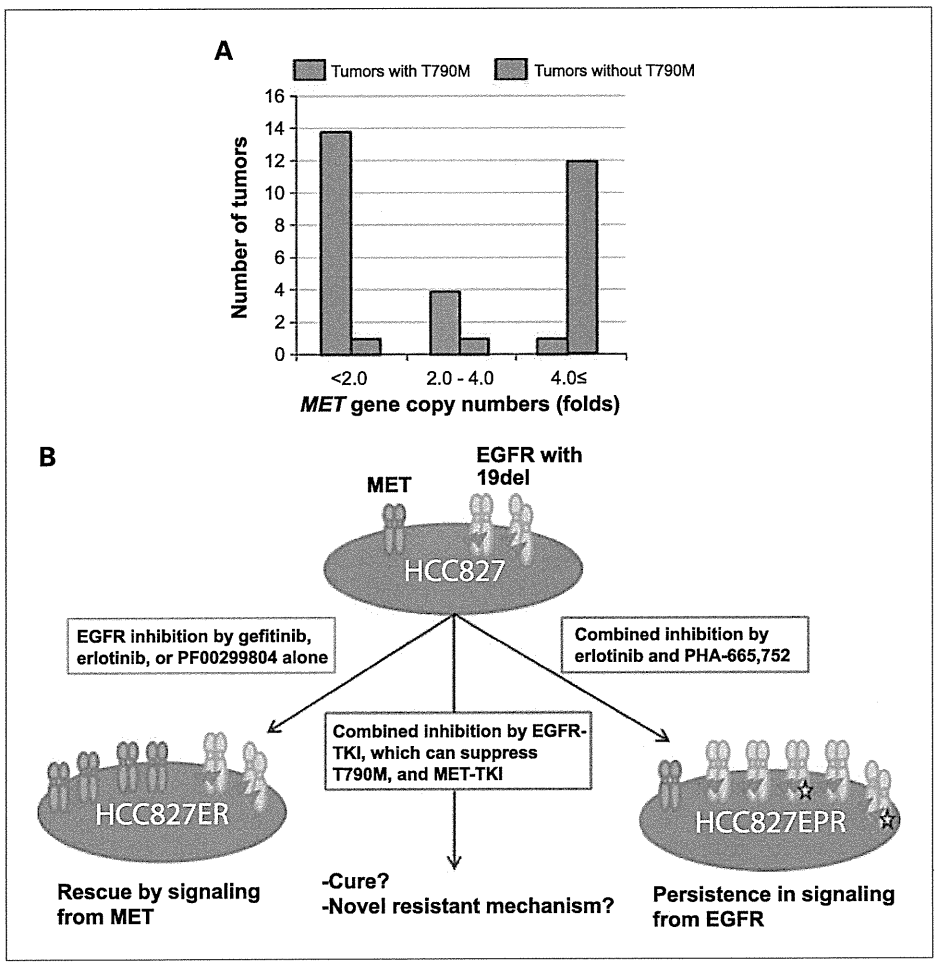

Fig. 4. Reciprocal relationship between *MET* amplification and T790M mutation. A, tumor numbers with or without T790M mutation by *MET* gene copy numbers in gefitinib-refractory tumors obtained from autopsy. B, schema of the difference of escape hatches of HCC827 cells depending on the selection pressure differences by molecular target drug(s). All amplified EGFR alleles harbor exon 19 deletion mutation in HCC827 cells, and a part of them in HCC827EPR cells acquired T790M mutation (yellow stars).

acquired resistance to gefitinib (11) or to an irreversible pan-ERBB kinase inhibitor, PF00299804 (14). We also found that the *MET* CNG increased in proportion to erlotinib resistance and that a ≥4-fold *MET* CNG compared with normal DNA was an apparent threshold for the development of clinically relevant TKI resistance. This observation is consistent with the observation that a moderate *MET* CNG (<4-fold) could coexist with T790M but that *MET* gene amplification (≥4-fold) and T790M were almost mutually exclusive in our autopsy analysis.

On the other hand, PC9 (exon 19 deletion) and H3255 (L858R) are known to develop resistance to EGFR-TKIs through T790M (18–20, 31). These phenomena are explained by the existence of minor clones with such alterations before EGFR-TKI treatment. Supporting this hypothesis further, Turke et al. found 0.06% to 0.14% of *MET*-amplified minor subclones in HCC827 but not in PC9 or H3255 cells (14). In addition, Inukai and Maheswaran showed that patients with *EGFR* mutations had shorter progression-free survival when the tumor had a very small amount of T790M before the EGFR-TKI therapy (13, 32). Thus, it seems that these cell lines are destined to use either mechanism to overcome EGFR-TKIs. It is interesting that HCC827 cells developed the T790M mutation when exposed to increasing concentrations of erlotinib under the inhibition of MET signaling (Fig. 4B), although it took about 1.5 times longer compared with erlotinib alone. The origin of the T790M allele in HCC827 cells is not clear, although this is the case with other *in vitro* gefitinib-resistant models used to develop the T790M mutation (PC9 and H3255 cell lines; refs. 18, 31). More sensitive methods might be able to detect the presence of minor clones with the T790M mutation in these cell lines before the start of EGFR-TKI treatment.

No studies have investigated the mechanisms responsible for the acquired resistance to EGFR-TKI therapy in multiple sites of metastases obtained from autopsy. The autopsy samples allowed us to see the ultimate pictures of resistance and to examine multiple organ sites simultaneously. Thirty-one of 33 lesions harbored the T790M mutation and/or *MET* amplification. We also found an inverse relationship between the presence of T790M and *MET* gene copy number, suggesting a complementary role of the two mechanisms in the acquisition of resistance. This is consistent with a previous report; one of the patients with acquired resistance to EGFR-TKI harbored

two tumors, one with *MET* amplification only and the other with moderate *MET* CNG and T790M (12). The incidence of the T790M mutation and *MET* amplification as mechanisms responsible for the acquired resistance to EGFR-TKIs was reported to be ~50% and ~20%, respectively (33). However, our present results suggest that the incidence of these two mechanisms is higher in the later phase and that overcoming these two mechanisms would be the key to improving patient outcomes further.

The factors that determine which mechanism will be used by tumor cells for overcoming EGFR-TKIs are not clear. One may speculate that the balance between positive and negative regulators of the MET pathway in the microenvironment of the tumor cells determines the mechanisms of resistance. Hepatocyte growth factor, a ligand for MET, has been shown recently to induce transient and reversible resistance to EGFR-TKIs (34, 35) and to facilitate *in vitro MET* amplification in the development of stable acquired resistance to EGFR-TKIs (14). On the other hand, there are several negative regulators of the HGF-MET axis. One example is to increase MET degradation by Cbl-mediated ubiquitination or another mechanism. Overexpression of LRIG1, a transmembrane leucine-rich repeat and immunoglobulin-like domain-containing protein, destabilizes MET and impairs the ability to respond to hepatocyte growth factor (36). Another possibility is a negative regulator of MET-induced cell behavior, such as Abl tyrosine kinase, which functions as a negative regulator of MET-induced cell motility via phosphorylation of the adapter protein CrkII (37).

In conclusion, we observed a reciprocal, complementary relationship between *MET* amplification and the *EGFR* T790M mutation in both an *in vitro* erlotinib-resistant model (illustrated in Fig. 4B) and in our analysis of gefitinib-refractory tumors obtained from autopsy samples. Molecular target therapy prolongs the overall survival in lung cancer patients with an *EGFR* mutation (38), and the development of the concurrent inhibition therapy might be essential for the further improvement.

Disclosure of Potential Conflicts of Interest

T. Mitsudomi has received lecture fees from AstraZeneca and Chugai. The other authors declare no conflict of interest.

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Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial

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Summary

Background **Patients with non-small-cell lung cancer harbouring mutations in the epidermal growth factor receptor (*EGFR*) gene respond well to the EGFR-specific tyrosine kinase inhibitor gefitinib. However, whether gefitinib is better than standard platinum doublet chemotherapy in patients selected by EGFR mutation is uncertain.**

Methods **We did an open label, phase 3 study (WJTOG3405) with recruitment between March 31, 2006, and June 22, 2009, at 36 centres in Japan. 177 chemotherapy-naive patients aged 75 years or younger and diagnosed with stage IIIB/IV non-small-cell lung cancer or postoperative recurrence harbouring *EGFR* mutations (either the exon 19 deletion or L858R point mutation) were randomly assigned, using a minimisation technique, to receive either gefitinib (250 mg/day orally; n=88) or cisplatin (80 mg/m², intravenously) plus docetaxel (60 mg/m², intravenously; n=89), administered every 21 days for three to six cycles. The primary endpoint was progression-free survival. Survival analysis was done with the modified intention-to-treat population. This study is registered with UMIN (University Hospital Medical Information Network in Japan), number 000000539.**

Findings **Five patients were excluded (two patients were found to have thyroid and colon cancer after randomisation, one patient had an exon 18 mutation, one patient had insufficient consent, and one patient showed acute allergic reaction to docetaxel). Thus, 172 patients (86 in each group) were included in the survival analyses. The gefitinib group had significantly longer progression-free survival compared with the cisplatin plus docetaxel goup, with a median progression-free survival time of 9·2 months (95% CI 8·0–13·9) versus 6·3 months (5·8–7·8; HR 0·489, 95% CI 0·336–0·710, log-rank p<0·0001). Myelosuppression, alopecia, and fatigue were more frequent in the cisplatin plus docetaxel group, but skin toxicity, liver dysfunction, and diarrhoea were more frequent in the gefitinib group. Two patients in the gefitinib group developed interstitial lung disease (incidence 2·3%), one of whom died.**

Interpretation **Patients with lung cancer who are selected by EGFR mutations have longer progression-free survival if they are treated with gefitinib than if they are treated with cisplatin plus docetaxel.**

Funding **West Japan Oncology Group (WJOG): a non-profit organisation supported by unrestricted donations from several pharmaceutical companies.**

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Introduction

Lung cancer is a major cause of cancer-related mortality worldwide.[1] However, current standard platinum doublet therapy seems to have reached a therapeutic plateau,[2] although it has recently been shown that patients with non-squamous histology who are treated with pemetrexed disodium have better survival than if they are treated with older drugs.[3]

Targeted therapies are actively being developed to improve efficacy in selected patient populations.[4] Small-molecule tyrosine kinase inhibitors (TKIs) that target the epidermal growth factor receptor (EGFR), such as gefitinib and erlotinib, are the first targeted drugs to enter clinical use for the treatment of lung cancer. Subgroups of patients of east-Asian origin, female sex, adenocarcinoma, and no history of smoking have been shown to be significantly associated with a favourable response to EGFR TKIs.[5,6] In 2004, researchers noted that activating mutations of the *EGFR* gene present predominantly in patients with the above-mentioned clinical characteristics, and determine sensitivity to EGFR TKIs.[7,8] *EGFR* mutations are present in the first four exons of the tyrosine kinase domain of the *EGFR* gene, and about 90% of these *EGFR* mutations are either short in-frame deletions in exon 19, or point mutations that result in a substitution of arginine for leucine at aminoacid 858 (L858R).[7–9] Subsequent retrospective and prospective trials confirmed that the response rate to gefitinib or erlotinib in patients with *EGFR* mutations is about 70–80%.[10–13] Furthermore, patients with *EGFR* mutations have a significantly longer survival than those with wild-type *EGFR* when treated

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with EGFR TKIs.[14,15] We proposed that the absence of any survival advantage conferred by gefitinib monotherapy in previous studies[16–18] is due at least in part to a lack of patient selection, and that gefitinib would confer a survival advantage compared with platinum doublet chemotherapy in a first-line setting if eligible patients were selected on the basis of *EGFR* mutation status. To address this issue, we did a phase 3 trial that compared gefitinib with cisplatin plus docetaxel in patients with an *EGFR* mutation.

Methods

Patients

This study (WJTOG 3405) was a multicentre, randomised, open-label, phase 3, trial of first-line treatment with gefitinib versus cisplatin plus docetaxel for patients with advanced or recurrent non-small-cell lung cancer (NSCLC) harbouring an activating mutation of the *EGFR* gene. We recruited patients between March 31, 2006, and June 22, 2009, at 36 centres in Japan. All centres were members of the West Japan Oncology Group (WJOG), which is a Japanese non-profit organisation for oncological clinical trials (formerly the West Japan Thoracic Oncology Group, or WJTOG).

Initially, only patients with postoperative recurrence were eligible, because these surgical specimens were expected to ensure good sample quality. However, because of the initial slow accrual, the protocol was amended on July 10, 2006, to include patients with stage IIIB/IV disease. Patients were eligible if they had histologically or cytologically confirmed NSCLC, harbouring activating *EGFR* mutations (either exon 19 deletion or L858R in exon 21), were aged 75 years or younger, had WHO performance status 0–1, had measurable or non-measurable disease according to the Response Evaluation Criteria in Solid Tumours (RECIST), and had adequate organ function. Patients with postoperative recurrence, treated with adjuvant therapy other than cisplatin plus docetaxel, were included when the interval between the end of adjuvant chemotherapy and registration exceeded 6 months for platinum-doublet

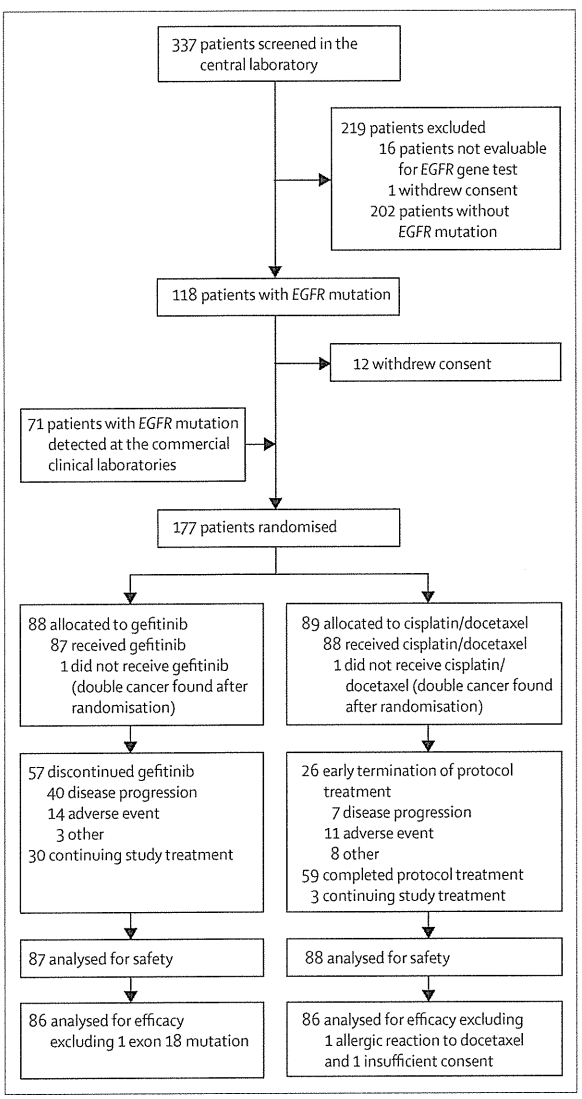

Figure 1: **Trial profile**

	Gefitinib (N=86)	Cisplatin plus docetaxel (N=86)
Sex		
Male	27	26
Female	59	60
Age (years; median; range)	64·0 (34–74)	64·0 (41–75)
Histological type		
Adenocarcinoma	83	84
Adenosquamous carcinoma	0	1
Squamous-cell carcinoma	1	0
Non-small-cell lung cancer; not otherwise specified	2	1
Smoking history		
Never	61	57
Former/current	25	29
Performance status		
0	56	52
1	30	34
Stage		
Postoperative recurrence	35	36
With postoperative adjuvant chemotherapy	19	23
Without postoperative adjuvant chemotherapy	16	13
IIIB	10	9
IV	41	41
EGFR mutation		
Exon 19 deletion	50	37
L858R	36	49

Table 1: **Demographic and baseline characteristics of the modified intention-to-treat population**

therapy and more than 1 month for oral tegafur plus uracil therapy. Patients were not eligible if they had received previous drug therapy that had targeted EGFR, had a history of interstitial lung disease, severe drug allergy, active infection or other serious disease condition, symptomatic brain metastases, poorly controlled pleural effusion, pericardial effusion or ascites necessitating drainage, active double cancer, or severe hypersensitivity to drugs containing polysolvate 80. Patients in pregnancy or lactation, or whose participation in the trial was judged to be inappropriate by the attending doctor, were not eligible. All patients provided written informed consent. Study approval was obtained from independent ethics committees at every institution. The study was undertaken in accordance with the Declaration of Helsinki.

Procedures

Patients were randomly assigned in a 1:1 ratio to receive gefitinib (250 mg/day, administered orally), or docetaxel (60 mg/m^2, administered intravenously over a 1 h period) followed by cisplatin (80 mg/m^2, administered intravenously over a 90-min period), with adequate hydration, in cycles of once every 21 days for three to six cycles.Treatment continued until progression of the disease, development of unacceptable toxic effects, a request by the patient to discontinue treatment, serious non-compliance with the protocol, or completion of three to six chemotherapy cycles. Further therapy after progression of the disease was at the physician's discretion. The primary endpoint was progression-free survival. Secondary endpoints included overall survival and response rate. Tertiary endpoints were disease control rate, safety, and mutation-type-specific survival.

Initially, patients were screened for *EGFR* mutation in a central laboratory at the Department of Molecular Diagnostics, Aichi Cancer Centre Hospital, Nagoya, Japan. The exon 19 deletion mutation was screened by fragment analysis and the L858R point mutation was screened by the Cycleave method, as described previously,[19] followed by confirmation by direct sequencing. On Feb 16, 2008, the protocol was amended to allow outsourcing of *EGFR* genetic testing from each institution to commercial clinical laboratories, either at SRL in Tokyo (direct sequencing), Mitsubishi Chemical Medience in Tokyo (peptide nucleic acid-locked nucleic acid PCR clamp[20]), or BML in Tokyo (PCR invader[21]), as this amendment would further facilitate patient accrual. The sensitivity of direct sequencing was anticipated to be less than that of other methods; however, false negativity was not a problem in this trial, since patients judged to lack EGFR mutations were not randomly allocated to a treatment.

Progression-free survival was assessed from the date of randomisation to the earliest sign of disease progression as determined by CT or MRI imaging using RECIST criteria, or death from any cause. Overall survival was assessed from the date of randomisation until death from any cause. Tumour response was assessed every 2 months

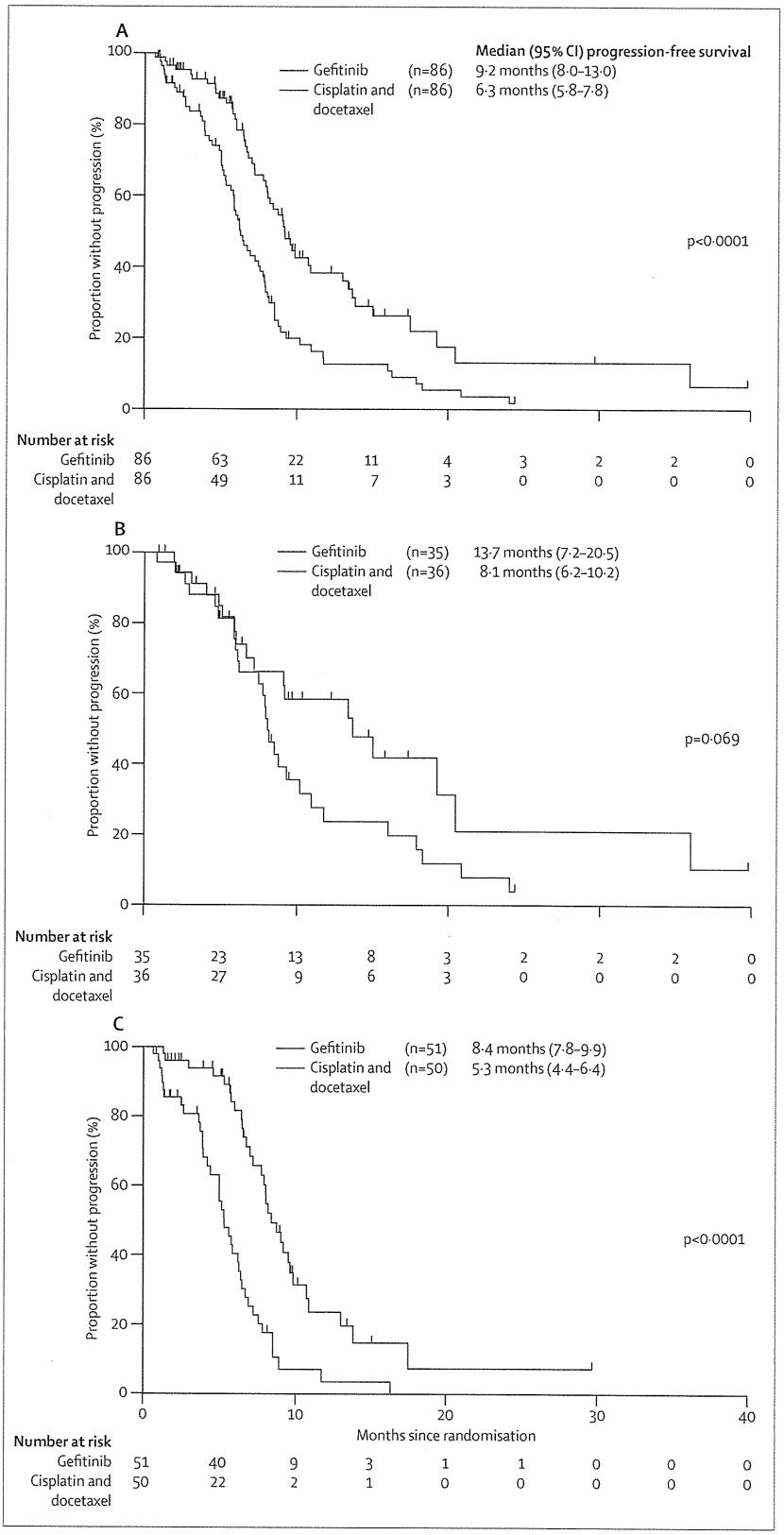

Figure 2: **Progression-free survival in the overall population (A), in patients with postoperative recurrence (B), and in patients with stage IIIB/IV disease (C)**

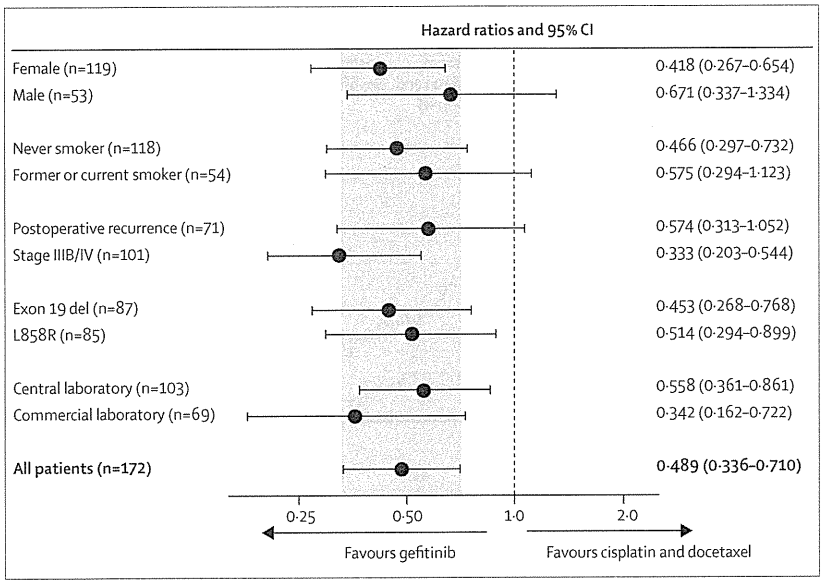

Figure 3: **Hazard ratios for progression-free survival using subgroup analysis in the overall population**
The shaded band represents the 95% CI of the hazard ratio for the overall population of patients.

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p	HR (95% CI)	p
Group (gefitinib/cisplatin plus docetaxel)	0·489 (0·336–0·710)	0·0002	0·258 (0·385–0·575)	<0·0001
Sex (male/female)	0·935 (0·625–1·398)	0·742	0·628 (0·361–1·092)	0·099
Age (<65 years /≥65 years)	1·091 (0·757–1·572)	0·641	1·183 (0·813–1·721)	0·380
Smoking history (never/former or current)	0·801 (0·541–1·186)	0·268	0·646 (0·378–1·105)	0·111
Stage (recurrence/IIIB–IV)	0·463 (0·220–0·976)	0·043	0·433 (0·290–0·649)	<0·0001
Mutation (exon 19 del/L858R)	1·001 (0·694–1·444)	0·996	1·135 (0·777–1·658)	0·514

Table 2: **Univariate and multivariate analysis of progression-free survival**

during the first year after randomisation, every 3 months between 12 and 18 months, and thereafter the interval of assessment was at the physician's discretion. Safety and tolerability were assessed according to National Cancer Institute Common Terminology Criteria (CTC) for Adverse Events, version 3.0. All events were confirmed via source-document verification at site visits to each participating institution by members of the WJOG data centre and the investigators.

Randomisation and masking

The investigator provided the necessary information to personnel at the WJOG data centre by fax. After an eligibility check, patients were allocated at the WJOG data centre to each treatment group using a desktop computer programmed for the minimisation method.[22] In this way, patient allocation was concealed from the investigator.

Because of the nature of treatment in each group, the study was open label. Stratification factors were: institution; postoperative adjuvant chemotherapy (presence *vs* absence); interval between surgery and recurrence (≥1 *vs* <1 year) for patients with postoperative recurrent disease; and institution; stage (IIIB *vs* IV); and sex (male *vs* female) for patients with stage IIIB/IV disease.

Statistical analysis

In previous studies the progression-free survival of patients harbouring EGFR mutations and treated with gefitinib was reported as 12·6 months,[15] compared with 6·6 months for patients harbouring EGFR mutations treated with carboplatin plus paclitaxel.[23] Assuming a progression-free survival for gefitinib and platinum doublet chemotherapy of 12·5 and 7 months, respectively, would yield a hazard ratio (HR) of 0·56. Taking this HR into consideration, 146 patients would be required to achieve 90% power to show superiority with α=0·05 (two-sided). Therefore, sample size was initially set at 200 patients. While this trial was ongoing, the results of the Iressa Pan-Asia Study (IPASS) were presented at the annual meeting of the European Society for Medical Oncology (Stockholm, Sweden, Sept 12–16, 2008), and were later published.[24] Subgroup analysis of patients with EGFR mutations using about a third of the patients showed that the HR of gefitinib compared with carboplatin plus paclitaxel for progression-free survival was 0·48. Similarly, the HR of gefitinib compared with carboplatin plus paclitaxel for progression-free survival in patients with EGFR mutations was 0·36 in the study done by the North East Japan (NEJ) 002 Gefitinib Study Group, which was presented at the annual meeting of the American Society of Clinical Oncology (Orlando, FL, USA, May 29–June 2, 2009).[25] NEJ 002 was a phase 3 trial that analysed 198 patients with EGFR mutation randomised either to gefitinib or carboplatin plus paclitaxel. 177 patients had been randomised in our trial as of June 13, 2009, and 79 events had been noted during the regular monitoring done in March, 2009. The number of events needed to detect a conservative HR of 0·48 was calculated to be 78, based on normal approximation of the logarithm of the hazard ratio under α=0·05 (two-sided) and 90% power. Therefore, further accrual of patients was considered to be futile and potentially unethical. Although interim analysis was originally planned to analyse progression-free survival, this analysis was not done. Instead, the steering committee held on June 13, 2009, proposed the amendment of the sample size and the final analyses be done using available data. This proposal was approved by the independent data and safety monitoring committee on Aug 28, 2009. The data were locked on June 30, 2009. Patient follow-up for safety and survival will continue until 1·5 years after the last patient entry, as originally described in the study protocol.

Progression-free and overall survival were analysed for the modified intention-to-treat population as defined previously.[26] They were analysed using the Kaplan-Meier method, and were compared using the log-rank test. Hazard ratios in the overall population and in patient

subsets were calculated using the Cox proportional hazards model. The χ^2 test was used to compare proportions. Differences were considered significant at a two-sided p value of 0·05 or less. All statistical analyses were done with SAS version 9.1. This study is registered with UMIN (University Hospital Medical Information Network in Japan), number 000000539.

Role of the funding source

There was no sole study sponsor for this trial. The WJOG designed and did the trial independently of any pharmaceutical company. The report was written by the corresponding author, who had unrestricted access to the study data and is responsible for the accuracy and completeness of the reported analyses. The corresponding author had final responsibility for the decision to submit for publication.

Results

118 patients were positive for EGFR mutation at the central laboratory, 106 of whom were randomly allocated a treatment together with 71 patients with EGFR mutations who were tested at the commercial laboratories, giving a modified intention-to-treat population of 172 patients (figure 1). Baseline characteristics were well balanced between the two treatment groups (table 1), with the exception that the gefitinib group had an excess of exon 19 deletion mutations (50 of 86; 58·1%) compared with the cisplatin plus docetaxel group (37 of 86; 43·0%). Most of the patients had adenocarcinoma. 71 of 172 (41·3%) patients had postoperative recurrent disease, and 54 of 172 (31·4%) of the patients had a history of smoking. At the data collection cut-off time, the median follow-up was 81 days (range 74–1253 days), the median exposure to gefitinib was 165 days (range 22–1100 days), and the median number of cycles of cisplatin plus docetaxel chemotherapy was four, or 64 days (range one to six cycles, or 1–106 days).

Median progression-free survival was 9·2 months (95% CI 8·0–13·9) in the gefitinib group and 6·3 months (5·8–7·8) in the cisplatin plus docetaxel group (p<0·0001; figure 2A). Gefitinib treatment resulted in significantly longer progression-free survival than cisplatin plus docetaxel (HR 0·489; 95% CI 0·336–0·710; p<0·0001). Progression-free survival can be affected by the schedule of clinic visits and the interpretation of evidence of disease progression. We were able to confirm that the time schedule for clinic visits was almost the same in the two treatment groups (data not shown). In our trial, 71 patients had postoperative recurrent disease, and the remaining 101 patients had stage IIIB/IV disease. In both patient subsets, progression-free survival in the gefitinib group was longer than that in the cisplatin plus docetaxel group (figure 2B, 2C), although this was not a pre-specified analysis and was non-significant for those patients with postoperative recurrence. We noted that curves for each treatment group in the postoperative recurrence subgroup (figure 2B) overlapped during the first 6 months, while the separation was clear during this time in the stage IIIB/IV group (figure 2C).

Patients treated with gefitinib had better progression-free survival than patients treated with cisplatin plus docetaxel in all subgroup analyses (figure 3). Additionally, gefitinib was better than cisplatin plus docetaxel, irrespective of where EGFR genetic testing was done. Exploratory analyses for progression-free survival showed that, in addition to the treatment group, patients with postoperative recurrent disease had a significantly better prognosis than those with stage IIIB/IV disease (table 2). We did a pre-planned comparison of exon 19 deletion with L858R in each treatment group. As shown in figure 4, mutation type was not prognostic. Therefore,

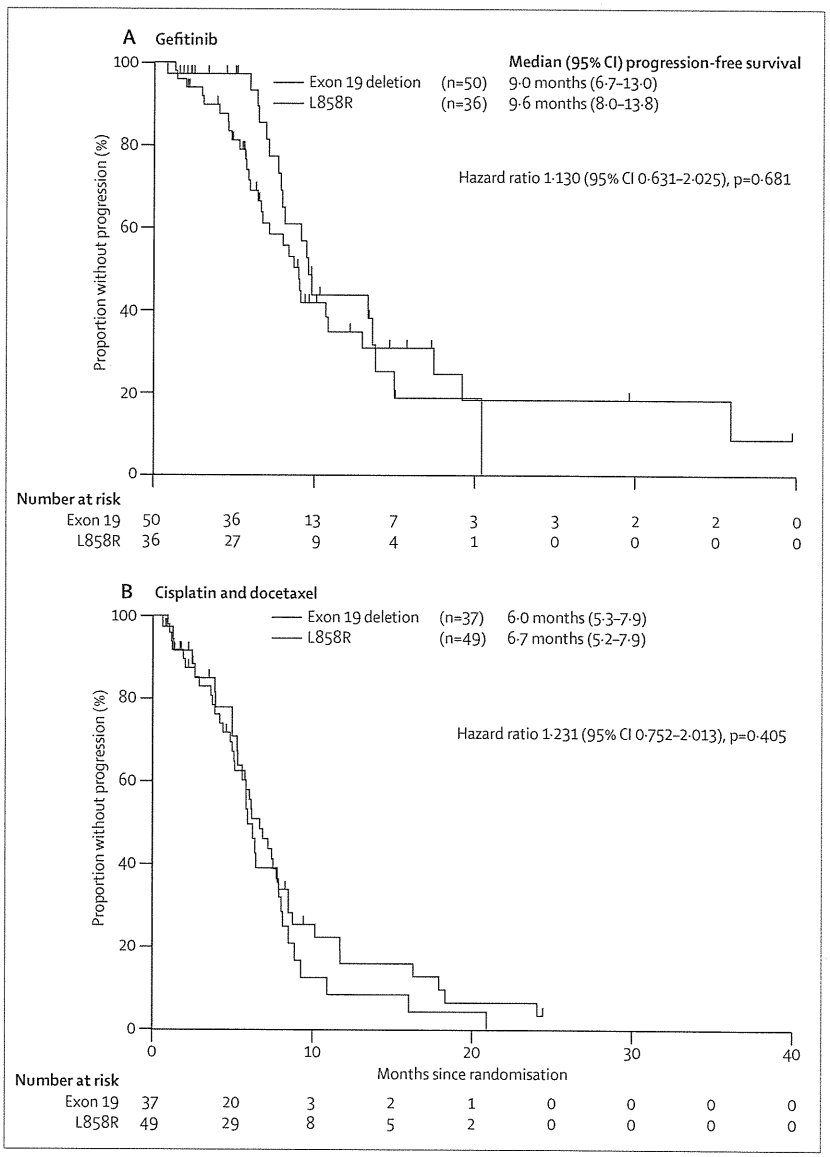

Figure 4: **Progression-free survival in (A) the gefitinib group and (B) the cisplatin plus docetaxel group according to type of the EGFR mutation**

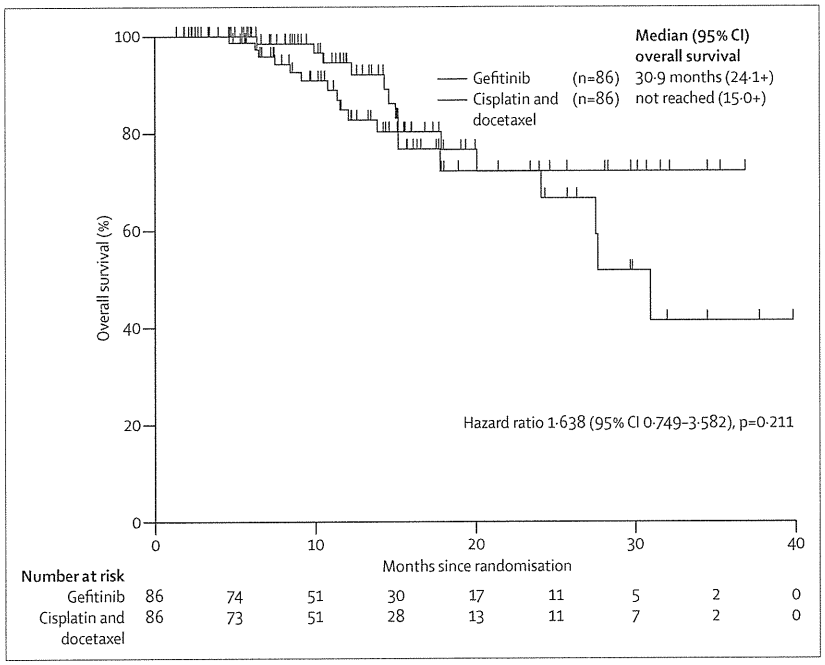

Figure 5: **Overall survival in the overall population**

See Online for webappendix

	Gefitinib (n=87)		Cisplatin plus docetaxel (n=88)	
	All	CTC grade ≥3	All	CTC grade ≥3
Non-haematological toxicity				
Rash*	74	2	7	0
AST*	61	14	17	1
ALT*	61	24	35	2
Dry skin*	47	0	3	0
Diarrhoea	47	1	35	0
Fatigue*	34	2	73	2
Paronychia*	28	1	1	0
Stomatitis	19	0	13	0
Nausea*	15	1	83	3
Constipation*	14	0	39	0
Alopecia*	8	0	67	0
Sensory disturbance*	7	1	23	0
Haematological toxicity				
Leucocytopenia*	13	0	82	43
Thrombocytopenia*	12	0	29	0
Neutropenia*	7	0	81	74
Anaemia*	33	0	79	15

ALT=alanine aminotransferase. AST=aspartate aminotransferase. CTC=National Cancer Institute Common Terminology Criteria. *p<0·001.

Table 3: **Adverse events occurring in more than 10% of either of the treatment groups listed according to incidence in the gefitinib group**

imbalance of mutation types was not likely to affect the interpretation of the overall results.

The objective response rate in the overall population with measurable disease (n=117) was 62·1% (36 of 58 patients) in the gefitinib group and 32·2% (19 of 59 patients) in the cisplatin plus docetaxel group (p<0·0001). The difference was significant (29·9%, 95% CI 12·6–47·1%; p<0·0001). The disease control rate was also higher in the gefitinib group (54/58, 93·1%) than in the cisplatin plus docetaxel group (46/59, 78·0%; difference in disease control rate 15·1%, 95% CI 2·7–27·6, p=0·020; webappendix). Because of frequent and detailed postoperative follow-up, which is standard practice in Japan, only 28 of 71 patients were found to have recurrent disease that met criteria for RECIST—ie, greater than 1 cm in the largest diameter. At the data cut-off, only 27 patients (15·7%) had died. Therefore, data for overall survival were immature, with follow-up still ongoing; 17 events (deaths) in the gefitinib group versus 10 events in the chemotherapy group—with an HR for gefitinib of 1·638 (95% CI, 0·75–3·58; figure 5). 51 patients in the chemotherapy group received an EGFR-TKI after they completed the study; 17 patients in the gefitinib group received post-protocol platinum doublet chemotherapy.

Adverse events occurring in more than 10% of either of the treatment groups are listed (table 3). The most common adverse event in the gefitinib group was skin rash followed by liver dysfunction, dry skin, and diarrhoea. However, adverse events with CTC grade 3 or more were infrequent, with the exception of liver dysfunction. By contrast, the most common adverse events in the cisplatin plus docetaxel group, which occurred in more than half of patients, were nausea, myelosuppression, fatigue, and alopecia.

Other potentially treatment-related toxicities included allergic reaction (one in gefitinib group, four in cisplatin plus docetaxel group) and oedema (one in gefitinib group, seven in the cisplatin plus docetaxel group). Two patients in the gefitinib group developed interstitial lung disease. There was one treatment-related death in the gefitinib group due to interstitial lung disease; there were no deaths in the cisplatin plus docetaxel group. There were no other serious adverse events.

Discussion

Our results show that first-line treatment with gefitinib conferred longer progression-free survival than treatment with cisplatin plus docetaxel in a molecularly defined (ie, EGFR mutation positive) group of patients with NSCLC.

In the IPASS study for patients with lung adenocarcinoma with no or former light smoking history, the progression-free survival of patients treated with gefitinib was significantly longer.[25] However, the curves crossed at the 6-month timepoint (initially chemotherapy was better, while gefitinib was better later). Molecular analysis for about a third of the patients suggested that the benefit of gefitinib was limited to patients with EGFR mutations with an HR of 0·48 (95% CI 0·36–0·64) and that gefitinib treatment was detrimental for patients without mutations (HR 2·85).[25] This result might seem similar to ours; however, the primary objective of the IPASS study was to assess gefitinib treatment in clinically selected patients,

Patient group		N	Median progression-free survival (months)			Median overall survival (months)	
			Gefitinib	Chemotherapy	HR (95% CI)	Gefitinib	Chemotherapy
Non-randomised pooled analysis							
I-CAMP[11]	Japanese, *EGFR* mutation	148	10·7	6·0	0·35 (0·23–0·52)	27·7	25·7
Subset analyses of the phase 3 trials for patients selected according to clinical backgrounds							
IPASS[25]	East Asian, light-non-smoker, adenocarcinoma	261	9·5	6·3	0·48 (0·36–0·64)	~20	~20
First SIGNAL[33]	Korean, non-smoker, adenocarcinoma	42	8·4	6·7	0·61 (0·31–1·22)	30·6	26·5
Phase 3 trials of patients selected according to *EGFR* mutation status							
NEJ 002[26]	Japanese, *EGFR* mutation	194	10·4	5·5	0·357(0·252–0·507)	28·0	23·6
WJTOG3405	Japanese, *EGFR* mutation	172	9·2	6·3	0·489 (0·336–0·710)	..	..

Table 4: Recent clinical trials assessing EGFR mutations as predictors of efficacy of gefitinib compared with chemotherapy

and not in molecularly selected patients, as was the case in our trial. In this context, a HR of 0·36 (95% CI 0·25–0·51)[26] for gefitinib compared with carboplatin plus paclitaxel in patients selected by EGFR mutation is highly relevant. Furthermore, our pooled analyses based on individual patient data from seven Japanese phase 2 studies that assessed prospectively the efficacy of gefitinib for patients with EGFR mutations (I-CAMP study)[11] and the pooled analysis of 1006 patients enrolled in a phase 3 trial of gefitinib[27] also showed similar progression-free survival of about 10 months for patients harbouring an EGFR mutation who were treated with gefitinb, while the median progression-free survival of patients treated with chemotherapy was 6·0 months (table 4).[11] These results strongly suggest that the presence of EGFR mutations, and not the clinical background of patients, determines clinical efficacy, and this knowledge should lead to molecularly based, personalised treatment of lung cancer.

Since the median duration of each treatment was quite different (165 days for gefitinib compared with 64 days for chemotherapy), one interpretation might be that a maintenance effect of gefitinib therapy contributed to the positive progression-free survival outcome, at least in part. Indeed, the progression-free survival curves of both groups in IPASS were initially similar, and then separate at about the time that chemotherapy stops. However, this was not the case in our trial, especially in patients with stage IIIB/IV disease. Furthermore, the SATURN[28] and the FAST-ACT[29] trials that tested maintenance erlotinib after chemotherapy showed that progression-free survival (both trials) and overall survival (SATURN) was prolonged. The benefit was much greater in patients with an *EGFR* mutation than in those without it in the SATURN trial.[28]

According to analyses of five US and European clinical trials that assessed first-line TKI treatment,[12] patients with the exon 19 deletion have a significantly longer progression-free and overall survival than patients with L858R (30·8 *vs* 14·8 months; p<0·0001). A similar trend was shown in a recent Spanish study.[13] In IPASS, the HR for progression-free survival for gefitinib versus chemotherapy was 0·38 (95% CI 0·25–0·56) in the subgroup of patients with exon 19 deletions, and 0·55 (95% CI 0·35–0·87) in the L858R mutation subgroup, although a direct comparison between exon 19 deletion and L858R in the gefitinib group was not done.[30] However, recent Japanese trials, including I-CAMP[11] and this study, did not detect any difference. The reason for this discrepancy is not clear, although it might be attributable to ethnic differences or difference of EGFR-TKI used between study populations.

Two patients in the gefitinib group (2·3%) developed interstitial lung disease, one of whom died. This incidence was low compared with previous Japanese reports of 4·0% (59/1482)[31] and 3·5% (70/1976).[32] Selecting patients according to EGFR mutation status is expected to reduce the risk of interstitial lung disease, because risk factors for interstitial lung disease include smoking, male sex, and squamous histology, all of which are negative predictors of the presence of EGFR mutations.[31,32]

Our study indicates that EGFR genetic testing is feasible and should be done when possible. Although patients without EGFR mutations were not included in our study, potential harm of first-line gefitinib therapy compared with chemotherapy for patients without EGFR mutation shown in the IPASS[25] and the First-SIGNAL[33] study indicate the necessity of patient selection by EGFR mutation.

Clinical background might help identify patients who have a higher chance of carrying EGFR mutations. However, it should be noted that in a previous study,[9] eight of 37 (22%) patients with lung adenocarcinoma with a history of heavy smoking (>50 pack-years) harboured EGFR mutations.[9]

In conclusion, gefitinib significantly prolonged the progression-free survival of patients with NSCLC who carry EGFR mutations compared with cisplatin plus docetaxel. It is not yet known whether the prolonged progression-free survival conferred by gefitinib will translate into prolonged overall survival; we will continue to carefully follow-up our patients to determine its long-term effects. Considering the efficacy and toxicity of gefitinib, it is a reasonable option for the first-line treatment of patients with activating EGFR mutations.

Contributors

TM, SM, SN, TS, MS, NK, and KN were involved in the conception and design of the study. KN and MF supervised the study. TM, IO, TS, MS, HT, TH, KA, NK, MT, HY, KS, SK, ES, HS, and ST were involved in the

provision of study material, patients, and data acquisition. TM, SM, YY, SN, IO, JT, TH, NK, MT, HY, KS, ES, HS, ST, and KN were involved in data analysis and interpretation. SM was in charge of the statistical design of the study. YY was in charge of *EGFR* gene testing at the central laboratory. All authors were involved in writing the report and approved the final version.

Conflicts of interest

TM has received lecture fees from AstraZeneca, Chugai, and Boehringer-Ingelheim. SN has received honoraria from AstraZeneca and Sanofi-Aventis. MS has received honoraria from AstraZeneca. HT has received honoraria from AstraZeneca and Sanofi-Aventis. ST has received honoraria from AstraZeneca and Chugai. KN has received lecture fees from AstraZeneca, Chugai, and Boehringer-Ingelheim. MF has received lecture fees from AstraZeneca, Chugai, and Boehringer-Ingelheim. All other authors declared that they have no conflicts of interest.

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Clinical Cancer Research

Immunohistochemical Detection of *EGFR* Mutation Using Mutation-Specific Antibodies in Lung Cancer

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Abstract

Purpose: Patients with mutations of *epidermal growth factor receptor (EGFR)* receive more benefit from *EGFR*-tyrosine kinase inhibitor treatment. However, usually such treatment is used to treat advanced lung cancer and only small biopsy samples are available for mutational analysis. We used immunohistochemistry to examine recently developed antibodies specific to major hotspot mutations of L858R and DEL E746-A750.

Experimental Design: We used five series of lung cancers: 47 non–small cell lung cancers (NSCLC) to evaluate various types of *EGFR* mutations, a consecutive series of 238 NSCLCs to study the sensitivity and specificity, 11 NSCLCs with both *EGFR* mutation and amplification to examine the spatial distribution, 32 patients treated with gefitinib to compare clinical responses, and 15 NSCLCs to explore changes associated with acquired T790M mutation.

Results: Each antibody specifically recognized the corresponding mutation but also recognized other types of mutations. Overall specificity and sensitivity were 96% and 47%, respectively. The positive reaction showed heterogeneous distribution that agreed with the expression of the total *EGFR* molecule, part of which was associated with gene amplification. A clinical response to gefitinib treatment correlated with the reaction, although one of the two patients with a positive reaction responded well despite having the wild-type *EGFR*. Acquired T790M mutation did not change the reaction to the antibodies.

Conclusions: On some characteristics, the positive reaction to mutation-specific antibodies differs from the molecular *EGFR* mutation. Therefore, this study revealed that not all patients with EGFR mutations can be selected using these mutation-specific antibodies. *Clin Cancer Res; 16(13); 3349–55. ©2010 AACR.*

Epidermal growth factor receptor (*EGFR*) is a receptor with a tyrosine kinase domain and plays essential roles in physiologic and neoplastic conditions (1, 2). Mutation in the tyrosine kinase domain has been identified in a subset of lung cancer (3–5), and its mutational status is associated with the clinical response to *EGFR*-tyrosine kinase inhibitor (TKI) treatment (6, 7). This association solved the enigma of why female, nonsmoking, adenocarcinoma patients of East Asian origin with lung cancers have a higher response to *EGFR*-TKIs; it is because patients with these characteristics have a higher incidence of *EGFR* mutation (8, 9). Similarly, much has been elucidated about the clinical response to the *EGFR*-TKIs and the characteristics of *EGFR* tyrosine kinase mutation (10, 11). Patients with *EGFR*-mutated lung cancers who initially achieve a marked response to *EGFR*-TKI treatment eventually develop progression of the disease during the course of treatment, which is caused, at least partly, by a secondary mutation, such as T790M (12, 13). The results of several clinical trials indicate that *EGFR* gene copy number is also a response predictor (6), and that an exaggerated form of increased copy number, *EGFR* amplification, is associated with progression of adenocarcinoma (14, 15).

Increasing clinical evidence suggests that patients with *EGFR* mutation receive more benefits from *EGFR*-TKI treatment (2, 6). *EGFR*-TKI treatment has been shown recently to be superior to carboplatin-paclitaxel as an initial treatment (16). Therefore, mutation analysis plays an important role in the initial treatment in many patients with advanced lung cancers. Many techniques are available to examine *EGFR* mutation, including direct sequencing, Scopion amplified refractory mutation system (ARMS), polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP), peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp, smart amplification process (SMAP), and cycleave PCR (17). Because *EGFR*-TKI treatment is used mainly to treat advanced stages of lung cancer, mutation analysis is done with a small biopsy sample, which is embedded in paraffin in most cases. However, DNA extracted from paraffin-embedded tissues is often degraded and mixed with a significant amount of

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Translational Relevance

Mutation analysis of *epidermal growth factor receptor (EGFR)* plays an important role in the initial treatment in many patients with advanced lung cancers, in which only small biopsy samples are usually available. Immunohistochemistry has an advantage in examining such small biopsy specimens. In this study, we evaluated recently developed antibodies, which were generated to recognize major hotspot mutations of L858R and DEL E746-A750. Each antibody was specific to the corresponding mutation. However, the positive reaction showed some characteristics that differed from those of the molecular mutation, including recognition of different mutation types, association with expression of the total *EGFR* molecule, and heterogeneous distribution of the positive reaction. A positive reaction correlated generally with a clinical response to *EGFR*-TKI treatment, but not all patients with EGFR mutations can be selected using these mutation-specific antibodies.

normal tissue. Yu et al. (18) recently developed mutation-specific rabbit monoclonal antibodies against *EGFR* with the E746-A750 deletion in exon 19 or the L858R point mutation and showed that these antibodies can be applied to the immunohistochemical detection of the mutations using paraffin-embedded tissue. Because these antibodies are available commercially, we applied them in lung cancer samples with various *EGFR* mutation types. We also analyzed tissues from a cohort of patients with lung cancer in a region with a high incidence of *EGFR* mutation. Furthermore, we correlated these data with the clinical response to *EGFR*-TKI treatment and in lung cancers with *EGFR* amplification and secondary T790M mutation.

Materials and Methods

Patients and samples

We used five independent data sets in this study. To evaluate various types of *EGFR* mutations, 47 cases of non–small cell lung cancer (NSCLC) were selected from a database held at the Department of Pathology and Molecular Diagnostics, Aichi Cancer Center, Nagoya, Japan. To determine the sensitivity and specificity in a cohort, we used tissue microarray data from a consecutive series of 238 NSCLCs that had been surgically resected from 2002 to 2003 in the Aichi Cancer Center. In this cohort, invasive tumors <1 cm in diameter were excluded because of difficulties in generating the tissue microarray. A part of this cohort has been reported (19), and the mutational status of the NSCLCs has been examined. To compare the immunohistochemical reactions with the clinical response to gefitinib treatment, we used 32 patients, whose data were reported previously (7). Using 10 adenocarcinomas and an adenosquamous cell carcinoma, whose copy numbers have been reported, we compared the spatial distribution between a positive reaction to the mutation-specific antibodies and gene copy number. In each tumor, three independent portions were selected to reflect the morphologic characteristics, and the status of the *EGFR* mutation and amplification were determined (14). To analyze the T790M mutation, 15 NSCLCs from nine patients were selected from the database. Six were patients who were treated with gefitinib and subsequently developed resistance caused by a secondary T790M mutation (19, 20). In the other three patients, simultaneous mutation of L858R and T790M was detected in the surgically resected primary adenocarcinomas, but the patients were not treated with *EGFR*-TKI before surgery. All specimens examined in this study were obtained from primary lung tumors, except for the T790M analysis, in which recurrent tumors of skin and lymph nodes were used. This study was a part of a comprehensive lung cancer research program, which had been approved by an institutional review board. Written informed consent for participation in the program was obtained from each patient.

Immunohistochemistry

Immunohistochemical examination proceeded according to the standard avidin-biotin-peroxidase complex method using monoclonal rabbit antibodies against L858R and DEL E746-A750 (clone 43B2 and clone 6B6, respectively, Cell Signaling Technology). Antigens were retrieved by microwave for 15 minutes in EDTA buffer (pH 9.0). To access expression of total EGFR molecule, we used the EGFR pharmDX kit (DAKO). Briefly, 4-mm-thick formalin-fixed and paraffin-embedded tissue sections were deparaffinized in xylene, treated with 0.3% hydrogen peroxide in methanol for 20 minutes to block endogenous peroxidase activity, microwaved for antigen retrieval, and incubated with 10% normal goat serum for 30 minutes to block nonspecific binding. The rabbit monoclonal antibodies were then applied as the primary antibody at a dilution of 1:100 at 4°C overnight. The subsequent procedure followed the manufacturer's instructions (Vectastain ABC Elite kit, Vector Laboratories). The sections were incubated with biotinylated anti-rabbit antibody for 40 minutes at room temperature and then with the ABC complex for 30 minutes. Color was developed by the diaminobenzidine reaction. In between each incubation step, the sections were washed with cold PBS.

Tissue microarray slides stained with these antibodies were digitized using an Aperio ScanScope (Aperio Technologies, Inc.) and were then analyzed using the TMALab Microarray Analysis Tool. The tissue cores scoring 1+ or more based on membrane algorithms were reevaluated under a microscope, and a tissue core was considered positive when an appropriate positive reaction was confirmed. Other staining was done using normal-sized sections of the tumor. In these sections, the positive intensity was scored as 1+, 2+, and 3+, which were equivalent to the intensity based on the computer-quantified analysis of tissue

microarrays. A tumor was recorded as positive when 10% or more of the tumor cells had an intensity score of 1+ or more. The immunohistochemical reactions were evaluated by the last author noted (Y.Y.) and at least one of the other authors.

EGFR mutation and amplification

Mutation data for the *EGFR* gene were obtained from the database, which has been analyzed using direct sequencing of reverse transcription-PCR products for fresh frozen tissues, and/or a cycleave PCR technique (L858R and T790M) and fragment analysis (deletion of exon 19) of paraffin-embedded tissues. These methods have been described elsewhere (7, 19, 20). To access the amplification status of EGFR, we used data previously reported (14). Briefly, we examined three portions of each individual tumor using two methods: quantitative PCR with microdissected fragments and fluorescent *in situ* hybridization.

Results

Positive reaction among a spectrum of *EGFR* mutations

We first examined the specificity of the two antibodies specific to the L858R point mutation and in-frame deletion in exon 19 (DEL E746-A750) using lung cancers with the spectrum of *EGFR* mutations listed in Table 1. Anti-DEL antibody showed a positive reaction in 4 of 16 mutation types in exon 19 (Fig. 1). All of these involved E746-A750. DEL E746-A750insK was positive, whereas DEL E746-A750insRP and DEL L747-A750insP were negative. The other deletions in exon 19 were negative. In contrast, anti-L858R mutation–specific antibody reacted in all 12 lung cancers with the L858R mutation, which involves a different pattern of nucleotide substitution (CTG → CGT). Additional point mutations adjacent or near the L858R mutation (A859S and A861S) also showed a positive reaction. Of note, these antibodies were positive for other types of mutations, including G719C, DEL L747-T751insQ, and A769insASV with anti-L858R–specific antibody, and D770insSVD with anti-deletion–specific antibody (Fig. 1), although such positive reactions were not always seen in the tumors with these mutation types. In addition, anti-L858R–specific antibodies frequently showed a faint reaction in tumors with deletional mutation in exon 19.

Table 1. Positive reactions among a spectrum of *EGFR* mutations

Genotype	Immunohistochemistry		
	n	Anti-L858R Ab	Anti-DEL Ab
Exon 18 (*n* = 5)			
G719A	1	0	0
G719C	2	1	0
G719S	2	0	0
Exon 19 (*n* = 21)			
DEL E746-A750	3	0	3
DEL E746-A750	2	0	2
DEL E746-A750 (2253 A → G T751T)	1	0	1
DEL E746-A750 ins K	2	0	2
DEL E746-A750 ins RP	1	0	0
DEL L747-A750 ins P	1	0	0
DEL L747-P753 insS	1	0	0
DEL L747-S752	2	0	0
DEL L747-S752 insV	1	0	0
DEL L747-T751 ins A	1	0	0
DEL L747-T751 ins Q	1	1	0
DEL S721-I759	1	0	0
DEL T751-A755 DEL E758-I759 ins A	1	0	0
DEL T751-I759 ins S	1	0	0
E746 ins VPVAIK	1	0	0
K744 ins KIPVAI	1	0	0
Exon 20 (*n* = 7)			
A769 ins ASV	2	1	0
D770 ins SVD	3	0	1
H773 ins NPH	1	0	0
P772 ins A	1	0	0
Exon 21 (*n* = 14)			
L858R(CTG → CGG)	11	11	0
L858R(CTG → CGT)	1	1	0
L858R & A859S	1	1	0
L858R & A861S	1	1	0
Total	47	19	8

Correlation of the positive reaction with *EGFR* genotype

We then examined a consecutive series of 238 lung cancers, and the results are summarized in Table 2. The overall sensitivity and specificity using the two specific antibodies were 92% and 37%, respectively. The anti-DEL–specific antibody had higher specificity and sensitivity than the L858R-specific antibody (99% and 40% versus 97% and 36%, respectively). Four tumors with a false-positive reaction were reexamined using normal-sized sections but produced the same results. Because 226 of the 238 tumors (95%) were evaluated using at least three tissue microarray cores, the distribution of the positive reaction was compared between the cores of individual tumors. Of 33 NSCLCs with a positive reaction, the reaction was heterogeneously distributed in 15 (45%).

Gene amplification and mutation-specific antibody reaction

We recently reported that *EGFR* is amplified heterogeneously in individual tumors and that this amplification was associated with invasive growth. To test whether this heterogeneous amplification is associated with a similar pattern of distribution of the positive reaction to the mutation-specific antibodies, we examined the correlation between the amplification and the positive reaction with mutation-specific and conventional *EGFR* antibodies.

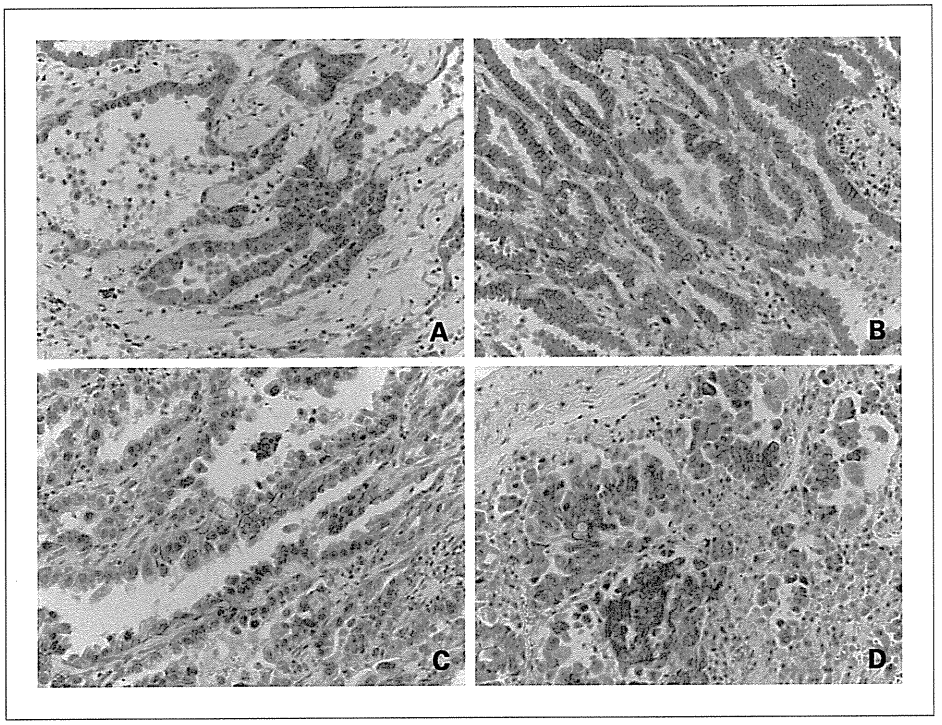

Fig. 1. Representative positive reactions of lung adenocarcinomas with DEL E746-A750 mutation stained with anti-DEL antibody (A), with L858R point mutation stained with anti-L858R antibody (B), with A769 ins ASV mutation in exon 20 stained with anti-L858R antibody (C), and with G719A mutation stained with anti-L858R antibody (D).

Using the amplification data of the individual tumors in the previous study (14), we compared the positive reaction with amplification status. The intensity of the positive reaction correlated significantly with the relative *EGFR* copy number *EGFR* (one-way ANOVA, $P < 0.01$; Fig. 2), whereas the amplification status did not correlate with the expression of total *EGFR* (one-way ANOVA, $P = 0.355$). Both expressions of mutation-specific and total *EGFR* was associated (one-way ANOVA, $P < 0.05$). These findings suggest that the positive reaction to mutation-specific antibodies is associated with *EGFR* amplification but that the amplification is not the sole determinant of a positive reaction.

Prediction of the *EGFR*-TKI response using mutation-specific antibodies

We expected that the positive reaction would be associated with a clinical response to *EGFR*-TKI treatment because the positive reaction was seen nearly exclusively in lung cancer with the *EGFR* mutation. In a series of 32 patients treated with gefitinib, 12 (63%) of 19 lung cancers with *EGFR* mutation were positive for mutation-specific antibodies, and 10 (71%) of 14 lung cancers with a positive reaction responded clinically to gefitinib treatment (Table 3). Of note, one patient whose lung cancer was positive for the mutation-specific antibodies showed good

Table 2. Relationship between genotype and positive reaction to the mutation-specific antibodies

Histologic subtype	Genotype				Total
	Wild-type (n = 151)	DEL Exon19 (n = 41)	L858R (n = 37)	Other type (n = 9)	
Adenocarcinoma	96	39	37	9	181
Other types*	55	2	0	0	57
Immunohistochemical reaction					
Negative	147	24	25	9	205
Positive	4	17	12	0	33
Anti-DEL antibody	0	16	1	0	17
Anti-L858R antibody	4	2	12	0	18
Heterogeneous†	1	9	5	0	15

*Other histologic types include 11 large cell carcinoma, four small cell lung cancer, five adenosquamous carcinoma, and one carcinoid tumor.
†Heterogeneous denotes different staining intensity in at least one of the tissue microarray cores in individual tumors.

response to the gefitinib treatment despite having the wild-type *EGFR* gene.

Reaction of tumors with secondary resistant mutations

Our data suggest that conformational changes of *EGFR* protein similar to L858R or E746-A750 in other mutation types might affect the positive reaction because both anti-L858R and anti-DEL antibodies reacted to the other types of mutations, such as G719C and D770insSVD (Table 1). Conversely, secondary mutations acquired in association with drug resistance might affect the reaction pattern. We examined lung cancers with T790M mutation in addition to the classic L858R and deletional mutations. Six lung adenocarcinomas with acquired resistance to gefitinib treatment had a positive reaction, and this finding is similar to that found in the primary lung adenocarcinomas examined in five patients (Table 4). The reaction in three primary lung adenocarcinomas with both L858R and T790M point mutations was similarly positive. These findings suggest that the secondary T790M mutation did not affect the positive reaction.

Discussion

Similar to the original report by Yu et al. (18), our data confirm the specificity of the mutation-specific antibodies. In this study, data from tissue microarray and gefitinib-treated group revealed that the overall specificity of these antibodies to *EGFR* mutation was 96%. In contrast, the overall sensitivity of 47% based on both data sets was much lower than the 92% reported by Yu et al (18). A possible explanation for this discrepancy may be the relatively low detection rate for deletional mutation in exon 19. As shown in Table 1, anti-DEL–specific antibody detected only the mutation type of DEL E746-A750 and that

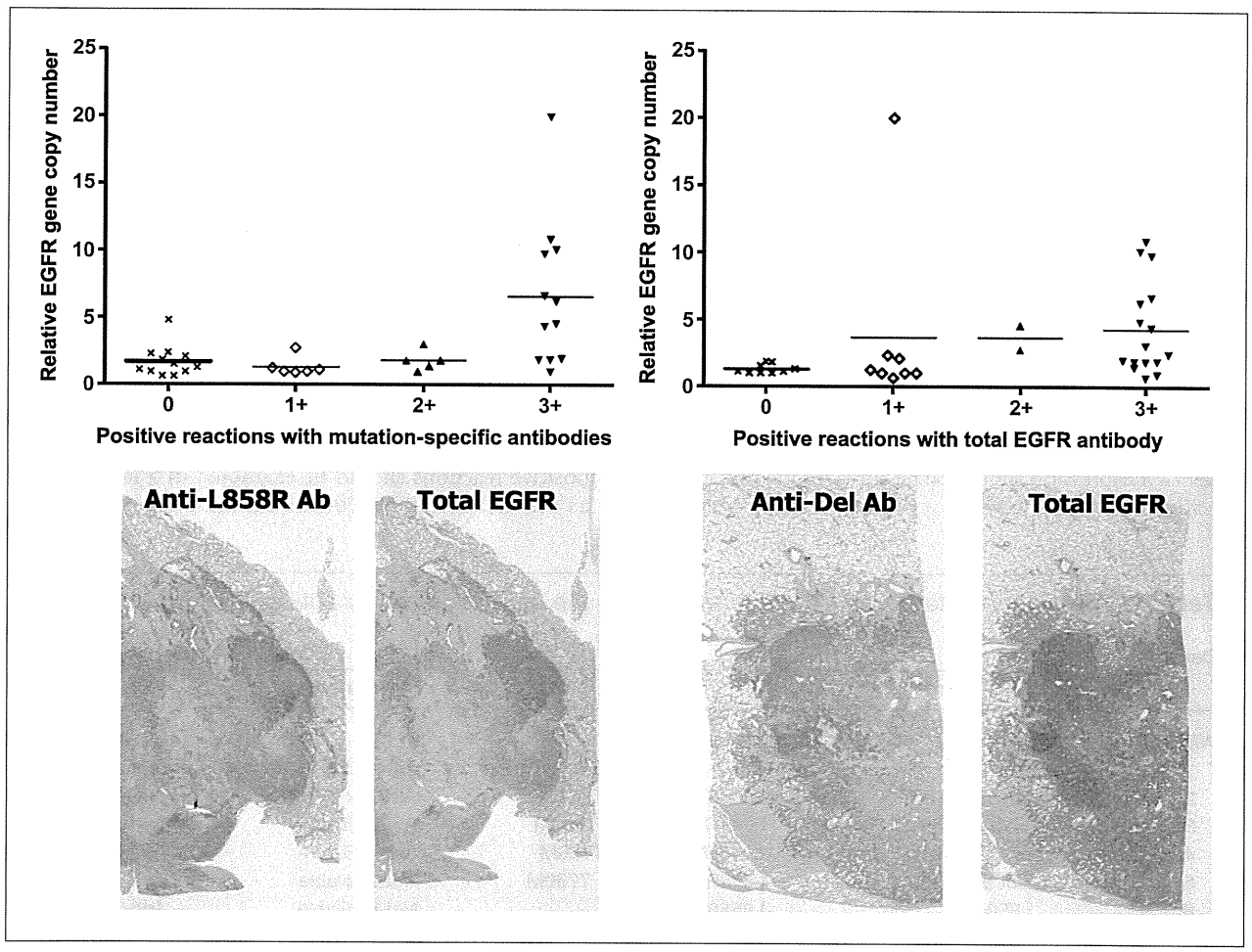

Fig. 2. Positive reaction of mutation-specific antibodies (Ab) and gene copy number. Top, the relationship of the gene copy number with the intensity of the positive reaction to mutation-specific antibodies (left) and total *EGFR* antibody (right). An intense positive reaction (score 3+) tended to show gene amplification with both antibodies. Similar to *EGFR* amplification, the positive reaction was also distributed heterogeneously. In general, a positive reaction correlated with the expression of the total *EGFR* molecule (bottom left), but this was not always the case (bottom right).

Table 3. Response to gefitinib treatment and positive reaction to the mutation-specific antibodies

Response to gefitinib	Mutation-specific antibodies	
	Positive	Negative
Responded	10	7
EGFR, wild-type	1	1
EGFR, mutated	9	6
Did not respond	4	11
EGFR, wild-type	1	10
EGFR, mutated	3	1

with minor modification at the edge of the deleted region, including DEL E746-A750insK. In our cohort of 511 patients with NSCLCs from 2000 to 2006, DEL E746-A750 and its minor variants, which were expected to be positive for anti-DEL antibody, comprised 53% of the 105 deletions in exon 19 (data not shown). However, anti-L858R antibody also showed a lower sensitivity (40%), suggesting that other mechanisms may be involved.

Because these two antibodies reacted to the corresponding mutated form of *EGFR* protein, the positive reaction should be reflected in the expression status of *EGFR*. In general, *EGFR* expression is associated with increased *EGFR* copy number but not with mutational status (21, 22), and this finding was confirmed in our study. It is likely that the positive reaction to the mutation-specific antibodies is also affected by the *EGFR* gene copy number. A strongly positive reaction was found almost exclusively in tumors with *EGFR* amplification, as shown in Fig. 2. Similarly, a low-level expression of mutated protein is associated with the expression of total *EGFR*, and thus, tumors without detectable *EGFR* expression should be negative for the mutation-specific antibodies. However, a positive reaction was not detected in some tumors, even when total *EGFR* protein was expressed, as shown in Fig. 2. Some other mechanisms, such as differences in posttranscriptional modifications or in the detection thresholds, might be involved.

Interestingly, although these two antibodies were generated to be specific for the L858R point mutation or for the deletion in exon 19, these antibodies reacted with other mutations (Fig. 1C and D). Because the antibody recognizes a part of the conformational composition, the positive reaction suggested that other mutation types generate or form a conformational composition similar to that of L858R and the deletion in exon 19. We examined whether conformational changes caused by T790M secondary resistant mutation lose such conformational composition, but we found that T790M did not change the composition recognized by these mutation-specific antibodies. Another point of interest is that one of two lung cancers with a positive reaction, which did not harbor *EGFR* mutation, showed a clinical response to gefitinib treatment. About 10% of *EGFR*-TKI responders do not have *EGFR* mutation (7, 23), and analysis of lung cancer with this false-positive reaction may provide a clue to identifying another mechanism responsible for the response to *EGFR*-TKI treatment.

This study reveals some pitfalls in applying these antibodies in place of a mutation test. First, the immunohistochemical results have clinical significance only when the reaction to the antibodies was positive. The overall sensitivity was relatively low (47%), and one fourth of immunohistochemically negative lung cancer may have had the *EGFR* mutation. Second, the antibodies may react to insertion mutation in exon 20, which is resistant to *EGFR*-TKI treatment. In this study, two (29%) of seven lung cancers with an insertion in exon 20 showed a positive reaction. Third, even when the antibodies showed a positive reaction, ~10% of such lung cancers may not have had an *EGFR* mutation. The clinical significance of these false-positive reactions should be examined in a larger cohort, as mentioned above. Fourth, tumor heterogeneity may

Table 4. Secondary mutation and positive reaction to the mutation-specific antibodies

Case ID	Genotype		Immunophenotype	
	Primary	Secondary	Primary	Secondary
With gefitinib treatment				
1	DEL	DEL + T790M	Anti-DEL: 1+	Anti-DEL: 2+
2	L858R+V834L	L858R + V834L + T790M	Anti-L858R: 2+	Anti-L858R: 3+
3	E747-753 insS	E747-753 ins S + T790M	Anti-DEL: 3+	Anti-DEL: 2+
4	E745-750 insK	E745-750 insK + T790M	Anti-DEL: 3+	Anti-DEL: 3+
5	Not available	L858R + H776R + T790M	Not available	Anti-L858R: 2+
6	L858R	L858R + T790M	Not available	Anti-L858R: 3+
Without gefitinib treatment				
1	L858R + T790M			Anti-L858R: 2+
2	L858R + T790M			Anti-L858R: 2+
3	L858R + T790M			Anti-L858R: 1+

cause a false-negative result. The heterogeneous distribution is crucial especially in the diagnosis of biopsy specimens. Table 2 is a summary of the results of the tissue microarray analysis, which used tissue cores to simulate real biopsy samples. The microarray analysis showed that 46% of lung cancer samples with a positive reaction showed a different reaction in at least one of the three or four cores. Taken together, these findings suggest that the clinical utility of these antibodies may be less than expected.

In summary, we examined *EGFR* mutation–specific antibodies. The antibodies showed specific reactions to *EGFR* mutations. However, the positive reaction showed some characteristics that differed from those of the molecular mutation, including recognition of different mutation types, association with expression of the total *EGFR* molecule, and heterogeneous distribution of the positive reaction. Despite a limited number of patients examined, a positive reaction correlated generally with a clinical response to *EGFR*-TKI treatment. Although these antibodies were relatively specific, not all patients with EGFR mutations can be selected using these mutation-specific antibodies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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BRIEF REPORT

EML4-ALK Mutations in Lung Cancer That Confer Resistance to ALK Inhibitors

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SUMMARY

The EML4 (echinoderm microtubule-associated protein-like 4)–ALK (anaplastic lymphoma kinase) fusion-type tyrosine kinase is an oncoprotein found in 4 to 5% of non–small-cell lung cancers, and clinical trials of specific inhibitors of ALK for the treatment of such tumors are currently under way. Here, we report the discovery of two secondary mutations within the kinase domain of EML4-ALK in tumor cells isolated from a patient during the relapse phase of treatment with an ALK inhibitor. Each mutation developed independently in subclones of the tumor and conferred marked resistance to two different ALK inhibitors.

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EML4-ALK IS A FUSION-TYPE PROTEIN TYROSINE KINASE THAT IS PRESENT in 4 to 5% of cases of non–small-cell lung cancer and is generated as a result of a small inversion within the short arm of human chromosome 2.[1-3] EML4-ALK undergoes constitutive dimerization through interaction between the coiled-coil domain within the EML4 region of each monomer, thereby activating ALK and generating oncogenic activity. In transgenic mice that express EML4-ALK specifically in lung epithelial cells, hundreds of adenocarcinoma nodules develop in both lungs soon after birth, and oral administration of a specific inhibitor of ALK tyrosine kinase activity rapidly eradicates such nodules from the lungs.[4] These observations reveal the essential role of EML4-ALK in the carcinogenesis of non–small-cell lung cancer harboring this fusion kinase. Furthermore, clinical trials are investigating crizotinib (PF-02341066), an inhibitor of the tyrosine kinase activity of both ALK and the met proto-oncogene (MET), for the treatment of EML4-ALK–positive non–small-cell lung cancer.

In addition to crizotinib, other tyrosine kinase inhibitors have been shown to have pronounced therapeutic activity in patients with cancer. For instance, imatinib mesylate and gefitinib, tyrosine kinase inhibitors for the c-abl oncogene 1 nonreceptor tyrosine kinase (ABL) and epidermal growth factor receptor (EGFR), improve the outcome for patients who have chronic myeloid leukemia that is positive for the BCR (breakpoint cluster region protein)–ABL fusion kinase[5] and patients who have non–small-cell lung cancer that is associated with EGFR activation,[6]

respectively. Unfortunately, however, a fraction of the target tumors are either refractory to corresponding tyrosine kinase inhibitors from the start of treatment or become resistant after an initial response.

In a case of EML4-ALK–positive non–small-cell lung cancer that became resistant to crizotinib after successful treatment for 5 months, we have discovered two de novo mutations in EML4-ALK, each of which confers resistance to the drug.

CASE REPORT

The patient was a 28-year-old man without a history of smoking who had received a diagnosis of lung adenocarcinoma, at a tumor–node–metestasis (TNM) clinical stage of T4N3M1, in April 2008. Given that the tumor did not harbor any EGFR mutations, the patient was treated with conventional chemotherapy. However, his tumor progressed after six cycles of three two-drug combinations. In November 2008, the presence of *EML4-ALK* variant 1 messenger RNA (mRNA)[1] in the tumor was confirmed by means of reverse transcription–polymerase-chain-reaction (PCR) analysis of a sputum sample. At this stage, the patient had large tumor nodules in the hilum of the right lung, multiple enlarged lymph nodes in the mediastinum, atelectasis in the right lung, and a massive effusion in the right pleural cavity (Fig. 1 in the Supplementary Appendix, available with the full text of this article at NEJM.org).

The patient was enrolled in the A8081001 study of crizotinib (ClinicalTrials.gov number, NCT00585195) on November 28, 2008, with oral administration of the drug at a dose of 250 mg twice per day. Within 1 week after the start of crizotinib treatment, his symptoms improved markedly. Although he had a partial response to the treatment, his pleural effusion was not completely eradicated (Fig. 1 in the Supplementary Appendix). After 5 months of treatment, however, the tumor abruptly started to grow again, resulting in a rapid expansion of the pleural effusion and in the development of tumors in both lungs (Fig. 1 in the Supplementary Appendix). The patient was withdrawn from the trial on May 25, 2009, and a sample of the pleural effusion in the right lung was then obtained for molecular analysis.

METHODS

DNA sequencing and characterization of the EML4-ALK mutants are described in detail in the Supplementary Appendix.

RESULTS

Because our patient's tumor resumed growth despite sustained administration of the ALK inhibitor crizotinib, we speculated that it might have acquired secondary genetic changes that confer resistance to the drug. Furthermore, given that resistance to tyrosine kinase inhibitors often results from acquired mutations within the target kinases,[7-9] we first examined the possibility that EML4-ALK itself had undergone amino acid changes.

Sputum specimens were obtained before crizotinib treatment, and pleural-effusion specimens were obtained after relapse, when treatment was stopped, for molecular analysis. Given that the proportion of tumor cells in the two types of specimens may have differed, we performed deep (high-coverage) sequencing of *EML4-ALK* complementary DNA (cDNA) derived from the specimens, using a high-throughput sequencer (Genome Analyzer II, Illumina) (Fig. 2 in the Supplementary Appendix). The sensitivity of our sequencing system, examined with the use of cDNA corresponding to the Janus kinase 3 (JAK3) amino acid mutation V674A[10] as a control, revealed that the maximum detection sensitivity was no more than one mismatched read per 6.50×10^5 total reads (Table 1 in the Supplementary Appendix).

Using deep sequencing, we detected a known single-nucleotide polymorphism, rs3795850, in the cDNA from the four specimens that were positive for *EML4-ALK* (Table 2 and Fig. 3 in the Supplementary Appendix). In addition, a T→C change at a position corresponding to nucleotide 4230 of human wild-type *ALK* cDNA (GenBank accession number, NM_004304) was detected at a low frequency (8.9%) in the sputum cDNA from our patient. Furthermore, two new alterations, G→A and C→A changes at positions corresponding to nucleotides 4374 and 4493 of wild-type *ALK* cDNA, were detected at frequencies of 41.8% and 14.0%, respectively, in the patient's pleural-effusion cDNA. There were no other recurrent alterations (present in 5% of reads) in the kinase-domain cDNA derived from any of the specimens.