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,

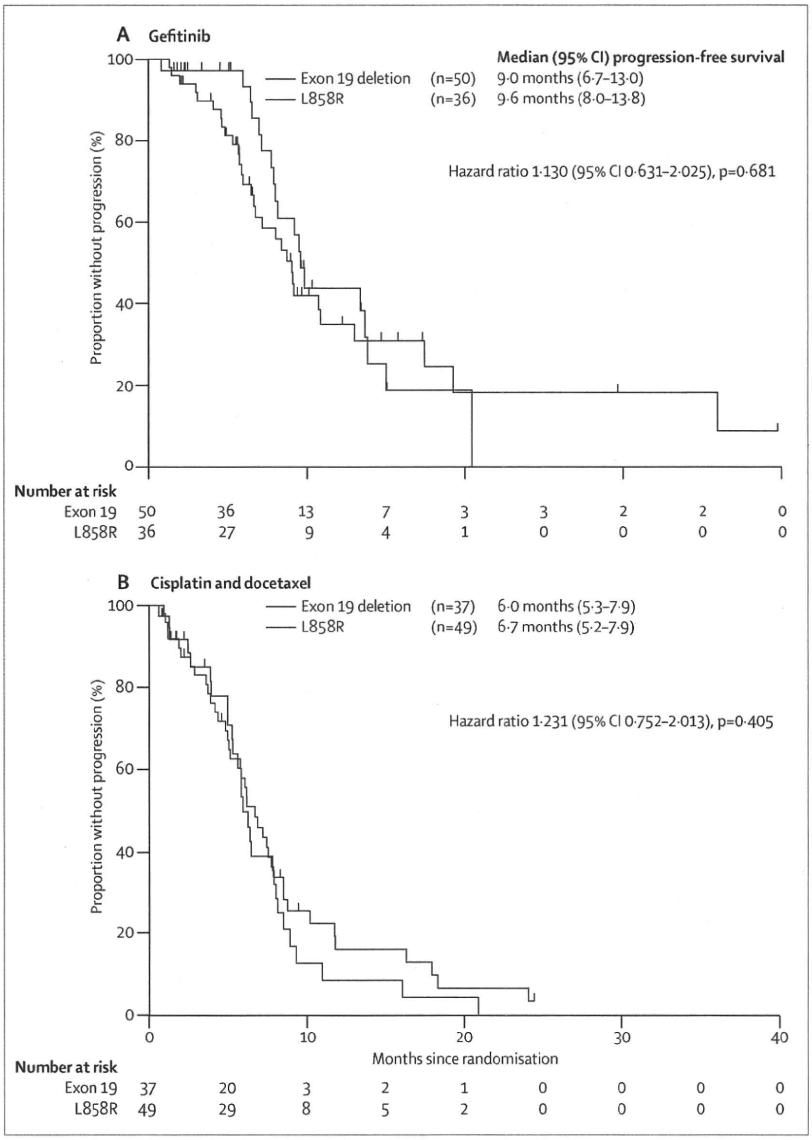

Figure 4: **Progression-free survival in (A) the gefitinib group and (B) the cisplatin plus docetaxel group according to type of the EGFR mutation**

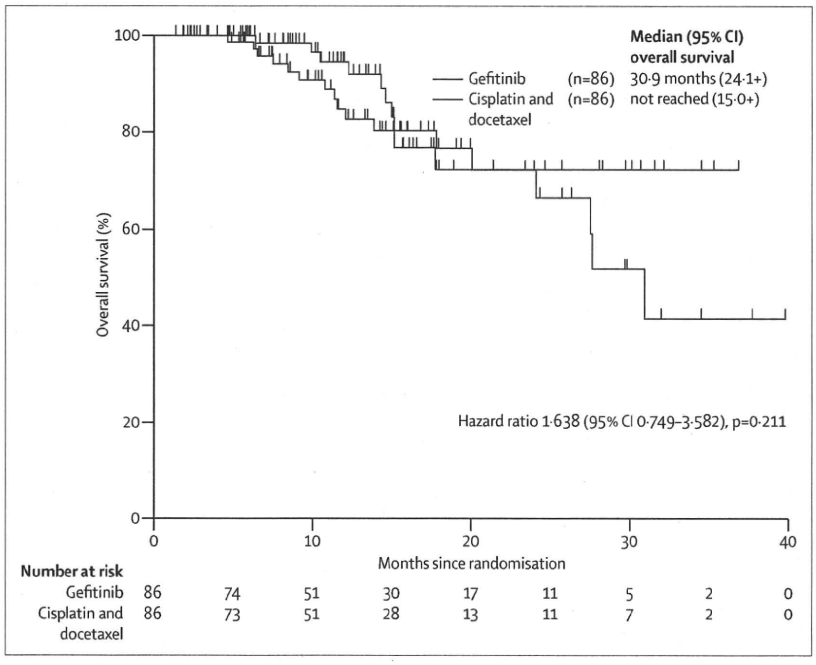

Figure 5: **Overall survival in the overall population**

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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Review Article: Molecular Target Treatment

Advances in Target Therapy for Lung Cancer

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Recent progress in molecular biology has shown that cancer cells acquire common phenotypes such as self-sufficiency of growth signals, resistance to anti-proliferative and apoptotic signals through the accumulation of genetic and epigenetic changes. Recently developed anticancer drugs target these molecular mechanisms and good results have been reported for various cancer types. In lung cancer, tyrosine kinase inhibitors specific for the epidermal growth factor receptor such as gefitinib and erlotinib have changed clinical practice dramatically. About half of the Japanese patients with lung cancers harbor an activating mutation of the epidermal growth factor receptor gene and they are very sensitive to epidermal growth factor receptor tyrosine kinase inhibitors. Progression-free survival of such patients is ~10 months when treated with gefitinib, whereas the survival for those treated with platinum doublet therapy is ~6 months. Target therapies against echinoderm microtubule-associated protein-like 4–anaplastic lymphoma kinase fusion protein or a mutated ERBB2 (v-ERB-B avian erythroblastic leukemia viral oncogene homologue 2) present in ~5% and ~3% of the Japanese patients with adenocarcinomas, respectively, are currently under development. Addition of an anti-epidermal growth factor receptor antibody, cetuximab, or anti-vascular endothelial growth factor antibody, bevacizumab, to platinum doublet therapy significantly but modestly prolonged the survival in recent clinical trials. However, clinical development of small molecule multi-kinase inhibitors including those targeting vascular endothelial growth factor receptors, such as vandetanib, sunitinib and sorafenib, has not been very successful. Through these collaborations among clinicians, basic researchers and pharmaceutical companies, it should be possible to individualize lung cancer treatment to turn this fatal disease into a chronic disorder and, eventually, to cure it.

Key words: EGFR – tyrosine kinase inhibitor – antibody therapy – oncogene addiction – angiogenesis inhibitors

INTRODUCTION

Lung cancer is a major cause of cancer-related mortality worldwide. Although various chemotherapeutic agents were developed in the late 1980s and 1990s, platinum doublet therapy seems to have reached a therapeutic plateau with an objective response rate of 30–40% and a median survival time (MST) of about 1 year for patients with Stage IIIB or IV disease (1,2). To circumvent this situation, a new class of drugs that specifically targets certain molecular pathways leading to cancer phenotypes is being developed. Table 1 is a partial list of such target drugs that are in clinical use or are being tested. This review describes recent advances of target therapy of lung cancer with an emphasis on molecular markers.

Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors

Small molecules that specifically inhibit the tyrosine kinase activity of the epidermal growth factor receptor (EGFR), such as gefitinib and erlotinib, were the first drugs to become clinically available in the treatment of non-small cell lung cancer (NSCLC). Gefitinib was approved in Japan

Table 1. Target drugs for lung cancer that are currently in clinical use or in development

Target	Mechanism	Drug
EGFR	TK inhibition	Gefitinib (Iressa), erlotinib (Tarceva)
	Antibody	Cetuximab (Erbitux), panitumumab, matuzumab
EGFR + ERBB2	TK inhibition	BIBW2992, PF00299804
ERBB2	Antibody	Trastuzumab (Herceptin)
ALK	TK inhibitor	PF02341066
VEGF	Antibody	Bevacizumab (Avastin)
VEGFR + EGFR	TK inhibition	Vandetanib (Zactima)
VEGFR + KIT + RAF	TK inhibition	Sorafenib (Nexabar)
VEGFR + KIT + PDGFR	TK inhibition	Sunitinib (Sutent), axitinib, AZD2171 (cediranib)
IGF1R	Antibody	CP751, 871
MET	TK inhibition	ARQ197, XL184
mTOR		CCI-779, RAD009
HSP90		17-AAG, 17-DMAG, CNF1010
Vascular disrupting agent		ASA-404 (DMXAA)

As of October 2009, only gefitinib and erlotinib are approved for treating patients with lung cancer in Japan. EGFR, epidermal growth factor receptor; TK, tyrosine kinase; ALK, anaplastic lymphoma kinase; VEGF, vascular endothelial cell growth factor; IGF1R, insulin like growth factor 1 receptor; mTOR, mammalian target of rapamycin; HSP90, heat shock protein 90.

in 2002 for the first time in the world. Asian women with adenocarcinomas and no history of smoking were found remarkably sensitive to EGFR-tyrosine kinase inhibitors (EGFR-TKIs) during the early clinical development of these drugs (3,4). In 2004, the reason for this high response rate was shown to be a somatic mutation of the *EGFR* gene in this subgroup (5,6). About 90% of these *EGFR* mutations are either short in-frame deletions in exon 19 (usually five amino acids) or point mutations that result in a substitution of arginine for leucine at amino acid 858 (*L858R*) (7). Subsequent retrospective and prospective studies showed that the response rate to EGFR-TKIs of patients with *EGFR* mutations was 70–80% (7). Furthermore, patients with *EGFR* mutations showed a significantly longer survival than those with wild-type *EGFR* when treated with EGFR-TKIs (8–10). However, data on the predictors for survival were initially controversial. Some investigators claimed that *EGFR* mutations were prognostic rather than predictive and that the *EGFR* gene copy number was more important (11), until the results of the IPASS study was reported (12). This was a Phase III trial that compared gefitinib with standard chemotherapy as a first-line treatment for Asian patients with lung adenocarcinomas, with no smoking history or only a light usage (12). Progression-free survival (PFS) of patients treated with gefitinib was significantly superior. However, the Kaplan–Meier survival curves crossed at 6 months (initially chemotherapy was better but later the gefitinib therapy was better). Molecular subset analysis for about one-third of the patients showed that the benefit was limited to patients with an *EGFR* mutation and that gefitinib treatment was detrimental for those without mutations (12). Furthermore, two recent Japanese trials (NEJ002 and WJTOG3405) selected patients according to the presence of an *EGFR* mutation. These trials confirmed that the determinant of clinical efficacy is the presence of an *EGFR* mutation and not the clinical background of the patient (13,14). The PFS of patients with EGFR mutation treated with gefitinib was around 10 months, whereas the PFS for those treated with platinum doublet chemotherapy was around 6 months. These figures are highly reproducible (Table 2).

It is almost inevitable for patients to show progression in the disease after presenting with an initial good response. A secondary mutation resulting in a change from threonine to methionine at codon 790 (*T790M*) is responsible for this acquired resistance in at least half of the patients (15,16). Substitution of the threonine at codon 790 with methionine restores the affinity of the EGFR protein to ATP to be higher than the affinity of EGFR to EGFR-TKI, resulting in resistance to EGFR-TKI (17). In about 20% of the patients with acquired resistance, *MET* gene amplification is found. The MET protein activates ERBB3 and subsequently AKT, resulting in evasion of apoptosis (18,19). To circumvent this acquired resistance, irreversible EGFR-TKIs such as PF002998904 (20) and BIBW2992 (21) are currently in clinical trials for patients with *T790M*. Patients with *MET* amplification could be treated with an EGFR-TKI plus an MET inhibitor (19).

EGFR Antibody

Antibodies directed against the extracellular domain of EGFR (such as cetuximab, matuzumab and panitumumab) are also in development for treating patients with lung cancers. There have been several randomized trials

Table 2. Summary of results of the recent clinical trials evaluating *EGFR* mutations as a predictive factor of the efficacy of gefitinib compared with chemotherapy in a first-line setting

Study	Patient selection	Median PFS (months)		Median OS (months)	
		G	CTx	G	CTx
I-CAMP (42) ($n = 148$)	Pooled analysis of seven prospective non-randomized studies for patients with *EGFR* mutations	10.7, HR = 0.35 (0.23–0.52)	6.0	27.2	25.7
IPASS (12) ($n = 261$)	Subset of patients with *EGFR* mutations in a Phase III randomized study	9.5, HR = 0.48 (0.36–0.64)	6.3	~20	~20
NEJ002 (13) ($n = 194$)	Phase III randomized study for patients with *EGFR* mutations	10.4, HR = 0.357 (0.252–0.507)	5.5	28.0	23.6
WJTOG3405 (14) ($n = 172$)	Phase III randomized study for patients with *EGFR* mutations	9.2, HR = 0.489 (0.336–0.710)	6.3	N/A	N/A

PFS, progression-free survival; OS, overall survival; G, gefitinib; CTx, chemotherapy; HR, hazard ratio. Numbers in parentheses are 95% confidence intervals.

comparing chemotherapy with chemotherapy plus cetuximab. The FLEX study was the first to show significant survival advantage by the addition of cetuximab to cisplatin plus vinorelbine, although the difference in MST was not great [i.e. 11.3 vs. 10.1 months; hazard ratio, 0.871; $P = 0.044$] (22). In addition, exploratory subset analysis for Asian patients ($n = 121$) revealed that prolongation of overall survival was not seen in this group (i.e. 17.6 months for the cetuximab group vs. 20.4 months for the control group) (22). However, small sample size (10% of total) and differences in histology and post-study EGFR-TKI treatments do not allow us to draw definite conclusions at present.

In patients with colorectal cancer, it is now established that a *KRAS* mutation is a negative predictive marker for any benefit of cetuximab treatment (23,24). However, this negative impact of *KRAS* mutations was not observed in the FLEX study (25).

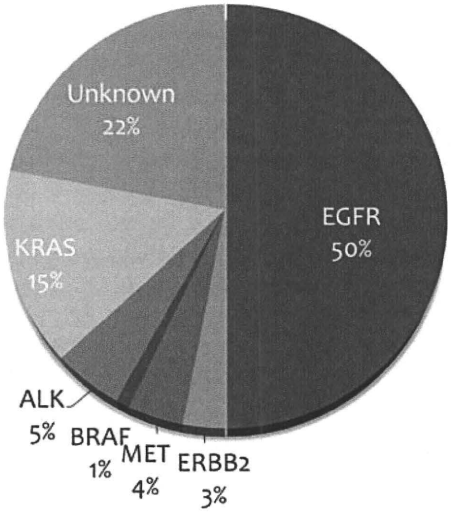

Figure 1. Molecular classification of lung adenocarcinomas according to the 'addicted oncogene'. EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma kinase.

Search for Another Achilles' Heel of NSCLC

In addition to *EGFR*, investigators have found molecular abnormalities in the oncogenes that occur in mutually exclusionary fashion each other, suggesting complementary roles of these mutations in lung carcinogenesis. These genes include *ERBB2*, *KRAS*, *BRAF*, and *MET* and translocation in anaplastic lymphoma kinase (*ALK*). Figure 1 shows the approximate incidence of these genetic disorders in Japanese patients with adenocarcinoma of the lung; more than three-quarters have at least one such mutation. It is anticipated that cancer cells usually contain multiple genetic and epigenetic abnormalities in each type of adenocarcinoma. For example, about 40% of adenocarcinoma harboring *EGFR* mutation also has *TP53* mutation (26). Despite this complexity, their growth and survival can often be impaired by the inactivation of a single oncogene, the phenomenon known as 'oncogene addiction' (27). A typical example is EGFR-TKI as described. Therefore, it is expected that other genes listed here can be promising target for therapy of lung cancer.

The *ERBB2* gene is mutated in a subset of patients with lung adenocarcinomas, although the frequency is low (28,29). These mutations are usually small in-frame insertions or duplications in exon 20 (28,29). Cells harboring the *G776insV_G/C* mutation in the *HER2*-encoded tyrosine kinase are shown to be sensitive to HKI-272, an irreversible dual-specific kinase inhibitor targeting both EGFR and ERBB2 (30). Similarly, lung cancers arising in female, non-smoking patients with an *HER2* mutation have been treated successfully using a combination of trastuzumab (anti-HER2 antibody) plus paclitaxel (31).

In 2007, Soda et al. identified the gene resulting from the fusion of that for echinoderm microtubule-associated protein-like 4 (*EML4*) and the gene for *ALK* as a transforming activity in mouse 3T3 fibroblasts from DNA of lung cancer in a Japanese man with a smoking history (32). This *EML4–ALK* fusion gene results from a small inversion within chromosome 2p. By fusing the coiled-coil domain of EML4 with the kinase domain of ALK, the ALK protein dimerizes without ligand binding, leading to oncogenic activation (32). A recent larger study has shown that an *ALK* translocation is

Table 3. Results of three randomized trials that evaluated the effects of addition of bevacizumab to platinum double chemotherapy

	AVAil			E4599		JO19907	
	G/P	G/P/7.5	G/P/15	C/T	C/T/15	C/T	C/T/15
Number of patients	347	345	351	433	417	59	121
RR	20%	34%*	30%*	15%	35%*	31%	61%*
PFS	6.1	6.7*	6.5*	4.5	6.2*	5.9	6.9*
OS	13.1	13.6	13.4	10.3	12.3*	N/A	N/A

AVAil and E4599 are Phase III studies, whereas JO19907 is a Phase II study. G/P, gemcitabine plus cisplatin; 7.5, 7.5 mg/kg bevacizumab; 15, 15 mg/kg bevacizumab; C/T, carboplatin plus paclitaxel; RR, response rate. *$P < 0.05$.

associated with patients without a smoking history, of younger age and acinar type adenocarcinomas (33). PF-02341066 is an orally available, potent and selective ATP competitive inhibitor of MET and ALK kinases and its clinical activity observed in a Phase I dose escalation trial for patients carrying activating ALK gene fusion was reported in ASCO 2009 (34). The results were certainly promising. The overall response rate was 53% (10/19 patients) and disease control rate at 8 weeks was 79% (15/19 patients), while four patients had progression at first evaluation (34).

Anti-angiogenesis Therapy

It is believed that tumor cells have to attract new blood vessels to bring nutrients and oxygen for them to grow over a certain size. Bevacizumab is a humanized antibody that targets vascular endothelial cell growth factor (VEGF), which is thought to play a pivotal role in tumor angiogenesis. In a Phase II trial, there was an increased risk of hemorrhage in patients with squamous cell carcinomas, especially those with a central location (35). Therefore, these patients were not eligible for subsequent clinical trials. There have been two randomized Phase III trials and one randomized Phase II trial conducted in Japan. The first Phase III trial, E4599, showed significant survival prolongation (MST: 10.2 vs. 12.5 months) by the addition of bevacizumab (15 mg/kg) to carboplatin/paclitaxel (36). However, the similarly designed AVAil study, in which addition of 7.5 or 15 mg/kg of bevacizumab to cisplatin/gemcitabine was evaluated, did not show any advantage in overall survival (37). Still, this trial showed significant prolongation of PFS, which was the primary endpoint of this study (37). In Japan, a randomized Phase II study (JO19907) has been conducted. This showed that the response rate was remarkably higher in patients treated with carboplatin/paclitaxel plus bevacizumab than in those without bevacizumab (i.e. 61 vs. 31%) (38). However, it is not clear why this high response did not translate into prolonged PFS (6.9 months for bevacizumab and 5.9 months; Table 3) (38).

Various small molecule VEGFR TKIs have been tested for their activity against NSCLCs. Most of them also inhibit other tyrosine kinases than VEGFR. For example, vandetanib is known to inhibit EGFR and RET in addition to VEGFR. In 2009, results of two large Phase III trials comparing chemotherapy plus vandetanib with chemotherapy were presented: ZODIAC comparing docetaxel plus vandetanib with docetaxel (39) and ZEAL comparing pemetrexed plus vandetanib with pemetrexed. Addition of vandetanib generated significantly better response in both trials; however, prolongation of PFS was significant only in the ZODIAC trial (39,40). Overall survival was not significant in either trial (39,40).

Sorafenib is a multiple kinase inhibitor that inhibits VEGFR, PDGFR and RAF. However, the ESCAPE study examined the effect of addition of sorafenib to standard carboplatin plus paclitaxel in a first-line setting. The result was negative with an MST for the sorafenib group of 10.7 vs. 10.6 months for controls. Addition of sorafenib was even detrimental for patients with squamous cell histology (41).

CONCLUSION

Here, I have reviewed recent progress in target drug therapy for patients with lung cancers. Identification of new molecular targets of the Achilles' heel type and development of their inhibitors as well as efficient patient screening by biomarkers will be the keys to novel cancer therapeutics in the twenty-first century.

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Conflict of interest statement

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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.

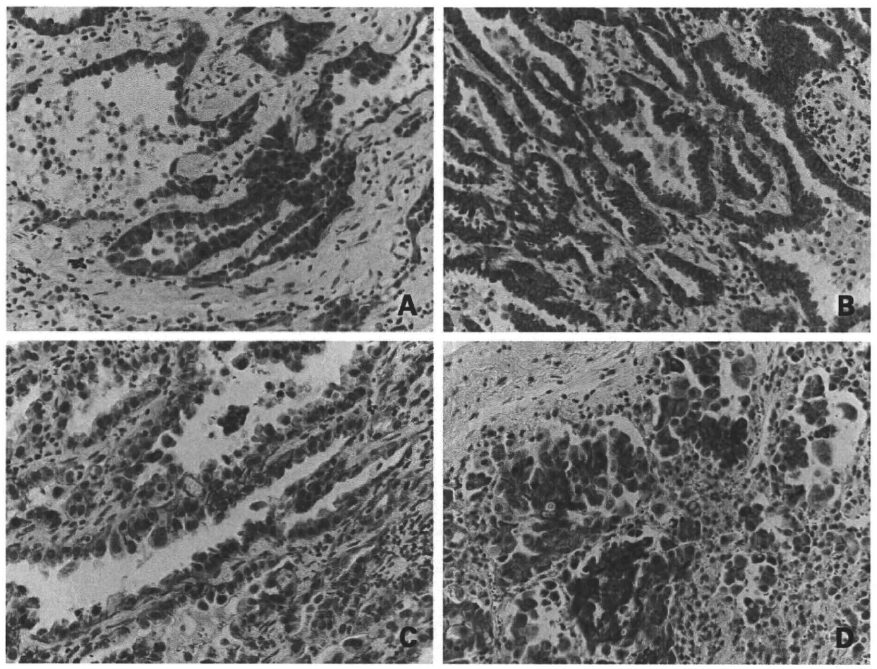

Fig. 1. Representative positive reactions of lung adenocarcinomas with DEL E746-A750 mutation stained with anti-DEL antibody (A), with L858R point mutation stained with anti-L858R antibody (B), with A769 ins ASV mutation in exon 20 stained with anti-L858R antibody (C), and with G719A mutation stained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

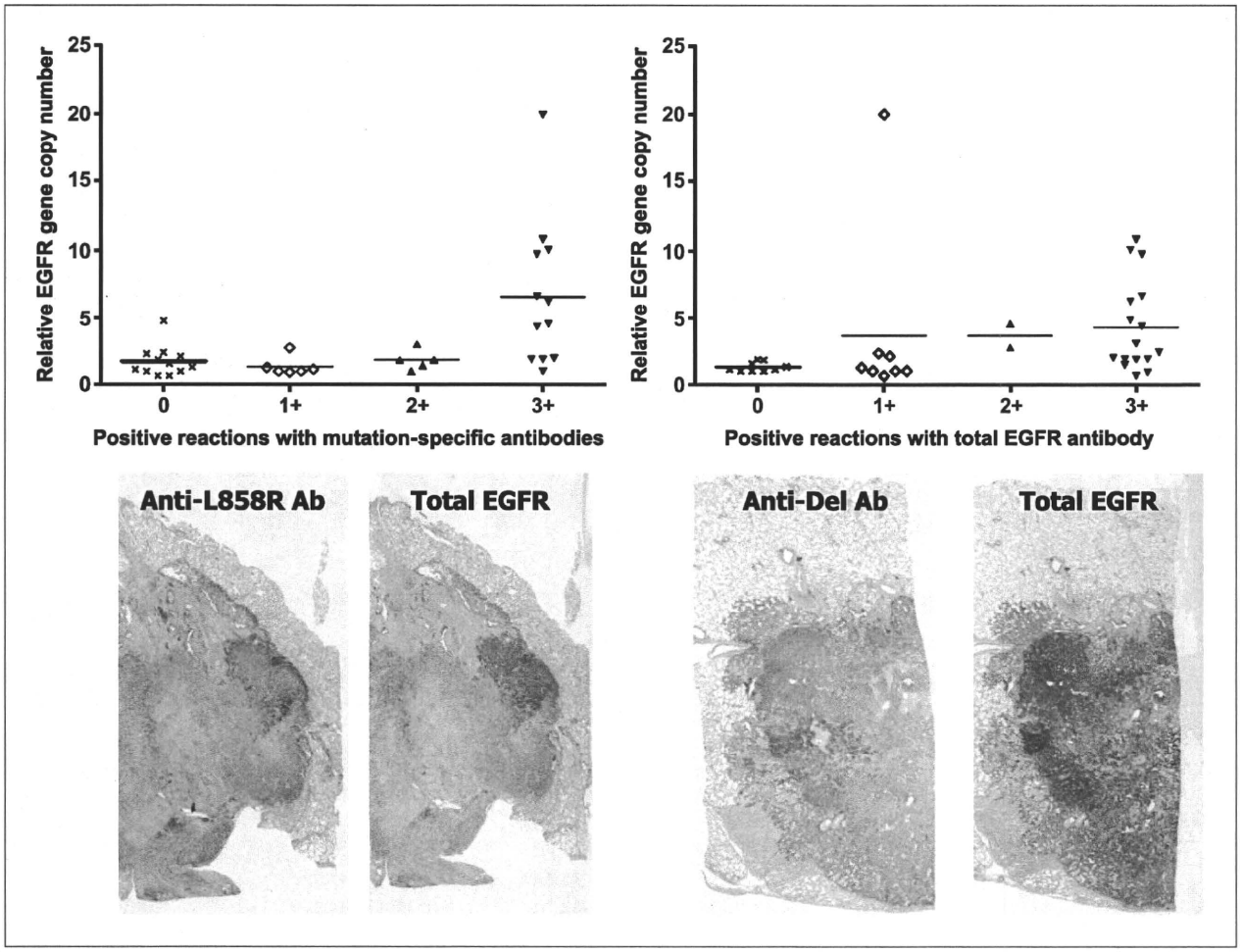

Fig. 2. Positive reaction of mutation-specific antibodies (Ab) and gene copy number. Top, the relationship of the gene copy number with the intensity of the positive reaction to mutation-specific antibodies (left) and total *EGFR* antibody (right). An intense positive reaction (score 3+) tended to show 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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Clinical Cancer Research

Molecular Diagnosis of Activating EGFR Mutations in Non–Small Cell Lung Cancer Using Mutation-Specific Antibodies for Immunohistochemical Analysis

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Abstract

Purpose: Therapeutic responses of non–small cell lung carcinoma (NSCLC) to epidermal growth factor receptor (EGFR)–targeted drugs, such as gefitinib and erlotinib, are closely associated with activating EGFR mutations. The most common mutations are delE746-A750 in exon 19 and L858R in exon 21, accounting for ~90% of all EGFR mutations. Recently, EGFR mutation-specific antibodies were developed and did well in immunohistochemical analysis, giving a sensitivity of ~90%. We have investigated whether this method detects activating EGFR mutations with sensitivity comparable with direct DNA sequencing, which is used to detect these mutations in NSCLC.

Experimental Design: We used antibodies specific for the E746-A750 deletion mutation in exon 19 and the L858R point mutation in exon 21 in Western blot analysis and immunohistochemistry to determine the presence of these mutations in NSCLC cell lines. We also examined these EGFR mutations in NSCLC tumor samples from 60 patients by immunohistochemically and direct DNA sequencing.

Results: We were able to identify EGFR mutations in NSCLC tumor samples immunohistochemically with a sensitivity of 79% using the anti–delE746-A750 antibody and 83% using the anti-L858R antibody. Additional DNA sequencing markedly improved the sensitivity obtained by immunohistochemistry.

Conclusions: This simple and rapid assay for detecting EGFR mutations, even in the small bronchial biopsies obtained in stage IV NSCLC patients, will be useful for diagnosing responsiveness to EGFR-targeted drugs in patients with NSCLC. Combining this with DNA sequencing is recommended for the development of improved personalized EGFR-targeted therapeutics. *Clin Cancer Res; 16(12); 3163–70. ©2010 AACR.*

Lung cancer is the most common cause of death from cancer worldwide. Non–small cell lung carcinoma (NSCLC) is the major type of lung cancer and is classified into three histologic types: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (1, 2). Since the introduction of the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor gefitinib and its approval for clinical use in the treatment of advanced NSCLC (3), a critical question has been how to optimize its therapeutic efficacy in NSCLC patients. Subsequent studies have shown a significant association between the presence of EGFR-activating mutations in lung tumors and their sensitivity to gefitinib and another EGFR tyrosine kinase inhibitor, erlotinib. Most of these mutations occur in exons 18 to 21 in the tyrosine kinase domain, the most common being deletions in exon 19, such as delE746-A750, and the L858R point mutation in exon 21 (4–6). These mutations are found more frequently in female patients, individuals who have never smoked, and patients of East Asian ethnicity (7–11).

Of the various molecular mechanisms that bring about EGFR activation and that affect responses to gefitinib, erlotinib, and other EGFR-targeted drugs (12), activating EGFR mutations, especially delE746-A750 and the L858R point mutation, are closely associated, with favorable clinical outcomes in ~80% of patients with NSCLC, especially in patients from East Asia (13, 14). The delE746-A750 mutation in exon 19 and the L858R mutation in exon 21 are the most common mutations found in NSCLC, accounting for ~90% of all EGFR mutations. The presence

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Translational Relevance

Activating mutations in the kinase domain of the epidermal growth factor receptor (*EGFR*) gene are critical for determining the therapeutic efficacy of EGFR-targeted drugs for patients with non–small cell lung carcinoma. DNA sequencing of the EGFR tyrosine kinase domain has been used to determine treatment strategies for these patients. Recently, mutation-specific anti-EGFR antibodies recognizing delE746-A750 in exon 19 and L858R in exon 21 have been developed and used immunohistochemically to identify EGFR mutations in cancer cells. The identification of EGFR mutations immunohistochemically and in Western blots is further investigated in this article. Our results suggest that a simple immunohistochemical diagnosis using these antibodies can provide important quantitative and tissue-specific expression data to complement DNA sequence results. We show that the sensitivities of the immunohistochemical and DNA tests are comparable and that the two methods show good correlation in determining the EGFR mutations present in non–small cell lung carcinoma in a Japanese population.

of these activating EGFR mutations is often determined by direct PCR-based sequencing of seven exons of the EGFR tyrosine kinase domain, exons 18 to 24. Yu and colleagues (15) have developed specific antibodies recognizing the delE746-A750 and L858R mutations, which can be used to identify the EGFR status of tumor samples and provide a simple immunohistochemical method for diagnosing EGFR mutations in human tissue. In this study, we have further investigated the use of these mutation-specific antibodies in immunohistochemistry and their application to the diagnostic screening of lung cancer patients and their responsiveness to EGFR-targeted drugs.

Materials and Methods

Cell lines and tissue culture

PC9 and QG56 cells were kindly provided by Dr. Yukito Ichinose (Kyushu Cancer Center, Fukuoka, Japan) and 11-18 cells were kindly provided by Dr. Kazuhiko Nakagawa (Kinki University, Osaka, Japan). LK2 cells were purchased from the Japanese Collection of Research Bioresources, and H1975 and HeLa cells were purchased from the American Type Culture Collection. PC9, QG56, LK2, H1975, and 11-18 cells were cultured in RPMI supplemented with 10% fetal bovine serum. HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum as described previously (16). The cells were maintained under standard cell culture conditions at 37°C in a humid environment in 5% CO_2.

Western blot analysis

Cells were rinsed with ice-cold PBS and lysed in 50 mmol/L HEPES, 150 mmol/L NaCl, 1% Triton X-100, and 10% glycerol containing 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L sodium orthovanadate (Triton X-100 buffer). Cell lysates were subjected to SDS-PAGE and transferred to Immobilon membranes (Millipore Corp.). After transfer, the membrane was incubated with blocking solution, probed with primary antibodies, and then washed. The primary antibodies were mutation-specific anti-EGFR antibodies recognizing the wild-type (WT) EGFR (D38B1; 16), the delE746-A750 mutation in exon 19 (6B6), and the L858R mutation (43B2) in exon 21 (15), all kindly provided by Cell Signaling Technology. The protein content was visualized using horseradish peroxidase–conjugated secondary antibodies, followed by enhanced chemiluminescence (Amersham).

Tumor samples

We retrospectively examined 45 primary NSCLC adenocarcinomas showing moderate to strong expression of total EGFR that had been completely removed surgically from patients at the Department of Surgery, Kurume University Hospital, between 1995 and 2005 (Kurume tumor samples). We also examined 15 primary NSCLC tumors surgically removed from patients at the Aichi Cancer Center (Nagoya tumor samples).

DNA extraction and direct DNA sequencing

Exon 19 (delE746-A750) and exon 21 (L858R) mutations in the *EGFR* gene were identified by direct DNA sequencing. In brief, genomic DNA was purified from paraffin-embedded tissues using a QIAamp DNA Micro kit (QIAGEN). The exon sequences of the EGFR kinase domain were amplified by nested PCR using specific primers, and exons 19 and 21 were done.

Immunohistochemistry for activating EGFR mutations in cultured lung cancer cells

Cells cultured on slides were rinsed with ice-cold PBS and fixed with 4% paraformaldehyde in PBS for 30 minutes. After fixation, the slides were washed briefly in water and boiled in a microwave for 30 minutes in 1 mmol/L EDTA (pH 9.0) target retrieval solution (DakoCytomation) to recover antigens. Intrinsic peroxidase activity was blocked by treatment with peroxidase-blocking reagent (DakoCytomation) for 5 minutes. After washing in TBS (DakoCytomation) for 5 minutes, primary antibodies, as used for Western blotting, were diluted 1:100 and applied to the cells. The slides were incubated at room temperature for 30 minutes, washed in TBS for 5 minutes, and incubated with labeled polymer–horseradish peroxidase secondary antibody (ChemMate ENVISION Kit, DakoCytomation) for 30 minutes at room temperature. After washing in TBS for 10 minutes, the slides were visualized using 3,3′-diaminobenzidine.

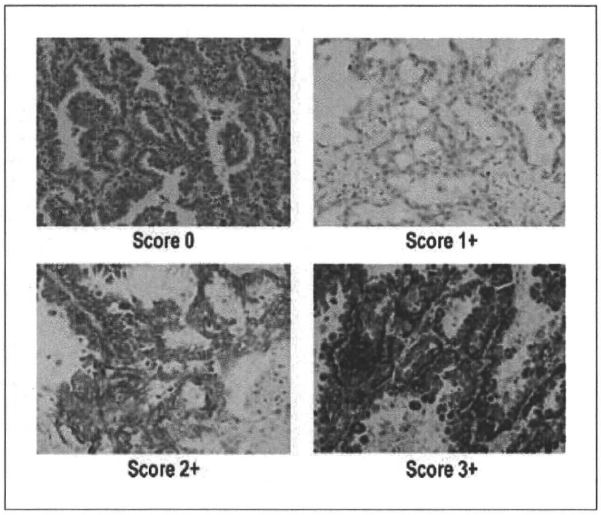

Fig. 1. Immunohistochemical staining and scores for NSCLC adenocarcinoma tumor samples labeled with anti–delE746-A750 EGFR antibody. Staining intensity was scored as 0, 1+, 2+, and 3+ (Materials and Methods).

Immunohistochemistry for activating EGFR mutations in clinical samples from NSCLC patients

Paraffin-embedded tissue samples of human lung cancer tissues were used to cut 4-μm sections, which were mounted on coated glass slides and incubated with the same mutation-specific anti-EGFR antibodies used for Western blotting 4°C overnight. We used DAKO autostainer (DakoCytomation). In evaluating the expression of EGFR mutations as biomarkers, we assumed that the staining intensity of the cancer cell membranes or cytoplasm with the mutation-specific antibodies represented the level of EGFR expression in the cancer specimens. The intensity of staining was scored using the following scale: no staining, 0; weak staining, 1+; moderate staining, 2+; and strong staining, 3+ in >10% of cancer cells (Fig. 1). We classified scores of 0 and +1 as negative and scores of 2+ and 3+ as positive.

Results

Immunocytochemical analysis of activating EGFR mutations in human lung cancer cell line

We first determined whether the mutation-specific antibodies can specifically recognize EGFR mutations in Western blots (Fig. 2) using five human lung cancer cell lines (QG56, LK2, PC9, 11-18, and H1975) and HeLa cells, a cervical cancer cell line. DNA sequence analysis showed that PC9 carried the delE746-A750 in exon 19 of the EGFR, and 11-18 and H1975 carried the L858R mutation in exon 21, whereas the QG56, LK2, and HeLa cell lines carried no EGFR mutations in either of these two exons. In Western blots, five of these cell lines showed comparable levels of EGFR expression when labeled with the control anti-EGFR antibody, but LK2 showed a lower level of expression, consistent with a previous study (16). The deletion-specific antibody recognized the mutant EGFR with a E746-A750 deletion in PC9 cells, whereas the antibody specific for the L858R mutation recognized EGFR in the 11-18 and H1975 cell lines (Fig. 2A). However, the deletion-specific antibody did not recognize EGFR in 11-18 and H1975 cells carrying the L858R mutation, and the antibody specific for the L858R mutation did not recognize EGFR in PC9 cells with the E746-A750 deletion,

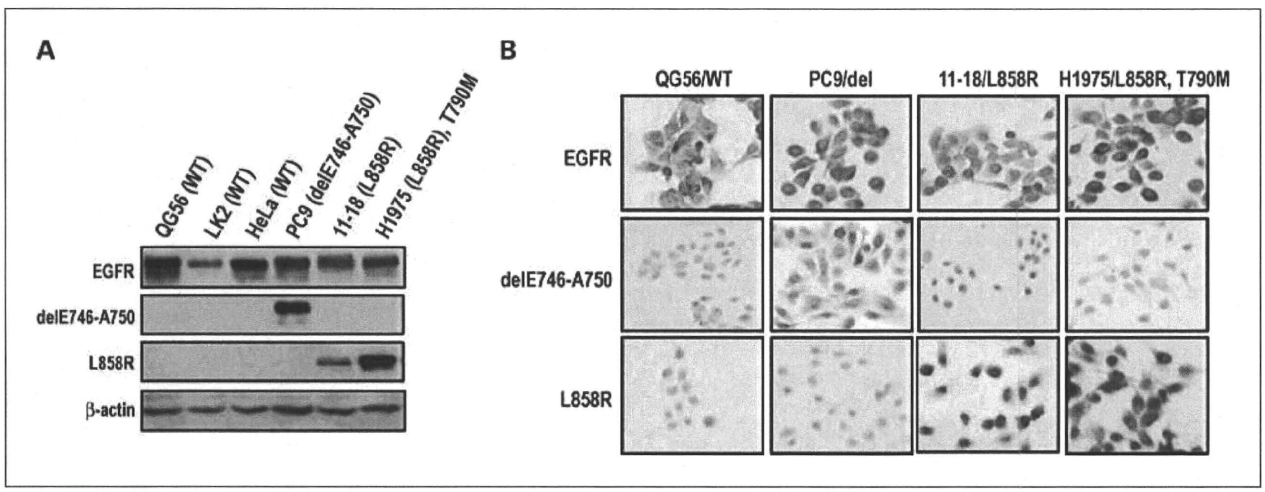

Fig. 2. Identification of the EGFR mutations delE746-A750 and L858R in NSCLC lines. The cervical cancer cell line HeLa was used as a control. A, Western blots showing the expression of EGFR, the delE746-A750 mutation, and L858R mutation in five NSCLC lines (QG56, LK2, PC9, 11-18, and H1975) under normal cell culture conditions. EGFR expression was identified using a control anti-WT EGFR antibody. Anti–delE746-A750 antibody labeled only PC9 carrying an exon 19 deletion. Anti-L858R antibody labeled 11-18 and H1975 cells carrying point mutations in exon 21. Expression of EGFR protein was determined in immunoblots using 100 μg protein of each cell lysate per lane. The loading control used was β-actin. B, immunohistochemical analysis of four NSCLC lines (QG56, PC9, 11-18, and H1975). Anti-WT EGFR antibody stained all cell lines, anti–delE746-A750 antibody stained only PC9 cells, and anti-L858R antibody stained 11-18 and H1975 cells.