5%) were wild-type for both ALK and EGFR. Objective response was observed in two out of six patients with ALK translocations, six out of nine with EGFR mutations, and in 11 (45.8%) wild-type patients. Conclusion: The combination of pemetrexed and cisplatin was effective and safe in Japanese patients with Nsq-NSCLC. We did not observe obvious differences in the efficacy of P-C between patients with ALK translocation or EGFR mutation and those with wild-type genotype.*

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Lung cancer is the major cause of cancer-related deaths worldwide. Approximately 85% of lung tumors are non-small cell lung cancers (NSCLC), 70% of which are either inoperable, locally advanced or metastatic (1). Two-drug combinations of a third-generation agent (docetaxel, paclitaxel, gemcitabine, vinorelbine, and pemetrexed) with a platinum compound (cisplatin and carboplatin) are the standard treatment options for advanced NSCLC (2-5).

Pemetrexed (Alimuta®; Eli Lilly and Company, Indianapolis, IN, USA) is a multitargeted antifolate that inhibits thymidylate synthase (TYMS), dihydrofolate reductase, glycinamide ribonucleotide formyltransferase, and aminoimidazole carboxamide ribonucleotide formyltransferase (6).

Randomized phase III clinical trials have demonstrated that pemetrexed is efficacious both in combination with cisplatin (P-C) for first-line treatment of NSCLC–having non-inferior efficacy and better tolerability than for the combination of gemcitabine and cisplatin (4)–and as a single-agent in second-line treatment (7). Moreover, in patients with non-squamous NSCLC (Nsq-NSCLC), pemetrexed has superior efficacy compared with other standard treatments, and the combination therapy with cisplatin leads to superior overall survival (OS) compared with gemcitabine and cisplatin, and is one of the most common regimens for treatment of metastatic Nsq-NSCLC (8).

Adenocarcinoma is the major histological type of Nsq-NSCLC. More than 75% East Asian never-smokers with lung adenocarcinoma harbor targetable oncogenic mutations, including epidermal growth factor receptor (*EGFR*) mutations, fusions of echinoderm microtubule-associated protein-like 4 (*EML4*) and anaplastic lymphoma kinase

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