epithelium of the cornea.[1] The management of filamentary keratitis can be clinically challenging. Current best-practice management of filamentary keratitis involves treatment of the underlying dry eye and specific treatments that are aimed at the corneal filaments.[2] Proposed treatments include lubricants, topical steroidal and nonsteroidal anti-inflammatory agents, and punctal plugs for aqueous-deficient dry eye, as well as mechanical removal of filaments, hypertonic saline, mucolytic agents, and bandage contact lenses for the filaments. However, the fundamental management strategy is treatment of the underlying cause of filament generation.

Cetuximab, a monoclonal antibody directed against the epidermal growth factor receptor (EGFR), has been demonstrated to improve overall and progression-free survival in patients with colorectal cancer that is refractory to traditional chemotherapy. EGFR inhibitors (EGFRIs) such as cetuximab typically induce adverse effects such as papulopustular rash, dry skin, itching, and hair and periungual alterations. It is not surprising that these adverse reactions occur as dermatologic symptoms because cutaneous tissues are critically dependent on EGFR signaling for normal function.[3]

We had two reasons for considering the cause of this patient's filamentary keratitis to be cetuximab, an EGFRI. First, EGFR signaling is known to play an important role in normal ocular homeostasis.[4] EGFRs are strongly expressed on the corneal epithelium, keratinocytes, and the endothelium. Endogenous EGF is found in high concentrations in tears, promoting the migration and proliferation of epithelial cells and thereby facilitating corneal epithelial wound healing.[4,5] Several clinical studies of topically applied EGF yielded promising results in terms of corneal epithelial healing after severe corneal damage, such as with corneal trauma. Recently, EGF eyedrops have been confirmed as an efficacious topical treatment for traumatic corneal ulcers[6] and herpetic corneal ulcers.[7] At present, human EGF eyedrops are an option, although off-label, for treating intractable corneal wounds.

Second, there is evidence that indicates that the inhibition of EGFR-mediated signaling pathways will evoke corneal damage in vivo.[8] For example, in preclinical toxicity studies, the systemic administration of gefitinib, an EGFR tyrosine kinase inhibitor, was reported to significantly delay the corneal epithelial healing and to decrease corneal epithelial cell proliferation in rats and dogs.[9,10] A small number of cases of EGFRI-associated corneal wounds in humans have also been reported.[11,12] Most required discontinuation of EGFRI because of exacerbation of symptoms. It is regrettable when EGFRI must be stopped because of adverse effects. Thus, treatment options for these adverse reactions are needed so that patients can continue their anticancer treatment as scheduled.

To our knowledge, this is the first report of filamentary keratitis associated with cetuximab that was successfully treated with EGF eyedrops. Although there is one report that describes the use of topical human EGF for the treatment of corneal damage during cetuximab treatment,[13] the filamentary keratitis was nonspecific in that case. Our case is noteworthy in that it was not necessary to stop antitumor treatment with cetuximab because of adverse effects. This raises the possibility that human EGF eyedrops are an effective therapy for EGFRI-associated corneal damage. It is important for patients to continue treatment with cetuximab as long as this agent remains effective. Use of EGF eyedrops for EGFRI-associated corneal damage is a reasonable treatment option, given that such eyedrops may augment endogenous EGF in tears and thereby locally reverse the inhibition of EGFR signaling by EGFRI. EGFR eyedrops also seem to be safe, given that there is no evidence of carcinogenesis associated with topical application of EGF. With the increasing use of cetuximab in cancer therapy, cetuximab-associated corneal damage may occur more frequently. Moreover, the topical EGF treatment administered to our patient might be applicable to serious adverse dermatologic reactions to EGFRI. Given that EGFRIs are such a promising anticancer therapy, we anticipate future studies aimed at establishing therapies for some of the adverse effects that typically occur with EGFRI administration.

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

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JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

Randomized Phase III Placebo-Controlled Trial of Carboplatin and Paclitaxel With or Without the Vascular Disrupting Agent Vadimezan (ASA404) in Advanced Non–Small-Cell Lung Cancer

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See accompanying editorial on page 2952

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ABSTRACT

Purpose
This phase III trial was conducted to test whether the novel vascular disrupting agent ASA404 (vadimezan), when combined with first-line platinum-based chemotherapy, improves survival in patients with advanced non–small-cell lung cancer (NSCLC) versus chemotherapy alone.

Patients and Methods
Patients with advanced stage IIIB or IV NSCLC, stratified by sex and tumor histology, were randomly assigned 1:1 to paclitaxel (200 mg/m^2) and carboplatin (area under the curve, 6.0) with or without ASA404 (1,800 mg m^2), given intravenously once every 3 weeks for six cycles followed by maintenance ASA404 or placebo. Primary end point was overall survival (OS); secondary end points included overall response rate (ORR) and progression-free survival (PFS).

Results
One thousand two hundred ninety-nine patients were randomly assigned. The trial was stopped for futility at interim analysis. At final analysis, there was no difference in OS seen between ASA404 (n = 649) and placebo (n = 650) arms: median OS was 13.4 and 12.7 months respectively (hazard ratio [HR], 1.01; 95% CI, 0.85 to 1.19; P = .535). Similarly, no OS difference was seen in the histologic (squamous or nonsquamous) and sex (male or female) strata. Median PFS was 5.5 months in both arms (HR, 1.04; P = .727), while ORR was 25% in both arms (P = 1.0). Overall rate of adverse events (AEs) was comparable between the ASA404 and placebo arms. Grade 4 neutropenia (27% *v* 19%) and infusion site pain (10% *v* 0.5%) were reported more frequently in the ASA404 arm.

Conclusion
The addition of ASA404 to carboplatin and paclitaxel, although generally well tolerated, failed to improve frontline efficacy in advanced NSCLC.

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INTRODUCTION

Vascular disruption of existing tumor blood vessels represents a novel antineoplastic strategy. In preclinical models, tumor vascular disrupting agents (VDAs) have been shown to selectively affect endothelial cells of established tumor blood vessels, resulting in ischemia in the central component of tumor masses, but with persistence of a viable layer of cancer cells in the periphery.[1-3] Because tumor VDAs predominantly target the tumor core—a hypoxic region in which cells are known to harbor resistance to traditional DNA-damaging chemotherapy—drug development has evolved to combine VDAs (targeting the core) with cytotoxic agents (targeting the viable rim) to achieve synergistic tumor kill.[4]

Among the tumor VDAs furthest along in development is ASA404 (vadimezan, 5,6-dimethylxanthenone-4-acetic acid), an analog of flavone acetic acid. Although the actual molecular target of ASA404 is unknown, its pharmacologic effects have been well described in preclinical models.[5] It has been shown to promote apoptosis of endothelial cells of tumor blood vessels, causing the release of von Willebrand's factor which then leads to blood clotting and vessel occlusion.

ASA404 has also been shown to trigger a local cascade of cytokines including serotonin and tumor necrosis factor. The direct and indirect effects of ASA404 culminate in the breakdown of vasculature and hemorrhagic tumor necrosis. ASA404 has also shown to have either additive or synergistic antitumor effects when combined with several cytotoxic chemotherapeutic agents, including paclitaxel.[6]

A randomized phase II trial of carboplatin (area under the curve [AUC], 6) and paclitaxel (175 mg/m^2) with or without ASA404 (at 1,200 mg/m^2) was conducted in 73 patients with advanced non–small-cell lung cancer (NSCLC),[7] a population in which standard platinum-based chemotherapy has traditionally yielded marginal outcomes, such as overall response rates (ORR) of lower than 30% and median overall survival (OS) times of approximately 8 to 10 months.[8,9] In that trial, ASA404 plus chemotherapy appeared to improve efficacy over chemotherapy alone in terms of ORR (31.3% *v* 22.2%), median time to progression (TTP, 5.4 *v* 4.4 months), and median OS (14.0 *v* 8.8 months). To further verify those results and to explore a dose-response relationship, a single-arm phase II extension trial of 31 patients with advanced NSCLC was performed to evaluate ASA404 at a higher dose of 1,800 mg/m^2, again in combination with carboplatin and paclitaxel. Tumor ORR was 37.9%, median TTP was 5.5 months, and median OS was 14.9 months.[10] In both studies, efficacy appeared to be improved with ASA404 regardless of tumor histology (squamous *v* nonsquamous), and there was no overt increase in serious adverse events.

These results led to this global, randomized, double-blind, placebo-controlled trial (Antivascular Targeted Therapy: Researching ASA404 in Cancer Treatment [ATTRACT-1]) of ASA404 plus carboplatin and paclitaxel versus placebo plus carboplatin and paclitaxel in patients with stage IIIB/IV NSCLC who had not previously received systemic therapy for metastatic disease. This trial was conducted at more than 200 sites in 20 countries.

PATIENTS AND METHODS

Patients

Eligible patients were ≥ 18 years of age with histologically confirmed NSCLC and WHO performance status 0 or 1 who had either newly-diagnosed stage IIIb disease (malignant pleural effusion or pericardial effusion) or stage IV disease.[11] No prior systemic antineoplastic treatment for advanced NSCLC was allowed; however, prior neoadjuvant or adjuvant chemotherapy for earlier stage I/II NSCLC was allowed if the last dose was 12 months or more before the baseline visit. Patients must have measurable or nonmeasurable disease per Response Evaluation Criteria in Solid Tumors (RECIST) and acceptable hematologic, renal, and hepatic end-organ function. Patients must have recovered from all prior anticancer therapies, including radiotherapy and major surgery.

Patients with symptomatic or uncontrolled central nervous metastases were excluded, as were those with a history of another primary malignancy ≤ 5 years, with the exception of nonmelanoma skin cancer or cervical cancer in situ. Prior exposure to tumor VDAs or other antiangiogenic agents was not allowed. Patients with uncontrolled hypertension (systolic blood pressure [BP] > 160 mmHg and/or diastolic BP > 90 mmHg), hemoptysis (> 1 teaspoon in a single episode within 4 weeks), or concurrent severe and/or uncontrolled medical, neurologic, or psychiatric disease were excluded. Because of the uncertain effects of protocol therapy on the developing fetus or nursing infant, pregnant or breast feeding females were excluded. Patients with pre-existing QT prolongation or relevant cardiac rhythm disorders at baseline were also excluded.

The study protocol was approved by the independent ethics committee or institutional review board of all participating study centers, and all patients gave written informed consent before any study-related procedures were performed. A list of all participating investigators and their countries of origin is provided in Appendix Table A1 (online only).

Study Design and Treatment Schedule

Patients received a 3-hour intravenous infusion of paclitaxel every 3 weeks. To be consistent with contemporary studies of paclitaxel-based therapy in NSCLC, paclitaxel dose was set at 200 mg/m^2 instead of 175 mg/m^2. Paclitaxel was followed by a 30- to 60-minute infusion of carboplatin AUC 6.0 on day 1. Calvert's formula using AUC and calculated glomerular filtration rate (Cockroft and Gault formula) was used to determine carboplatin dose. Patients also received an intravenous infusion of ASA404 1800 mg/m^2 or matched placebo (both with identical amber colored cover and tubing for ASA404 light sensitivity) over 20 minutes after the administration of chemotherapy on day 1. Any dose reduction or dose delay in chemotherapy was based on the severity of a related toxicity, as graded by National Cancer Institute Common Toxicity Criteria for Adverse Events version 3.0. Patients requiring a delay in study treatment for longer than 3 weeks or who had more than two dose reductions were discontinued from study treatment.

Study treatment was to be administered for 6 treatment cycles. Patients who completed the 6 cycles of study treatment without progressive disease (PD) continued to receive blinded study drug, either ASA404 1,800 mg/m^2 or placebo, as maintenance treatment until progression. Patients who discontinued study treatment before completing all 6 cycles were not eligible to continue on maintenance treatment but were observed until documented PD and then for survival.

Tumor response was evaluated according to the RECIST using computed tomography scans (or magnetic resonance imaging) with contrast of the chest and abdomen. All the patients were assessed radiographically every 6 weeks ± 3 days from the date of random assignment until PD. Patients who discontinued study treatment for reasons other than documented PD continued to have tumor assessments every 6 weeks until documented PD. All patients were followed every 6 weeks for survival following treatment discontinuation, or documented PD until either death or the data cutoff date was reached.

The European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire C30 questionnaire was used to evaluate the patient's symptoms, function, and quality of life. Questionnaires were administered before patients being assessed for response or informed about their disease status. Questionnaires were completed by the patient on day 1 of each odd cycle and at the end of treatment visit. Safety assessments consisted of monitoring and recording all AEs and serious AEs, with their severity and relationship to study drug, and regular monitoring of hematology, blood chemistry, urine, EKGs, vital signs, physical condition, and body weight.

Statistical Analysis

Random assignment was stratified by sex (male *v* female) and histology (squamous *v* nonsquamous). Institutional balancing was used to ensure that approximately the same numbers of patients were assigned to each treatment arm within the center. Sample size calculation was based on a two-look group sequential design with an overall type I error of $\alpha = .025$ (one sided) and a study power $1 - \beta = 90\%$ using the log-rank test. Assuming an hazard ratio (HR) of 0.80 (corresponding to a median OS of 9 months for the placebo plus carboplatin/paclitaxel arm and 11.25 months for the ASA404 plus carboplatin/paclitaxel arm), a 1:1 random assignment to ASA404 versus placebo and a preplanned interim analysis with 25% of the total number of deaths, a total of 950 deaths were required in the final analysis of OS. Assuming a recruitment time of 18 months and an additional follow-up of approximately 15 months, 1,200 patients were required. One interim analysis of OS was planned after the occurrence of 238 deaths (25% of the total deaths). The trial was to be stopped for futility if an observed HR (ASA404 *v* placebo) was greater than 0.9985, where a HR of lower than 1 meant better survival in the experimental arm than in the control arm. At the preplanned interim analysis conducted in March 2010, and the independent data safety monitoring committee recommended stopping the trial for futility.

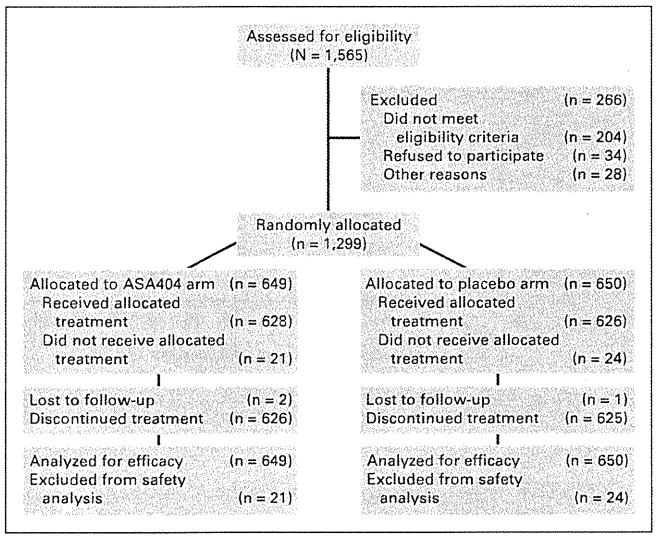

Fig 1. CONSORT diagram.

RESULTS

Patients

From April 2008 to October 2009, 1,299 patients were randomly assigned, 649 to the ASA404 arm and 650 to the placebo arm. Figure 1 summarizes the disposition of patients entered into the trial. Baseline demographics and disease characteristics are summarized in Table 1. The ASA404 and placebo treatment arms were well-balanced with regard to the demographic characteristics. The median age was 61 years, and the majority of the patients were white (approximately 72%). Most patients had nonsquamous tumor histology (75%), with adenocarcinoma (approximately 67%) being the most common subtype. The vast majority of patients (91%) had stage IV disease. The time from initial diagnosis to random assignment was ≤ 6 months for 92% of patients. There were no apparent differences between the arms in the proportion and type of subsequent systemic therapies after completion of protocol treatment, as summarized in Table 2.

Efficacy

Overall survival outcomes for all randomly assigned patients are summarized in Figure 2. The median OS for the ASA404 and placebo arms was 13.4 months (95% CI, 11.4 to 16.6) and 12.7 months (95% CI, 11.3 to 14.4), respectively. There was no statistically significant difference in OS between the two treatment arms, HR of 1.01 (95% CI, 0.85 to 1.19; one-sided $P = .535$). There were also no differences in OS between the two treatment arms with regards the primary stratification factors of histology and sex. Specifically, HRs for OS for the strata were as follows: patients with nonsquamous NSCLC (HR, 0.98; 95% CI, 0.80 to 1.19); squamous NSCLC patients (HR, 1.10; 95% CI, 0.79 to 1.52); male patients (HR, 1.02; 95% CI, 0.83 to 1.25); and female patients (HR, 0.98; 95% CI, 0.72 to 1.34).

Table 1. Patient Demographics and Disease Characteristics

Demographic or Characteristic	ASA404 + Carboplatin/Paclitaxel		Placebo + Carboplatin/Paclitaxel		All Patients	
	No.	%	No.	%	No.	%
No. of patients	649		650		1,299	
Age, years						
Median	62		61		61	
Range	29-87		23-85		23-87	
Sex						
Male	403	62.1	405	62.3	808	62.2
Race						
White	464	71.5	465	71.5	929	71.5
Asian	162	25.0	164	25.2	326	25.1
Other	23	3.5	21	3.3	44	3.4
Performance status						
0	266	41.0	258	39.7	524	40.3
1	381	58.7	384	59.1	765	58.9
Missing	2	0.3	8	1.2	10	0.8
Histology						
Squamous	132	20.3	133	20.5	265	20.4
Nonsquamous	494	76.1	484	74.5	978	75.3
Adenocarcinoma	432	66.5	436	67.1	868	66.8
Undifferentiated carcinoma	24	3.7	20	3.1	44	3.4
Adenosquamous cell carcinoma	7	1.1	4	0.6	11	0.8
Large-cell carcinoma	29	4.5	23	3.5	52	4.0
Other (mixed carcinoma or missing)	25	3.8	34	5.3	59	4.5
Stage						
IIIB	53	8.2	56	8.6	109	8.4
IV	596	91.8	591	90.9	1,187	91.4
Missing	0	0.0	3	0.5	3	0.2

Table 2. Systemic Antineoplastic Therapies Since Discontinuation of Study Treatment

Line of Treatment	ASA404 + Carboplatin/ Paclitaxel		Placebo + Carboplatin/ Paclitaxel	
	No.	%	No.	%
No. of patients	649		650	
Second line				
Any	364	56.1	368	56.6
Pemetrexed	115	17.7	113	17.4
Erlotinib	70	10.8	75	11.5
Carboplatin	64	9.9	56	8.6
Paclitaxel	42	6.5	41	6.3
Cisplatin	36	5.5	25	3.8
Docetaxel	35	5.4	44	6.8
Gemcitabine	34	5.2	38	5.9
Gefitinib	26	4.0	27	4.2
Investigational drug	22	3.4	28	4.3
Vinorelbine	20	3.1	16	2.5
Bevacizumab	9	1.4	9	1.4
Paclitaxel with carboplatin	4	0.6	2	0.3
Other cytotoxic chemotherapy	5	0.5	9	1.4
Other biologics	3	0.5	1	0.2
Third line				
Any	99	15.3	106	16.3
Erlotinib	35	5.4	31	4.8
Pemetrexed	28	4.3	29	4.5
Vinorelbine	9	1.4	7	1.1
Gemcitabine	7	1.1	5	0.8
Cisplatin	6	0.9	5	0.8
Docetaxel	6	0.9	16	2.5
Gemcitabine	6	0.9	2	0.3
Investigational drug	5	0.8	2	0.3
Bevacizumab	4	0.6	3	0.5
Carboplatin	3	0.5	1	0.2
Gefitinib	2	0.3	6	0.9
Other cytotoxic chemotherapy	5	0.8	6	0.9
Other biologics (cetuximab)	1	0.2	0	
Fourth line				
Any	31	4.8	28	4.3
Fifth line				
Any	6	0.9	5	0.8

Progression-free survival (as assessed by investigators) for all patients is summarized in Figure 3. The estimated rates of PFS at 12 months were 6.7% and 6.9% in the ASA404 and placebo arms, respectively. The median PFS was 5.5 months (95% CI, 5.2 to 5.6) for the ASA404 arm, and 5.5 months (95% CI, 5.4 to 5.6) for the placebo arm. The two treatments arms did not show a statistically significant difference in PFS (HR, 1.04; 95% CI, 0.91 to 1.19; one-sided $P = .727$). As with the OS analysis, none of the prespecified strata demonstrated any significant differences in PFS between the treatment arms (data not shown).

Overall response rate as per RECIST based on investigators' assessment demonstrated complete response (CR) in 2 (0.3%) and 3 (0.5%) patients and partial response (PR) in 158 (24.3%) and 157 (24.2%) in the ASA404 and placebo arms, respectively. Disease stabilization (39.6% *v* 39.5%) and PD (15.7% *v* 15.8%) were observed at similar rates between the ASA404 and placebo arms, respectively. The

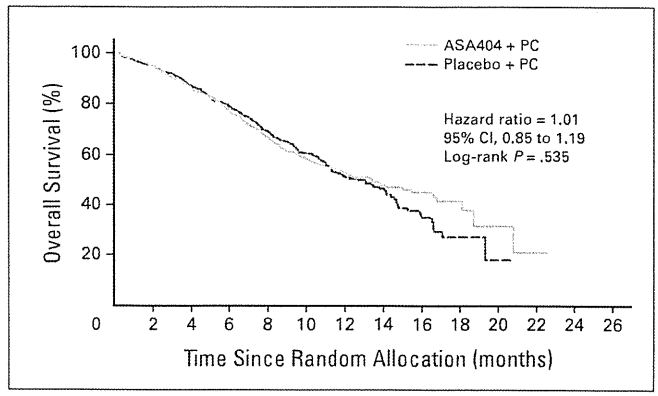

Fig 2. Kaplan-Meier curves for overall survival. PC, paclitaxel and carboplatin.

ORR (CR + PR) respectively between the ASA404 and placebo arms were 24.7% (95% CI, 21.4 to 28.2) and 24.6% (95% CI, 21.3 to 28.1).

Safety and Tolerability

The median number of cycles delivered for the combination treatment was 5 (range, 1 to 6) in both treatment arms. The median number of cycles for maintenance treatment was 3 (range, 1 to 17) and four (range, 1 to 16) in the ASA404 and placebo arms, respectively. Overall, there were no major variations between the two arms in the number of patients in each treatment cycle. Similarly, dose reductions and delays were comparable between both the treatment arms. The most common reasons for dose reduction were AEs (36.9% in the ASA404 arm *v* 31% in the placebo arm) and lab test abnormalities (21.6% *v* 20.8%), whereas the most common reasons for dose delay were AEs (23.5% *v* 22.2%) and scheduling conflicts (25.6% *v* 26.1%). Median cumulative dose, median dose intensity, and median relative dose intensity were also comparable between the ASA404 and placebo treatment arms (data not shown).

The incidence of AEs was similar between the arms and the majority of AEs were of grade 1 to 2 severity. Neutropenia, alopecia, nausea, and fatigue were the most frequently reported AEs, occurring with comparable incidence in both arms. Incidence of grade 4 neutropenia was higher in the ASA404 arm than the placebo arm (26.6% and 19%, respectively). Infusion site pain was also reported at a higher incidence in the ASA404 arm compared with the placebo arm (10.5%

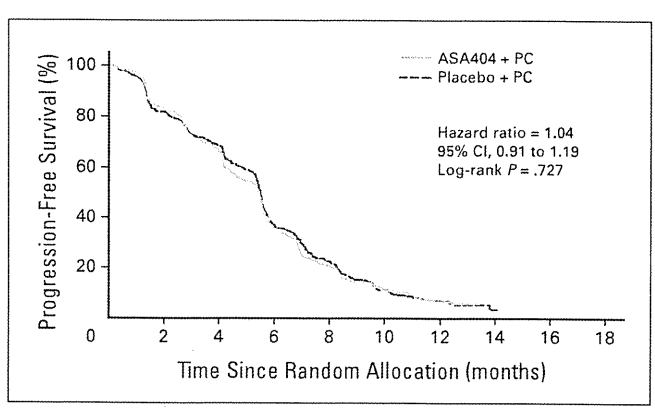

Fig 3. Kaplan-Meier curves for progression-free survival (investigator assessment). PC, paclitaxel and carboplatin.

Table 3. Adverse Events, Regardless of Study Drug Relationship, With at Least 10% Incidence of Any Grade Events in Either Arm by Preferred Term, Maximum Grade, and Treatment

	Treatment by Grade											
	ASA404 + PC (n = 629)						Placebo + PC (n = 62)					
	All		3		4		All		3		4	
Event	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Neutropenia	357	56.8	158	25.1	167	26.6	317	50.7	148	23.7	119	19.0
Alopecia	297	47.2	10	1.6	3	0.5	303	48.5	6	1.0	0	0.0
Nausea	250	39.7	14	2.2	1	0.2	251	40.2	13	2.1	0	0.0
Fatigue	224	35.6	22	3.5	1	0.2	219	35.0	21	3.4	0	0.0
Decreased appetite	195	31.0	13	2.1	0	0.0	166	26.6	12	1.9	0	0.0
Constipation	166	26.4	7	1.1	1	0.2	161	25.8	5	0.8	0	0.0
Anemia	155	24.6	29	4.6	5	0.8	156	25.0	28	4.5	2	0.3
Diarrhea	154	24.5	15	2.4	1	0.2	128	20.5	6	1.0	1	0.2
Arthralgia	153	24.3	7	1.1	0	0.0	146	23.4	13	2.1	2	0.3
Dyspnea	131	20.8	32	5.1	5	0.8	131	21.0	26	4.2	4	0.6
Myalgia	131	20.8	5	0.8	0	0.0	12	20.3	10	1.6	0	0.0
Vomiting	131	20.8	9	1.4	0	0.0	146	23.4	13	2.1	0	0.0
Peripheral neuropathy	115	18.3	8	1.3	2	0.3	124	19.8	10	1.6	1	0.2
Cough	104	16.5	11	1.7	0	0.0	106	17.0	7	1.1	0	0.0
Peripheral sensory neuropathy	100	15.9	6	1.0	1	0.2	99	15.8	8	1.3	1	0.2
Pain in extremity	93	14.8	9	1.4	1	0.2	69	11.0	4	0.6	1	0.2
Dizziness	92	14.6	1	0.2	0	0.0	67	10.7	0	0.0	0	0.0
Leucopenia	91	14.5	40	6.4	2	0.3	74	11.8	21	3.4	3	0.5
Insomnia	86	13.7	2	0.3	0	0.0	99	15.8	3	0.5	0	0.0
Pyrexia	86	13.7	1	0.2	0	0.0	92	14.7	5	0.8	0	0.0
Thrombocytopenia	85	13.5	22	3.5	6	1.0	84	13.4	22	3.5	4	0.6
Asthenia	83	13.2	8	1.3	0	0.0	76	12.2	6	1.0	1	0.2
Rash	76	12.1	0	0.0	0	0.0	79	12.6	2	0.3	0	0.0
Infusion site pain	66	10.5	3	0.5	0	0.0	7	1.1	0	0.0	0	0.0
Back pain	61	9.7	14	2.2	0	0.0	70	11.2	13	2.1	2	0.3
Paresthesia	60	9.5	3	0.5	0	0.0	69	11.0	2	0.3	0	0.0
Noncardiac chest pain	48	7.6	5	0.8	1	0.2	74	11.8	11	1.8	2	0.3

NOTE. Preferred terms are sorted by descending frequency of all grades in the ASA404 + PC arm. Adverse events occurring more than 28 days after last date of study treatment are not summarized.
Abbreviation: PC, paclitaxel and carboplatin.

and 1.1%, respectively). The other AEs reported with a slightly higher incidence in the ASA404 arm compared to placebo were dysgeusia, visual impairment, decreased appetite, pain in extremity, and dizziness. There was no overt increase in AEs relevant to VDAs, such as hemoptysis or cardiac toxicity. For example, hemoptysis (all grades) was observed in 6.4% in the ASA404 arm versus 6.2% in the placebo arm. Only one patient in each arm had grade 4 hemoptysis. A summary of AEs by treatment arm is presented in Table 3.

Overall, a similar number of on-treatment deaths were reported between the ASA404 and placebo treatment arms (28 patients and 25 patients, respectively). Three deaths were considered to be related to the study drug, one in the ASA404 arm (myocardial infarction) and two in the placebo arm (cerebrovascular accident in one and unknown in the other). There was no clustering of any specific type of events leading to death in any treatment arm. Notably, there was no evidence for enhanced vascular toxicities, such as bleeding or thrombosis with ASA404, even in the squamous cell cancer subset, in contrast to that seen with angiogenesis inhibitors such as bevacizumab.

Quality of Life

Summary of the changes in patient reported outcome scores assessed using the European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire C30 questionnaire by time point and treatment are presented in the Appendix Table A2 (online only). There was a decrease in the physical functioning domain across both treatment arms at the end of treatment. However, for the global health status/quality of life domain there was no change observed between the treatment arms over time.

DISCUSSION

This large randomized trial failed to demonstrate any efficacy advantage to the addition of the tumor VDA ASA404 to standard platinum-based chemotherapy for the first-line treatment of advanced NSCLC. As a result, further clinical development of this agent has been halted. This trial thus joins a long list of many like-designed negative studies that have tested the paradigm of chemotherapy with or without a novel targeted agent. Of the dozens of failed randomized phase III trials that employed this strategy in the recent past, only trials of bevacizumab plus carboplatin/paclitaxel (Eastern Cooperative Oncology Group trial 4599)[12] and arguably, cetuximab plus cisplatin/vinorelbine,[13] have yielded improvements in OS, albeit modest, in favor of the experimental arm. The ATTRACT-1 trial has now clearly demonstrated that the purported synergistic vascular disrupting activity of

ASA404 was insufficient to improve any of the efficacy measures in unselected patients. This was in contrast to the trends for improvement in efficacy variables of the preceding randomized phase II trial.

Why was there a disconnect between the encouraging results of the randomized phase II trial of carboplatin/paclitaxel with or without ASA404 and the negative results of this subsequent randomized phase III trial? The most likely explanation is that the smaller sample size of the phase II trial simply overestimated the treatment effect; the small number of events wrongly influenced the shape of the survival curves in favor of the experimental arm. This so-called random high yielded a false-positive signal that could only have been refuted by a larger clinical trial such as ATTRACT-1.[14] The lack of a placebo control and investigator/patient blinding in the preceding phase II trial may also have introduced biases that favored the experimental arm.

Surprisingly, the control arm of this phase III trial yielded a median survival time of 12.7 months, well above the a priori assumption of 9 months that was used in the ATTRACT-1 sample size and power calculations. In fact, the numerical median OS achieved in the control arm of this trial is higher than the median OS achieved with the bevacizumab plus carboplatin/paclitaxel regimen in the pivotal ECOG 4599 trial, which was 12.3 months.[12] The reason for this temporal upward drift in OS is unclear, but may be related to stage migration, the higher accrued proportion of Asian patients (25%) who typically have better outcomes compared to Western populations, and/or overall improvements in subsequent therapies for advanced NSCLC beyond initial platinum-based therapy.[15,16] For example, in Eastern Cooperative Oncology Group trial 4599, subsequent therapy was reported in 46% of patients in the bevacizumab arm and in 43% in the control arm, contrasting with the 56% rate in both arms of the current study. It must also be noted that a slightly higher dose of paclitaxel was used in ATTRACT 1 (200 mg/m^2) as compared with the predecessor phase II study where 175 mg/m^2 was used. Whether this change contributed to the higher than expected OS in the control arm is uncertain. Nevertheless, the a priori assumptions of the ATTRACT 1 trial may have confounded the expectations of benefit in both treatment arms.

It is notable that clinical evaluation of nonflavonoid (ie, tubulin directed) VDAs are still in progress. These agents include fosbretabulin, ABT-751, and NPI-2358, among others.[17] Interestingly, preliminary results of a randomized phase II trial of carboplatin, paclitaxel, and bevacizumab with or without fosbretabulin in advanced NSCLC demonstated enhanced OS in the fosbretabulin-containing arm.[18] However, it remains to be seen whether tubulin-directed VDAs in combination with chemotherapy and/or angiogenesis inhibitors will improve outcomes in the phase III context.

Finally, it is worth emphasizing that the precise molecular target of ASA404 remains elusive. This lack of understanding of the basic mechanisms of ASA404 drug action have hampered a more defined and ideal approach to clinical trial design wherein only patients with a high likelihood of benefiting from VDA therapy, as identified by some putative biomarker, are selectively accrued to a phase III randomized experiment. Molecular correlative studies on tumor and blood specimens collected from patients in this trial are ongoing and will be reported in a separate publication. If further development of this class of agents were to prosper, identification and validation of predictive biomarkers for VDA benefit are warranted.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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Duration of prior gefitinib treatment predicts survival potential in patients with lung adenocarcinoma receiving subsequent erlotinib

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ABSTRACT

Purpose: We investigated survival potential in patients receiving erlotinib after failure of gefitinib, focusing on response and time to progression (TTP) with gefitinib.
Methods: We retrospectively reviewed lung adenocarcinoma patients who received erlotinib after experiencing progression with gefitinib. Our primary objective was to evaluate the prognostic significance of erlotinib therapy.
Results: A total 42 lung adenocarcinoma patients were included in this study. Overall disease control rate was 59.5% (partial response [PR], 2.4%; stable disease [SD], 57.1%). Median overall survival was 7.1 months, and median progression-free survival was 3.4 months. The number of patients who achieved PR and non-PR (SD+ progressive disease [PD]) with gefitinib were 22 (52%) and 20 (48%), respectively. Patients with PR for gefitinib showed significantly longer survival times than those with non-PR (9.2 vs. 4.7 months; $p = 0.014$). In particular, among PR patients, those with TTP <12 months on gefitinib showed significantly longer survival times than those with TTP ≥12 months (10.3 vs. 6.4 months; $p = 0.04$).
Conclusions: Erlotinib may exert survival benefit for lung adenocarcinoma patients with less than 12 months of TTP of prior gefitinib who achieved PR for gefitinib.

1. Introduction

Gefitinib and erlotinib are oral epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs). Gefitinib has been reported to be effective in limited populations such as never smokers, Asians, and patients with adenocarcinoma, and is particularly effective in patients with EGFR mutations [1–3]. Erlotinib, which has a similar quinazoline frame to gefitinib, is the first EGFR-TKI shown to provide survival benefit in patients with non-small cell lung cancer (NSCLC) [4]: the BR.21 trial revealed significantly longer survival times among patients who received erlotinib compared with a placebo group [4]. In addition, these two EGFR-TKIs have been found to occasionally induce a particularly significant response in EGFR-mutant patients. However, despite this documented efficacy, most cancer clones acquire resistance to these particular compounds over time [5].

Previous studies have demonstrated that amplified MET oncogene and secondary EGFR T790M mutations are most commonly responsible for resistance to gefitinib and erlotinib [6,7]. Indeed, several previous studies showed that secondary EGFR T790M mutation and MET amplification occurred in nearly half and 20% of lung cancer specimens that had become resistant to EGFR-TKIs, respectively [8–11]. In addition, the majority of patients who showed secondary resistance had EGFR mutations such as exon 19 deletion mutations or L858R point mutation, which have been found to be sensitive to EGFR-TKIs [12,13].

Several reports have demonstrated clinical benefits when administering erlotinib to NSCLC patients following failure of gefitinib [14–18]; in contrast, one previous report has suggested that no erlotinib-derived clinical benefit can be expected in patients who failed gefitinib [19]. However, reports thus far have all had small sample sizes, and clear findings regarding efficacy of erlotinib in patients who failed gefitinib have yet to be obtained. Consequently, whether or not erlotinib is useful in these patients remains controversial.

We hypothesize that tumor clones may require exposure to gefitinib treatment with a positive response for a specific duration to acquire secondary common resistance to EGFR-TKIs. Even if a patient experiences tumor progression on gefitinib therapy, subsequent erlotinib therapy may nevertheless still be able to inhibit progression, provided the tumor clones did not acquire secondary resistance. As such, in positive-responder patients with confirmed progression within a specific duration of gefitinib treatment, some tumor clones may remain sensitive to erlotinib, and therefore these patients may still experience survival benefit with erlotinib treatment.

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Here, we conducted a retrospective study primarily aimed at assessing overall survival (OS) of patients who received erlotinib therapy after failure with gefitinib. We also attempted to characterize the clinical features of patients who benefited from erlotinib treatment.

2. Patients and methods

We retrospectively reviewed records for patients with histopathologically diagnosed lung adenocarcinoma who received erlotinib after experiencing progression on gefitinib at Kinki-chuo Chest Medical Center between December 2008 and October 2009. Responses were evaluated based on patient records and radiographic studies, such as chest roentgenograms and computed tomographic (CT) and magnetic resonance imaging (MRI) scans. We examined EGFR mutation status using the PCR-invader method with paraffin sections of biopsy specimens from patients.

Time to progression (TTP) with gefitinib was defined as the period from initiation of gefitinib therapy to the date when disease progression was confirmed. Overall survival was defined as the period from initiation of erlotinib therapy to the date of death or last follow-up. Disease control rate (DCR) was defined as complete response (CR) plus partial response (PR) plus stable disease (SD). Evaluation of response to gefitinib and erlotinib therapy by CT scan was performed according to the response evaluation criteria in solid tumors (RECIST). Stable disease plus progressive disease (PD) with prior gefitinib treatment was defined as "non-PR."

Categorical outcomes, including DCRs, were compared using the χ^2 test, and survival distribution was estimating using the Kaplan–Meier method. Overall survival and progression-free survival (PFS) were compared with regard to demographic factors such as gender, performance status, EGFR mutation status, response to gefitinib, TTP with gefitinib, and toxicity grade of skin rash, which may be associated with survival, using the log-rank test. Values were considered statistically significant for $p < 0.05$. A multivariate Cox-proportional-hazards model was used to determine the clinical variables which influenced OS. Statistical analyses were carried out using SPSS software ver. 11.0 for Windows (IBM, Chicago, IL, USA).

3. Results

3.1. Patient characteristics

Forty-two patients with lung adenocarcinoma were reviewed in the present study. All patients became refractory to gefitinib during the course of treatment and were subsequently switched to erlotinib therapy. Patient characteristics are described in detail in Table 1. Thirty patients (71%) had received 1 or 2 regimens before initiation of erlotinib and 2 (7%) had received gefitinib as first-line treatment.

Table 1
Patient characteristics.

	Number (%)
Median age, years (range)	65 (31–85)
Sex	
Male	13 (31)
Female	29 (69)
Smoking history	
Never	28 (67)
Former/current	14 (33)
ECOG score	
0–1	24 (57)
2–4	18 (43)
Cancer stage	
IIIB	8 (19)
IV	34 (81)
Number of previous treatments with erlotinib	
1–2	30[a] (71)
3≤	12 (29)
EGFR mutation	
Exon 19 deletion mutation	14 (33)
L858R	14 (33)
Exon 18 point mutation	1 (2)
Wild	13 (32)
TTP with gefitinib treatment, months (range)	8.1 (0.9–40.7)
<12	29 (69)
≤12	13 (31)
Response to gefitinib	
CR	0 (0)
PR	22 (53)
SD	17 (40)
PD	3 (7)

EGFR, epidermal growth factor receptor; TTP, time to progression; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; ECOG, Eastern Cooperative Oncology Group.
[a] Two patients received gefitinib as first-line treatment.

EGFR mutations were detected in 29 (69%) patients: 14 (33%) had exon 19 deletions, 14 (33%) had L858R mutations, and 1 (2%) had an exon 18 point mutation. The median TTP with gefitinib treatment was 8.1 months. Thirteen (31%) patients had TTPs of 12 months or more, while 29 (69%) had TTPs of less than 12 months. Twenty-two (53%) patients receiving gefitinib achieved PR, and 17 (40%) achieved SD. None achieved CR while receiving gefitinib therapy. The response rate (RR) and DCR for gefitinib were 53% (22 of 42 patients) and 93% (39 of 42 patients), respectively. Of the 22 patients who achieved PR with gefitinib, 19 (86%) were found to have EGFR mutations. Of the 20 patients who had SD or PD (non-PR)

Table 2
Response to erlotinib according to the response to prior gefitinib and EGFR mutation status.

EGFR mutation		Response to gefitinib					
		PR ($n=22$)		Non-PR[a] ($n=20$)			
				SD ($n=17$)		PD ($n=3$)	
		Positive, n (%)	Negative[b], n (%)	Positive, n (%)	Negative, n (%)	Positive, n (%)	Negative, n (%)
Response to erlotinib	PR ($N=1$)	1 (4.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	SD ($N=24$)	14 (64)	1 (4.5)	3 (18)	5 (29)	0 (0)	1 (33)
	PD ($N=17$)	4 (18)	2 (9)	6 (35)	3 (18)	1 (33)	1 (33)

EGFR, epidermal growth factor receptor; PR, partial response; SD, stable disease; PD, progressive disease. Overall disease control rate (PR + SD) was 73% (EGFR mutation-positive: 15/22 [68%], EGFR mutation-negative: 1/22 [5%]) among patients who achieved PR with gefitinib and 45% (EGFR mutation-positive: 3/20 [15%], EGFR mutation-negative: 6/20 [30%]) among patients with non-PR (SD + PD for gefitinib) with gefitinib treatment. Overall disease control rate was 62% (PR for gefitinib: 15/29 [52%], non-PR for gefitinib: 3/29 [10%]) in EGFR mutation-positive patients and 54% (PR for gefitinib: 1/13 [8%], non-PR for gefitinib: 6/13 [46%]) in EGFR mutation-negative patients.
[a] Defined as SD plus PD with prior gefitinib therapy.
[b] EGFR wild-type.

Table 3
Response to erlotinib stratified by TTP with prior gefitinib treatment.

(A) TTP with gefitinib <12 months				
Response to gefitinib		TTP with gefitinib (months) <12 (n = 29)		
		PR (n = 11) n (%)	Non-PR (n = 18)	
			SD (n = 15) n (%)	PD (n = 3) n (%)
Response to erlotinib	PR (n = 1)	1[b] (9)	0 (0)	0 (0)
	SD (n = 17)	8[a,b] (73)	8[a] (44)	1 (6)
	PD (n = 11)	2 (18)	7 (39)	2 (11)
B. TTP with gefitinib ≥ 12 months				
Response to gefitinib		TTP with gefitinib (months) ≥12 (n = 13)		
		PR (n = 11) n (%)	Non-PR (n = 2)	
			SD (N = 2) n (%)	PD (N = 0) n (%)
Response to erlotinib	PR (n = 0)	0 (0)	0 (0)	0 (0)
	SD (n = 7)	7 (64)	0 (0)	0 (0)
	PD (n = 6)	4 (36)	2 (100)	0 (0)

EGFR, epidermal growth factor receptor; PR, partial response; SD, stable disease; PD, progressive disease; TTP, time to progression. Overall disease control rate was 62% (PR for gefitinib: 9/29 [31%], non-PR for gefitinib: 9/29 [31%]) in patients with TTP of gefitinib <12 months. Overall disease control rate was 54% (PR for gefitinib: 7/13 [54%], non-PR for gefitinib: 0/13 [0%]) in patients with TTP of gefitinib ≥12 months.

[a] Ten patients showed improvement of target lesions, but not to PR standards. Seven and three patients achieved PR and SD, respectively, with gefitinib treatment.

[b] A second biopsy from progression lesions was performed in three patients (one had PR and 2 had SD with erlotinib) who achieved PR with gefitinib. Exon 19 deletion mutations which were the same pattern as detected in first biopsy specimen for primary diagnosis of NSCLC were identified, whereas EGFR T790M mutation, which endowed secondary common resistance to EGFR-TKIs, was not identified in those biopsy specimens.

with gefitinib, EGFR mutations were detected in 10 (50%). Among patients with EGFR mutations, only one showed PD with gefitinib therapy, and RR and DCR in this group were 66% (19 of 29) and 97% (28 of 29), respectively.

3.2. Response

On erlotinib therapy, 1 of 42 patients achieved PR, and 24 had SD. No patients achieved CR with erlotinib. Overall RR and DCR for erlotinib were 2.4% (one of 42) and 59.5% (25 of 42), respectively.

Response to erlotinib categorized by response to prior gefitinib duration and EGFR mutation status is described in Table 2. Among patients who achieved PR with gefitinib, one achieved PR and 15 patients achieved SD with erlotinib therapy. Patients who achieved PR with gefitinib showed higher DCRs with erlotinib than patients who had non-PR with gefitinib (16 [73%] of 22 vs. 9 [45%] of 20), albeit without statistical significance ($p = 0.07$). In addition, EGFR mutation status was not found to be associated with response to erlotinib; in terms of DCR, no significant difference was noted between EGFR-mutant patients (18/29) and EGFR non-mutant patients group (7/13) (62% vs. 54%, $p = 0.616$).

Time to progression with prior administration of gefitinib was not found to be associated with achieving a response with subsequent erlotinib. Details regarding response to erlotinib categorized by TTP with gefitinib are shown in Table 3. DCR among patients experiencing progression after less than 12 months of gefitinib therapy was 18/29 (62%). In contrast, DCR among patients with TTPs of 12 months or more was 7/13 (54%). No statistical significant difference in DCR was noted between these two groups according to TTP with prior administration of gefitinib ($p = 0.62$). Of the 24 patients who achieved SD with erlotinib therapy, 10 showed improvement in target lesions which had been exacerbated during gefitinib treatment; all 10 were EGFR-mutant patients (4 L858R, 5 exon 19 deletion mutations, and 1 exon 18 point mutation), and TTPs with gefitinib were all less than 12 months. Of the two patients who received gefitinib as first-line treatment, one had an EGFR L858R mutation and showed responses to gefitinib and subsequent erlotinib of PR and SD, respectively. While this particular patient showed a relatively long TTP (39.5 months) with gefitinib, disease progression was confirmed 4 months after initiation of erlotinib therapy, and OS was 58.6 months. The other patient who received gefitinib as first-line treatment had EGFR-wild type, and responses to both gefitinib and subsequent erlotinib treatment were PD. TTP and OS in this patient were 3 and 7.4 months, respectively.

A second biopsy of the progressed lesions was performed in three patients after gefitinib therapy failed. While exon 19 deletion mutations of the same pattern as noted in the first biopsy specimen for primary diagnosis were also detected on this second biopsy, we noted no EGFR T790M mutations. Of note, however, was the fact that imaging findings for lesions after erlotinib therapy were improved on the second biopsy (Table 3).

3.3. Survival

Median OS and median progression-free survival (PFS) were 7.1 months (95% confidence interval [CI]: 4.4–9.8 months) and 3.4 months (95% CI: 1.1–5.7 months), respectively (Fig. 1). Multivariate analysis of prognostic factors was performed using a Cox proportional hazards model to determine which clinical variables were most strongly associated with OS (Table 4). Response to gefitinib

Table 4
Multivariate analysis of prognostic variables for OS by use of a Cox proportional-hazards model.

	Multivariate analysis		
	p[a]	Hazard ratio	95% CI
Sex	0.51	1.35	0.55–3.31
ECOG score	0.19	0.58	0.25–1.31
EGFR mutation	0.78	1.13	0.48–2.70
Response to gefitinib	0.005	0.23	0.80–0.64
TTP of gefitinib	0.05	0.34	0.12–1.01
Grade of skin rash	0.29	0.64	0.27–1.47

EGFR, epidermal growth factor receptor; TTP, time to progression; CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; PR, partial response. Response to gefitinib was the only independent prognostic factor. TTP with gefitinib showed borderline significance. Variables were compared as paired categories: sex (female vs. male), ECOG score (0–1 vs. 2–4), response to gefitinib (PR vs. non-PR), TTP of gefitinib (<12 months vs. ≥12 months), grade of skin rash (3 vs. 1–2).

[a] $p < 0.05$ was considered significant.

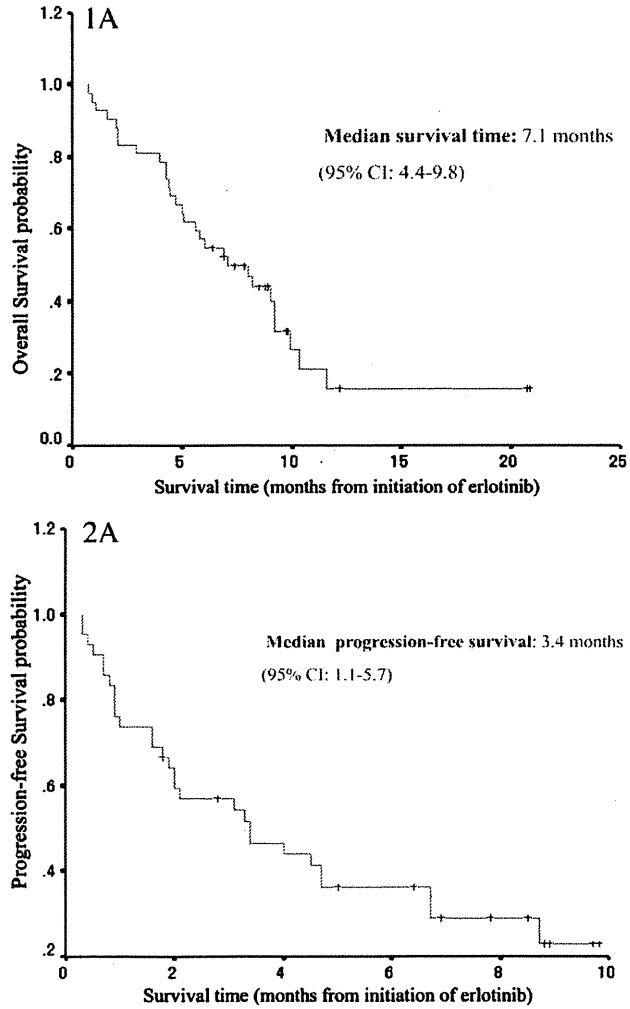

Fig. 1. Kaplan–Meier plot of survival time with erlotinib. (A) Overall survival rates and (B) progression-free survival rates of 42 patients. MST: median survival time; mPFS: median progression-free survival.

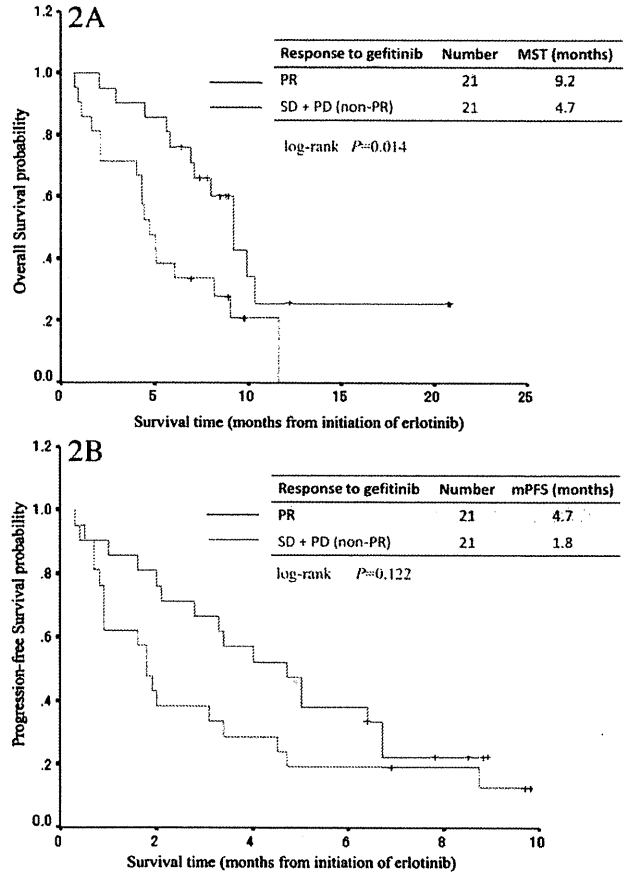

Fig. 2. Kaplan–Meier plot of survival time with erlotinib. (A) Overall survival rates and (B) progression-free survival rates stratified by response to prior gefitinib. Non-PR is defined as SD plus PD with gefitinib therapy.

was found to be the only independent prognostic factor (hazard ratio = 0.23; 95% CI: 0.08–0.64, $p = 0.005$), and time to progression with gefitinib showed borderline significance (hazard ratio = 0.34; 95% CI: 0.12–1.01, $p = 0.05$).

Kaplan–Meier curves of survival time according to response to prior gefitinib therapy are shown in Fig. 2. Patients who achieved PR while receiving gefitinib therapy showed significantly longer OS ($p = 0.014$). However, no significant difference was noted in PFS between patients with PR for gefitinib and those with non-PR (4.7 months [95% CI: 2.9–6.5 months] vs. 1.8 months [95% CI: 1.4–2.2 months]; $p = 0.122$). Time to progression with gefitinib showed a borderline significant impact on survival with erlotinib therapy. However, among patients who achieved PR with gefitinib, TTP with gefitinib therapy was strongly correlated with survival time. Kaplan–Meier curves of survival time for patients who achieved PR with gefitinib stratified according to TTP are shown in Fig. 3. Patients with TTPs of less than 12 months with gefitinib therapy were found to have significantly longer OS (10.3 months [95% CI: 7.0–13.6 months] vs. 6.4 months [95% CI: 2.6–10.2 months]; $p = 0.04$) and longer PFS (6.4 months [95% CI: 3.6–9.2 months] vs. 3.4 months [95% CI: 1.2–5.6 months]; $p = 0.19$) than patients with TTPs of 12 months or more. However, no statistically significant difference was noted between the two groups in terms of PFS ($p = 0.19$).

In addition, we found that skin rash was not predictive of survival with erlotinib therapy. All patients in the present study were affected by rash of some grade while receiving erlotinib. The degree of skin rash toxicity due to erlotinib exceeded the grade noted during gefitinib treatment in 32 patients. Seven patients required dose reduction of erlotinib due to grade 3 skin rash. Using a Cox proportional hazard model, we determined that skin rash grade had no impact on survival (hazard ratio = 0.64 [95% CI: 0.27–1.47]; $p = 0.29$).

4. Discussion

Here, we investigated survival potential in patients receiving erlotinib after failure of gefitinib, focusing on response and TTP with gefitinib. Our findings suggest that administration of erlotinib subsequent to gefitinib may exert survival benefit in former gefitinib-positive responders. Further, among those former responders, most with TTP <12 months may not yet have secondary resistance to EGFR-TKIs. Our findings suggest little chance for patients to achieve a high response with erlotinib therapy after experiencing progression with gefitinib therapy. This observation may be due to these two EGFR TKIs sharing the same mechanism of EGFR blockade or to cross resistance [5].

Our retrospective study showed that response achieved with prior administration of gefitinib was the only prognostic factor for subsequent erlotinib therapy after experiencing progression on gefitinib therapy. In particular, among patients who achieved PR with gefitinib, patients with TTPs of less than 12 months with gefitinib therapy were found to have significantly longer OS than

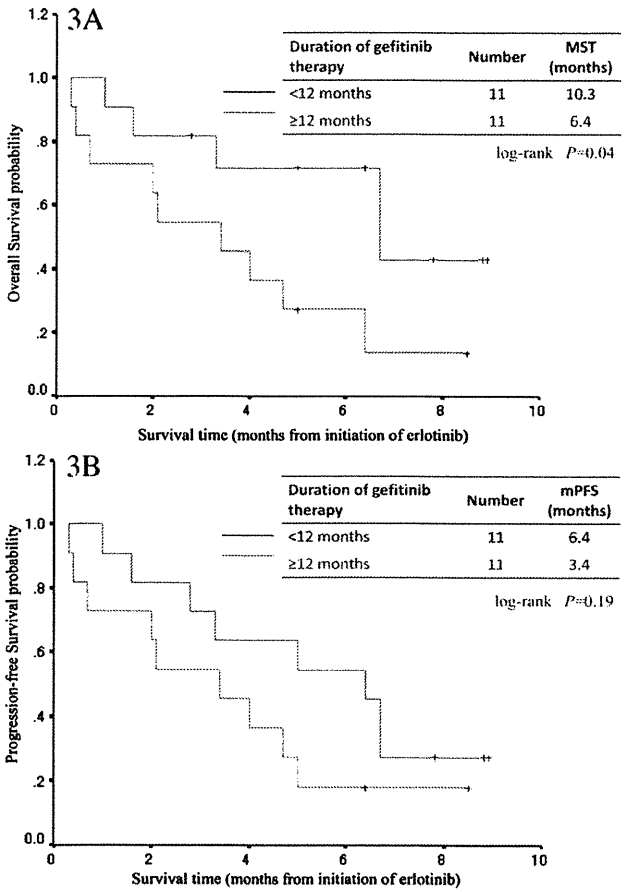

Fig. 3. Kaplan–Meier plot of survival time for patients who achieve PR with gefitinib. (A) Overall survival rates and (B) progression-free survival rates stratified by TTP with gefitinib.

patients with TTPs of 12 months or more. In addition, most of these patients showed some degree of improvement in image findings after subsequent erlotinib therapy. We noted no EGFR T790M mutations in any of three patients who underwent a second biopsy of their progressed lesions after failure with gefitinib therapy. We therefore supposed that most patients with TTP <12 months may have not yet acquired the EGFR T790M mutation. However, we only investigated the presence of a secondary EGFR T790M mutation in three patients in the present study. Validation of this hypothesis will require collection of more molecular information from patients who are no longer responsive to gefitinib in the future.

Shepherd et al. demonstrated that TTP was 2.6 months in NSCLC patients who had previously been treated with docetaxel therapy [20]. We observed that PFS was 3.4 months in patients with TTP ≥12 months who achieved PR in our study, a duration which appears improved over that demonstrated by Shepherd et al. Given these findings, we posited that, regardless of duration of gefitinib therapy, subsequent erlotinib may be able to prolong PFS compared to chemotherapy with cytotoxic agent provided the patients demonstrated a positive response with gefitinib. However, given that our results were obtained in a retrospective study with an extremely small sample population, a prospective study is warranted to clarify whether or not erlotinib administered subsequent to gefitinib can elicit greater survival benefit in gefitinib-positive responders than chemotherapy with cytotoxic agents.

We noted here that treatment with erlotinib following gefitinib resulted in more toxic grades of skin rash in patients, findings which suggest that erlotinib may have greater biological activity than gefitinib. Several other investigators have also suggested based on their own findings that erlotinib may have higher biological activity than gefitinib. Costa et al. showed that differing efficacy between gefitinib and erlotinib was due to differences in commonly administered dosages between the two drugs [21]. Gefitinib (250 mg per day) is typically administered at one third of its maximum-tolerated dose, whereas erlotinib (150 mg per day) is administered at its maximum tolerated dose. In vitro data showed that the mean concentration of gefitinib was 0.24 μg/ml at the 300-mg daily dose and 1.1 μg/ml at 1000 mg/day. In contrast, median concentration of erlotinib at 150 mg/day was 1.26 μg/ml. These previous findings suggest that erlotinib (150 mg/day) has a higher biological dose of EGFR inhibition than gefitinib (250 mg/day).

Recent studies have demonstrated that the increased biological activity of EGFR-TKIs is associated with control of tumor clones. Yoshimasu et al. reported observing a dose-response relationship between inhibition rates and gefitinib concentration [22]. Clarke et al. reported that high-dose erlotinib was effective in controlling leptomeningeal metastases progression while receiving standard erlotinib therapy in EGFR-mutant patients [23]. These authors demonstrated that a weekly 1200-mg dose of erlotinib controlled leptomeningeal metastases in a patient who was no longer responsive to a standard daily dose of erlotinib (150 mg).

Our findings here suggest that a treatment duration of 12 months of gefitinib therapy may be the borderline period for tumor clones to attain resistance to EGFR-TKIs. However, speculation as to whether or not previously EGFR-TKI-sensitive clones gradually grow resistant to EGFR-TKIs has not been resolved. Further studies are necessary to validate our findings.

In conclusion, gefitinib responders may achieve survival benefits from erlotinib therapy after experiencing progression with gefitinib. Among patients who have been receiving gefitinib therapy for less than 12 months, tumor clones may not yet have acquired a secondary mutation. However, further studies are needed to clarify precisely how tumor clones attain such secondary resistance to EGFR-TKIs.

Conflicts of interest statement

None declared.

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The usefulness of mutation-specific antibodies in detecting epidermal growth factor receptor mutations and in predicting response to tyrosine kinase inhibitor therapy in lung adenocarcinoma

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ABSTRACT

Introduction: Among the mutations of epidermal growth factor receptor (*EGFR*), deletions in exon 19 (DEL), and point mutations in exon 21 (L858R) predict the response to EGFR-tyrosine kinase inhibitors (TKIs) in primary lung adenocarcinoma. The ability to detecting such mutations using immunohistochemistry (IHC) would be advantageous.
Methods: The molecular-based and IHC-based *EGFR* mutations were analyzed in 577 lung adenocarcinomas using high resolution melting analysis (HRMA) and 2 mutation-specific antibodies, respectively.
Results: In the molecular-based analyses, DEL was detected in 135 cases (23%), and L858R was detected in 172 cases (30%). In the IHC-based analyses, a positive reaction was detected in 59 cases (10%) for the DEL-specific antibody, and in 139 cases (24%) for the L858R-specific antibody. With the molecular-based results set as the gold standard, the sensitivity and specificity of the DEL-specific antibody were 42.2% and 99.5%, respectively, while the sensitivity and specificity of the L858R-specific antibody were 75.6% and 97.8%, respectively. The antibody specificities improved when the threshold for the mutation-positive reactions was set as >50% of immunopositive tumor cells. The significant predictors of the clinical response to EGFR-TKI were molecular-based *EGFR* mutations ($p < 0.001$) and IHC-based *EGFR* mutations ($p = 0.001$). However, a multivariate analysis revealed that only molecular-based *EGFR* mutations were significantly correlated with the clinical response ($p < 0.001$).
Conclusions: Mutation-specific antibodies demonstrated extremely high specificities, but their sensitivities were not higher than those of molecular-based analyses. However, IHC should be performed before a molecular-based analysis, because it is more cost-effective and can effectively select candidates for EGFR-TKI therapy.

1. Introduction

Many human receptor tyrosine kinases mediate signals that promote the proliferation and survival of cancer cells. Activation of tyrosine kinases appears to be the causal event in many human malignancies [1]. The importance of this finding is reflected in the development of new anticancer drugs that specifically target these activated proteins. The clinical success of tyrosine kinase inhibitors (TKIs), such as imatinib for the treatment of chronic myeloid leukemia and gastrointestinal stromal tumors, has prompted intensive efforts to identify and target additional oncogene kinases as a broad therapeutic strategy for selected patient populations [2,3].

A subset of non-small cell lung cancer (NSCLC), particularly adenocarcinomas, has activating mutations in the *epidermal growth factor receptor* (*EGFR*) gene [4,5]. The most prevalent *EGFR* mutations are deletions in exon 19 (DEL) and a point mutation at codon 858 in exon 21 (L858R); together, these account for more than 90% of all *EGFR* mutations. These 2 types of *EGFR* mutations cause sustained activation of *EGFR*, followed by the selective activation of Akt and signal transduction, and the activation of

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transcription signaling pathways: altogether, these promote cell survival [4,6].

EGFR-TKIs are competitive inhibitors of the adenosine triphosphate-binding clefts within the tyrosine kinase domain of *EGFR* [7]; they effectively inhibit the critical antiapoptotic signals transduced by the mutant receptors [6]. The clinicopathologic parameters of female gender, East Asian ethnicity, adenocarcinoma histology, and nonsmoking status are strong predictors of the response to EGFR-TKIs [4,5,8,9]. Moreover, the DEL and L858R mutations were also revealed to be strong predictors [10–14]. Therefore, the detection of such mutations provides both patients and physicians with important information regarding the optimal choice for therapy.

Direct sequencing is the gold standard method to detect *EGFR* mutations. However, to obtain precise data, high-quality DNA extracted from an adequate amount of pure tumor cells is required, and this is expensive and time-consuming. Recently, other indirect methods were developed to detect *EGFR* mutations, including Scorpion ARMS, the peptide nucleic acid-locked nucleic acid PCR clamp, mutant-enriched PCR, the smart amplification process, and high-resolution melting analysis (HRMA) [15,16]. These methods have high sensitivities, and can be applied to specimens in which cancer cell content is low. However, they invariably require technical labor and sophisticated instruments, and are therefore, not applied in most pathology laboratories.

Compared to molecular techniques, immunohistochemistry (IHC) is a fast and cost-effective method that can be performed in most pathology laboratories on not only fresh, but also archival, formalin-fixed tissue samples. Recently, some authors revealed the correlation between EGFR mutations and EGFR phosphorylation detected by IHC [17,18]. Additionally, EGFR phosphorylation antibodies exhibited a correlation with response to EGFR-TKIs [18]. However, these antibodies recognize EGFR phosphorylation regardless of mutational status. More recently, highly sensitive and specific rabbit monoclonal antibodies against the 2 most common mutations were developed for detecting *EGFR* mutations [19–24].

The main purpose of the present study was to explore the use of the 2 mutation-specific antibodies for DEL and L858R for detecting *EGFR* mutations. Additionally, we compared the molecular-based and the IHC-based *EGFR* mutational status to the response to EGFR-TKI.

2. Materials and methods

2.1. Case selection

After obtaining institutional review board approval, the specimens used in the present study were obtained from 577 Japanese patients who underwent a surgical resection for primary lung adenocarcinoma at the National Cancer Center Hospital, Tokyo, Japan, between 1993 and 2009. Histological diagnosis was based on the latest World Health Organization classification of lung tumors [25].

2.2. Analysis of EGFR mutational status by molecular technique

The materials analyzed for the molecular-based mutational status were as follows: fresh frozen (in liquid nitrogen), surgically resected tissue specimens from 505 patients (88%); methanol-fixed, paraffin-embedded, surgically resected tissue specimens from 36 patients (6%); and ethanol-fixed, imprint cytologic smears obtained from the fresh-cut surface of resected tumor specimens from 36 patients (6%). We used HRMA for detecting the DEL and L858R mutations, routinely performed at our institution. HRMA is well validated, and has been previously shown to accurately reflect *EGFR* mutational status [15].

2.3. Tissue microarray construction

The representative tumor regions to be sampled for the tissue microarray (TMA), were carefully selected and marked on a hematoxylin-eosin-stained slide. The TMAs were assembled using a manual tissue-arraying instrument (Azumaya, Tokyo, Japan). Considering tumor heterogeneity, 2 replicate 2-mm cores were routinely sampled from different regions of each tumor.

2.4. Immunohistochemistry

For the immunohistochemical staining, the 4-μm-thick TMA sections were deparaffinized. A heat-induced epitope retrieval with Target Retrieval Solution (Dako, Carpinteria, CA, USA) was performed. The primary antibody used were a rabbit monoclonal antibody against human *EGFR* with the DEL (E746-A750del) mutation (1:100, clone 6B6, Cell Signaling Technology, Danvers, MA, USA) and a rabbit monoclonal antibody against human *EGFR* with the L858R mutation (1:200, clone 43B2, Cell Signaling Technology). The antibodies were diluted in SignalStain (Cell Signaling Technology), and slides were incubated with each primary antibody for 1 h, at room temperature. The immunoreactions were detected using the EnVision Plus system (Dako) and 3,3′-diaminobenzidine, followed by counterstaining with hematoxylin. We used positive and negative controls for the IHC that previously confirmed the mutational status by using molecular analyses.

2.5. Immunohistochemical scoring system for mutation-specific antibodies

The immunoreactivity for each mutation-specific antibody was evaluated by using light microscopy at magnifications of 4 and 10× with objective lenses. Immunoreactivity was classified on the basis of cytoplasmic intensity. The following scoring system was used: negative intensity, 0 (defined as no immunoreactivity with any intensity); weak intensity, 1 (defined as the immunoreactivity only observed in 10× objective lenses); moderate intensity, 2 (defined as the immunoreactivity easily detected in 4× objective lenses, but less intense than the positive control); and strong intensity, 3 (defined as immunoreactivity equal to or stronger than the positive control; Fig. 1A and B). We also evaluated the extent of each intensity as a percentage (0–100%). Next, an expression score was obtained by multiplying the intensity by the percentage values (range, 0–300) for each core. Finally, the staining scores obtained in 2 cores were averaged, and the result was used as the representative score for each case. In the case of loss of tumor cells in 1 of the 2 cores during IHC, the staining score for the other core was used. We set the threshold at a staining score of 10; therefore, a staining score <10 was categorized as negative and a score ≥10 was categorized as positive. Additionally, we set another threshold for positive cases, defined as >50% of immunopositive tumor cells with any intensity.

2.6. Evaluation of the response to EGFR-TKI

Of the 577 patients, 116 received systemic therapy with EGFR-TKI gefitinib (250 mg daily) after tumor relapse. The therapeutic effect of gefitinib was complete response (CR) in 3, partial response (PR) in 61, stable disease (SD) in 13, and progressive disease (PD) in 37. Two patients were not evaluable for the clinical response due to the withdrawal of gefitinib caused by drug-induced liver dysfunction. The clinical response to gefitinib was determined using standard bidimensional measurements [26]. Responders were defined as patients with CR or PR, and non-responders were defined as patients with SD or PD.

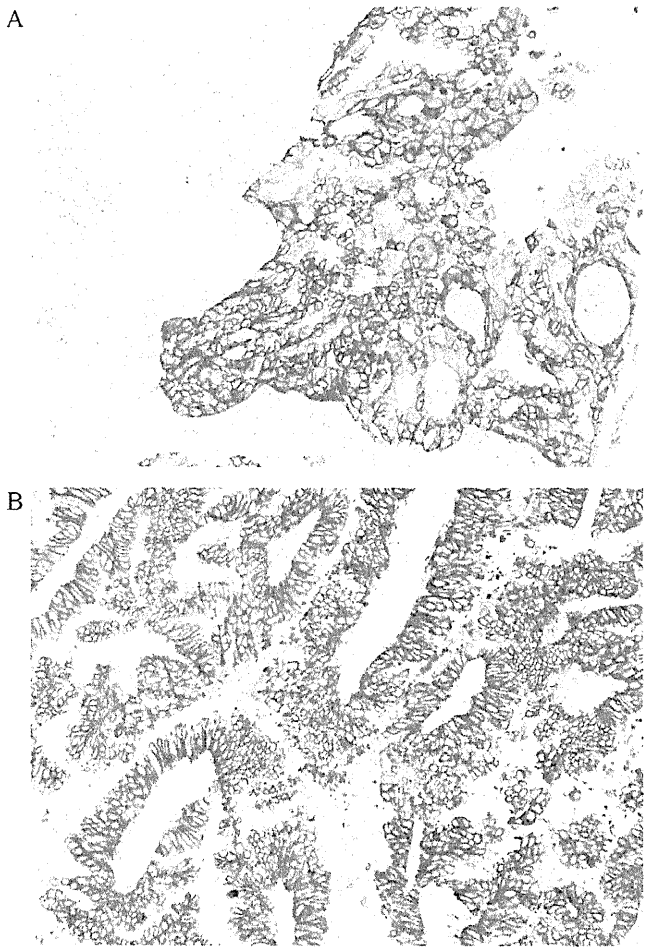

Fig. 1. A representative immunohistochemistry staining of intensity 3 for the DEL-specific antibody (1A, top) and the L858R-specific antibody (1B, bottom). The case 1A/1B harbored the molecular based DEL/L858R status.

2.7. Statistical analyses

Statistical analyses were performed using SPSS 12.0 for Windows (SPSS, Chicago, IL, USA). Chi-square tests for categorical variables were used and $p < 0.05$ was regarded as statistically significant.

3. Results

3.1. Clinicopathologic parameters

There were 319 males and 258 females with median age at surgery being 60 years (range, 30–82). A total of 343 patients had never/light smoking status with Brinkman index of <400, and 234 patients had smoking status with Brinkman index of ≥400. The pathological tumor stage (p-stage) was I in 331, II in 74, III in 164, and IV in 8 cases.

3.2. Molecular-based EGFR mutational status

After analyzing the *EGFR* mutational status by HRMA, DEL (m-DEL) was detected in 135 cases (23%), and L858R (m-L858R) was detected in 172 cases (30%). The remaining 270 cases (47%) were regarded as wild-type (m-WT), because neither the DEL nor the L858R mutation was detected.

Table 1A
Usefulness of DEL-specific antibody in detecting *EGFR* mutation of DEL under the threshold for the mutation-positive defined as staining score ≥10 and >50% of immunopositive tumor cells.

IHC-based *EGFR* mutation of DEL	Molecular-based *EGFR* mutation of DEL	
Staining score ≥10	(+)	(−)
(+)	57	2
(−)	78	440
Sensitivity = 42.2%; specificity = 99.5%		
>50% of immunopositive tumor cells		
	(+)	(−)
(+)	28	0
(−)	107	442
Sensitivity = 20.7%; specificity = 100.0%		

EGFR, epidermal growth factor receptor; DEL, deletions in exon 19; IHC, immunohistochemistry.

3.3. IHC-based EGFR mutational status

Although the tumor tissues of 52 of the 2308 cores (2.3%) were lost during the IHC procedure, at least 1 of the 2 cores contained tumor tissue in all cases. A positive immunoreactivity for the DEL-specific antibody was observed in 59 cases (10%). A positive immunoreactivity for the L858R-specific antibody was observed in 139 cases (24%). The remaining 379 cases were regarded as negative because neither the DEL- nor the L858R-specific antibody was positive. The immunohistochemical expression using DEL- and L858R-specific antibodies was mutually exclusive.

3.4. Correlation between the molecular-based and the IHC-based EGFR mutational status

We compared the molecular-based and IHC-based mutational status using molecular-based mutational status as the gold standard. The 59 cases that were positive for the DEL-specific antibody consisted of 57 cases with m-DEL, and 2 cases with m-WT. The sensitivity and specificity for the DEL-specific antibody was 42.2% and 99.5%, respectively (Table 1A). The 139 cases that were positive for the L858R-specific antibody consisted of 130 cases with m-L858R, and 9 cases with m-WT. The sensitivity and specificity for the L858R-specific antibody was 75.6% and 97.8%, respectively (Table 1B). Combining the results using these 2 antibodies, the overall sensitivity and specificity were 60.9% and 98.7%, respectively.

Table 1B
Usefulness of L858R-specific antibody in detecting *EGFR* mutation of L858R under the threshold for the mutation-positive defined as staining score ≥10 and >50% of immunopositive tumor cells.

IHC-based *EGFR* mutation of L858R	Molecular-based *EGFR* mutation of L858R	
Staining score ≥10	(+)	(−)
(+)	130	9
(−)	42	396
Sensitivity = 75.6%; specificity = 97.8%		
>50% of immunopositive tumor cells		
	(+)	(−)
(+)	83	5
(−)	89	400
Sensitivity = 48.3%; specificity = 98.8%		

EGFR, epidermal growth factor receptor; L858R, L858R mutation in exon 21; IHC, immunohistochemistry.

Table 2
Correlation between the clinicopathologic parameters of 577 patients and the response to EGFR-TKI.

	Responder (CR + PR, $n = 64$)	Non-responder (SD + PD, $n = 50$)	p-Value
Age			
≥65	20	18	0.690
<65	44	32	
Gender			
Male	35	27	1.000
Female	29	23	
Smoking status			
Brinkman index <400	45	33	0.687
Brinkman index ≥400	19	17	
p-Stage			
IA–IIB	26	19	0.848
IIIA–IV	38	31	

EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

3.5. Correlation between the molecular-based and IHC-based EGFR mutational status under another threshold

Positive immunoreactive cases for the DEL- or the L858R-specific antibody exhibited lower sensitivities and higher specificities when the threshold for the mutation-positive cases was restricted to >50% of the immunopositive tumor cells with any intensity. The incidence of positive immunoreactive cases for the DEL-specific antibody decreased from 59 to 28 cases—all of which were m-DEL (sensitivity, 20.7%; specificity, 100.0%; Table 1A). The incidence of positive immunoreactive cases for the L858R-specific antibody decreased from 139 to 88 cases, with 83 m-L858R cases and 5 m-WT cases (sensitivity, 48.3%; specificity, 98.8%; Table 1B).

3.6. Comparison of the molecular-based and IHC-based EGFR mutational status and the response to EGFR-TKI

A total of 114 patients were evaluable for the clinical response to EGFR-TKI. They consisted of 38, 39, and 37 patients with tumors harboring m-DEL, m-L858R, and m-WT, respectively; therefore, 68% of patients harbored the molecular-based *EGFR* mutations, and the remaining 32% harbored wild-type *EGFR*. The correlation between the conventional clinicopathologic parameters and the response to EGFR-TKI is shown in Table 2. In the present study, none of these parameters were significantly correlated with the response to EGFR-TKI.

Among the 77 patients harboring the molecular-based *EGFR* mutations, 59 (77%) were responders. In contrast, among the 37 patients without molecular-based *EGFR* mutations, only 5 (14%) were responders. Among the 55 patients with the IHC-based *EGFR* mutations, 40 (73%) were responders. In contrast, among the 59 cases without IHC-based *EGFR* mutations, 24 (41%) were responders (Table 3). Both the molecular- and IHC-based mutational statuses were significantly correlated with the response to EGFR-TKI ($p < 0.001$ and $p = 0.001$, respectively). We analyzed another threshold of the mutation-specific antibodies, defined as mutation-positive in >50% of the immunopositive tumor cells with any intensity. However, this threshold resulted in a slightly weaker correlation between the IHC-based mutational status and the response to EGFR-TKI ($p = 0.012$, Table 3).

3.7. Multivariate analysis of the response to EGFR-TKI

A multivariate analysis of the response to EGFR-TKI with 2 variables (molecular-based mutational status and IHC-based mutational status), which showed a significant correlation by univariate analysis, was performed; only the molecular-based mutational status was significantly correlated with the response to EGFR-TKI ($p < 0.001$). The IHC-based mutational status ($p = 0.211$) was not significantly correlated (Table 4).

Table 3
Comparison of the molecular-based and IHC-based *EGFR* mutational status and the response to EGFR-TKI.

	Responder (CR + PR, $n = 64$)	Non-responder (SD + PD, $n = 50$)	p-Value
Molecular-based *EGFR* mutation			
(+)	59	18	<0.001
(−)	5	32	
IHC-based *EGFR* mutation			
Staining score ≥10	40	15	0.001
Staining score <10	24	35	
Immunopositive tumor cells >50%	24	8	0.012
Immunopositive tumor cells ≤50%	40	42	

EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; IHC, immunohistochemistry.

4. Discussion

In the present study, we investigated the clinical usefulness of IHC using 2 rabbit monoclonal antibodies against specific mutant *EGFRs* in lung adenocarcinomas. We found that the IHC-based *EGFR* mutational status detected by these antibodies was significantly correlated with the molecular-based *EGFR* mutational status. Furthermore, the IHC-based mutational status showed a significant correlation with the clinical response of tumors in conjunction with EGFR-TKI therapy.

The overall specificity of the 2 mutation-specific antibodies was 99%, and this specificity was consistent with that reported previously [19–24]. There were 11 cases in which the results of IHC examination were positive and those of molecular testing were negative. These false-positive cases might harbor other types of mutations that induce conformational changes in the *EGFR* protein, similar to DEL and L858R [24]. Since none of these 11 patients received EGFR-TKI therapy, the clinical significance of these mutations was not analyzed in the present study.

Despite the significant correlation between clinical response and immunoreactivity, the overall sensitivity of the 2 mutation-specific antibodies was 61%. This sensitivity was the lowest compared to values previously reported by others, which ranged from 78% to 92% [19,21–23]. One possible reason for the lower sensitivity in the present study was the methodological difference in the analysis of the molecular-based *EGFR* mutational status. HRMA, which was used for the molecular *EGFR* mutation analysis in the present study, was more sensitive than direct sequencing. HRMA has been shown to be a highly sensitive method for detecting DEL and L858R in prospective studies, and the detection sensitivity of this assay was reported to be 0.1–10%[15,27,28]. Conversely, the direct sequencing used in previous reports [19,21–23] required the presence of at least 20–25% *EGFR*-mutant cells to detect the DEL and L858R mutations. In the other 2 reports that validated the mutation-specific antibodies by correlating them with the *EGFR* mutational status by using highly sensitive molecular assays (mass spectrometry-based DNA analysis, cycleave PCR, and frag-

Table 4
Multivariate analysis of the response to EGFR-TKI.

	Odds ratio	95% CI	p-Value
Molecular based mutation	40.533	8.691–189.035	<0.001
IHC based mutation	0.421	0.109–1.632	0.211

EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; CI, confidence interval; IHC, immunohistochemistry.

ment analysis), the reported sensitivities of IHC-based mutations were lower and partially similar to ours. Brevet et al. have reported that the sensitivity of the DEL-specific antibody was 67%, and that of the L858R-specific antibody was 76%, with the threshold for positive cases defined as moderate staining [20]; Kitamura et al. have reported that the overall sensitivity of these 2 mutation-specific antibodies was 47%, with almost the same threshold for positive cases as our staining score of 10 [24]. Although highly sensitive methods sometimes elicit false positive results, we showed that the response rate to EGFR-TKI in patients with lung tumors harboring HRMA-detected *EGFR* mutations was 77%, and this was consistent with 2 previous reports (82% and 83%) [12,29]. Therefore, HRMA was not likely to have overestimated the *EGFR* mutations.

Most of the extracted DNA in the present study was isolated from fresh frozen tissues or ethanol-fixed imprint cytologic smears, whereas in other reports concerning mutation-specific antibodies, DNA extracted from formalin-fixed, paraffin-embedded tissues was used for molecular *EGFR* mutation analysis [19–22]. Formalin-fixed tissues exhibit non-reproducible sequence alterations more frequently than DNA isolated from frozen tissues. This is because formalin can cross-link cytosine nucleotides on either strand [30]. However, ethanol causes very little chemical change, and therefore preserves nucleic acids better than formalin [30]. Taken together, these data suggest that using a highly sensitive molecular assay and high-quality DNA can reduce false-negative cases. Therefore, the sensitivity of the 2 novel mutation-specific antibodies used in the present study, was decreased.

Another possibility was that the immunopositive tumor cells for the mutation-specific antibodies were not diffusely distributed. When the threshold for mutation-positive was set as >50% of immunopositive tumor cells, the positive cases for the DEL- and L858R-specific antibodies decreased from 59 to 28 cases (47%), and from 139 to 88 cases (63%), respectively. From these decreased rates, the immunopositive tumor cells for DEL were distributed more sparsely and/or focally than those for L858R. These findings, detected by IHC analysis, suggested the presence of heterogeneity in the *EGFR*-mutant cells. Other molecular methods for detecting *EGFR* mutations also revealed the heterogeneous distribution of *EGFR* mutant cells [31–33].

In the present study, the predictors of the EGFR-TKI response were molecular-based (HRMA) *EGFR* mutations ($p < 0.001$), and IHC-based *EGFR* mutations ($p = 0.001$). Two novel mutation-specific antibodies served as the predictors of EGFR-TKI response in the univariate analysis. However, the multivariate analysis revealed that only molecular-based *EGFR* mutations were significantly correlated with the response to EGFR-TKI. Among 6 previous reports on mutation-specific antibodies, 3 analyzed the correlation of IHC-based *EGFR* mutational status with the response to EGFR-TKI, and a significant correlation was found in 2 of these studies [21,24]. The sensitivity and specificity of IHC-based *EGFR* mutations to the EGFR-TKI response calculated in this study were 63% and 70%, respectively. In 2 previous reports, IHC-based *EGFR* mutations showed a sensitivity ranging from 59% to 89% and a specificity ranging from 73% to 96% to the EGFR-TKI response. The last report showed an insignificant correlation between these parameters [22]. The role of IHC in predicting response to EGFR-TKI remains controversial [34]. It is necessary to prospectively study a larger number of cases to determine the usefulness of IHC for the response to EGFR-TKI.

The amount of immunopositive tumor cells did not affect the EGFR-TKI response in the present study. The threshold for mutation-positive, defined as >50% of immunopositive tumor cells, was less significantly correlated with the clinical response to EGFR-TKI than when using judgments by the expression score of 10 ($p = 0.012$). Further discussion regarding whether the percentage of immunopositive tumor cells is correlated with the response to EGFR-TKI, is necessary. The present results showed, for the first time, that the presence of diffusely immunopositive cells does not necessarily predict a response to EGFR-TKI therapy. Therefore, in clinical practice, a threshold for mutation-positive of expression score of 10 should be adopted.

Although the mutation-specific antibodies are not superior to the highly sensitive molecular techniques in detecting *EGFR* mutations, they have some potential advantages. Their excellent specificities [19–24] will serve as the first screening for *EGFR* mutational status, including the human epidermal growth factor 2 status for breast carcinoma [35,36]. In clinical settings, the first screening of IHC enables the omission of molecular *EGFR* mutational analysis in IHC-positive cases. IHC saves time, is cost-effective, and can be performed in most pathology laboratories. Another advantage of IHC over molecular techniques is that it can distinguish between tumor morphology and mutation-bearing cells by light microscopy.

In summary, the mutation-specific antibodies exhibited extremely high specificities, but did not show high sensitivities compared to the highly sensitive molecular method. In clinical practice, IHC using these 2 antibodies is a cost-effective and simple method for detecting *EGFR* mutations in most pathology laboratories, and can quickly evaluate patients for EGFR-TKI therapy.

Conflict of interest statement

All authors have no financial or personal relationship with other people or organization that could inappropriately influence our work.

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