reaction. Despite the limited usefulness of FDG-PET in the differential diagnosis of a pulmonary mass, the information obtained from this case should prove useful for obtaining a better understanding of this rare condition.

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Asymptomatic Endobronchial Metastases of Typical Carcinoid 15 Years after Curative Resection

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The patient was a 71-year-old man who had undergone curative left upper lobectomy and mediastinal lymph node dissection for typical endobronchial carcinoid 15 years previously. Pathologic examination revealed that the tumor was 2.5 × 2.5 cm, polypoid, and located from B^4 to the distal part of the left upper bronchus with microscopic invasion into the adjacent alveolar region. The postoperative pathological stage was IA, and no vascular or lymphatic invasion was detected. Thereafter, follow-up had been performed periodically without adjuvant therapy. The patient presented with no symptoms or abnormal findings on routine chest radiography, but he requested examination for recurrence by computed tomographic scan (CT) of the chest 7 years after the last follow-up CT, or 15 years after resection. The chest CT revealed only slight irregularity in the wall of the lower trachea. Unexpectedly, fiberoptic bronchoscopic examination revealed multiple red polypoid tumors with marked vascularity and various sizes on the wall from the lower trachea to the left main and the right upper bronchi (Fig. 1), but not in the stump of the left upper bronchus. Pathologic examination of biopsied specimens revealed these tumors to be endobronchial metastases of typical carcinoid. The patient refused treatment but has been carefully followed.

Carcinoid tumors, which comprise typical and atypical carcinoid, account for 0.5 to 1% of all tumors of bronchial origin.[1–4] Most typical carcinoids (80–90%) present as stage I lesions, and they rarely exhibit vascular invasion or distant metastasis. Patients with typical carcinoid have an excellent prognosis after adequate resection, with 5- and 10-year survival rates greater than 85 and 80%, respectively. However, the present case suggests that thoracic surgeons and pulmonary oncologists should bear in mind the possibility of asymptomatic

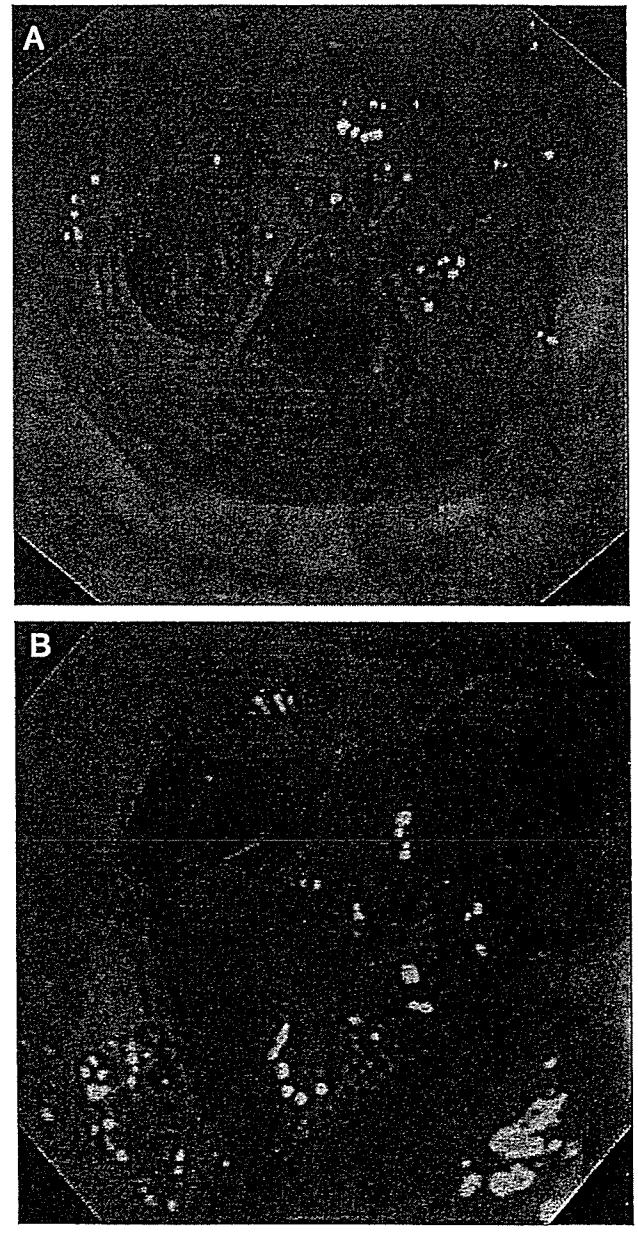

FIGURE 1. (*A*) Multiple red polypoid lesions were found on the anterior wall from the lower trachea to the carina; (*B*) these lesions also involved the right upper bronchus.

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endobronchial metastasis of typical carcinoid and that long-term follow-up after surgery for typical carcinoid is necessary.

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Risk factors differ for non-small-cell lung cancers with and without *EGFR* mutation: assessment of smoking and sex by a case-control study in Japanese

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The present study aimed to assess the impact of smoking and sex for the risk of non-small-cell lung cancer (NSCLC) with or without epidermal growth factor receptor (*EGFR*) mutation. We conducted a case-control study using 152 patients with *EGFR*-mutated ($EGFR^{mut}$) NSCLC, 283 with *EGFR*-wild-type ($EGFR^{wt}$) NSCLC and 2175 age- and sex-frequency-matched controls. Smoking was a significant risk factor for $EGFR^{wt}$ NSCLC (odds ratio [OR] for ever-smokers, 4.05; 95% confidence interval [CI], 2.79–5.88) but not for $EGFR^{mut}$ NSCLC (OR, 0.73; CI, 0.46–1.14). Sex did not affect this association. The association was observed consistently with other smoking-related parameters including pack-years. Sex was the sole risk factor for $EGFR^{mut}$ NSCLC (OR for women relative to men, 2.19; CI, 1.41–3.39) and there was no significant interaction between women and smoking. In contrast, sex, smoking and their interaction were significant in $EGFR^{wt}$ NSCLC. The impact of sex on *EGFR* mutation status was assessed by several indicators of reproductive history among women. Total fertile years showed a significant positive association with $EGFR^{mut}$ NSCLC but not with $EGFR^{wt}$ NSCLC. Other indicators showed similar trends and this result may partly explain the sexual difference in the acquisition of *EGFR* mutation. In conclusion, our case-control study clearly demonstrated that the impacts of smoking and sex on the risk of $EGFR^{mut}$ NSCLC are different from those for $EGFR^{wt}$ NSCLC. Further epidemiological evaluation is warranted. (*Cancer Sci* 2007; 98: 96–101)

Lung cancer has been pathologically classified into several categories, such as small-cell carcinoma, adenocarcinoma, squamous-cell carcinoma and large-cell carcinoma, and those other than small-cell carcinoma are categorized as non-small-cell lung cancer (NSCLC). Due to differences in the sensitivity to chemotherapy between small-cell carcinoma and NSCLC, it is clinically important to differentiate them.[1]

Recently, the epidermal growth factor receptor (EGFR) was identified. It is a receptor tyrosine kinase activating several signaling pathways resulting in cell proliferation, escape from apoptosis, invasion or metastasis, all of which are associated with cancer phenotypes.[2] Elevated levels of EGFR are seen frequently in a variety of epithelial tumors,[3] including NSCLC.[4,5] Activating mutations of *EGFR* have been reported in a subset of NSCLC and those with *EGFR* mutations are usually highly sensitive to kinase inhibitors such as gefinitive and erlotinib.[6–8] This suggests that NSCLC can be further classified into two distinct groups, namely *EGFR*-mutated ($EGFR^{mut}$) NSCLC and *EGFR*-wild-type ($EGFR^{wt}$) NSCLC, as is the case with estrogen–progesterone receptor status in breast cancer treatment.[9] Recent cross-sectional studies among NSCLC cases have revealed that the frequency of non-smoker is significantly different between $EGFR^{mut}$ and $EGFR^{wt}$ NSCLC.[6–8,10,11] These observation also implicate that NSCLC can be divided into two groups in reference to *EGFR* mutation status.

Taken together, it is natural to hypothesize that risk factors may differ between $EGFR^{mut}$ NSCLC and $EGFR^{wt}$ NSCLC. However, to our knowledge, there has been no report assessing the impact of smoking by case-control or cohort design, which is more desirable than cross-sectional study in terms of risk factor evaluation. Here, to assess the significance of smoking on the risk of NSCLC with or without *EGFR* mutation in consideration of confounding between smoking status and sex, we conducted a case-control study in 435 patients with NSCLC and 2175 matched controls in a Japanese population.

Materials and Methods

Study subjects. A total of 435 patients diagnosed as having primary NSCLC at the Department of Thoracic Surgery, Aichi Cancer Center Hospital (ACCH) and treated between January 2000 and February 2005 were examined. A further 2175 age- and sex-frequency-matched (1:5 case-control ratio) non-cancer patients who visited ACCH during the same period were also examined. Cancer patients underwent potentially curative pulmonary resection at the Department of Thoracic Surgery. The distribution of histological subtypes for NSCLC among the 435 patients was 347 adenocarcinomas (79.7%), 18 large-cell carcinomas (4.4%), 60 squamous-cell carcinomas (13.8%) and 10 (2.3%) miscellaneous-type lesions. Control subjects were eligible if they had no past history or current diagnosis of cancer and were selected randomly from 26 550 potential controls.

All subjects were enrolled in the Hospital-based Epidemiological Research Program at ACCH (HERPACC) at the time of their first visit to ACCH. The study framework of this program has been detailed elsewhere.[12,13] Briefly, all first-visit outpatients at ACCH aged 18–79 years are asked to complete a self-administered questionnaire on lifestyle factors and were checked by trained interviewers. The questionnaire included items on demographic characteristics, medical history, smoking and drinking habits, regular physical exercise, as well as menstrual and reproductive history and dietary habits before the development of current symptoms. To date, approximately 95% of eligible subjects completed the questionnaire. All data were loaded into a HERPACC database and linked periodically with the hospital cancer registry system to update the data on cancer incidence. Like most general hospitals in Japan, the ACCH accepts new outpatients who visit of their own volition, with or without a doctor's referral. Thus, even though the ACCH is called a cancer hospital, only 19% of all new outpatients have cancer.[14] Among non-cancer outpatients, 45% present with no abnormal findings on clinical examination

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Table 1. Subject characteristics

Characerisitic	Controls	Non-small-cell lung cancer cases				
		$EGFR^{mut}$	Comparison with controls (*P*-value)	$EGFR^{wt}$	Comparison with controls (*P*-value)	Comparison by mutation status (*P*-value)
n	2175	152		283		
Sex (M:F, %)*	60.4:39.6	37.5:62.5	<0.001	72.6:27.4	<0.001	<0.001
Age (±SD)**	62.6 ± 9.4	62.7 ± 9.7	0.397	62.5 ± 9.4	0.546	0.278
Smoking*						
Never smoker	1064 (48.9%)	104 (68.4%)		63 (22.3%)		
Former smoker	521 (24.0%)	23 (15.1%)		74 (26.2%)		
Current smoker	587 (27.0%)	25 (16.5%)		146 (51.6%)		
Unknown	3 (0.1%)	0 (0.0%)	<0.001	0 (0.0%)	<0.001	<0.001
0 pack-years	1065 (49.0%)	104 (68.4%)		63 (22.3%)		
1–40 pack-years	653 (30.0%)	28 (18.4%)		73 (25.8%)		
>40 pack-years	426 (19.6%)	19 (12.5%)		142 (50.2%)		
Unknown	31 (1.4%)	1 (0.7%)	<0.0001	5 (1.8%)	<0.001	<0.001
Histopathology*						
Adenocarcinoma	147 (96.7%)			200 (70.7%)		
Squamous-cell carcinoma	3 (2.0%)			57 (20.1%)		
Large-cell carcinoma	1 (0.7%)			17 (6.0%)		
Others	1 (0.7%)			9 (3.2%)		<0.001

**P*-values were calculated for homogeneity across three groups. **Mann–Whitney ranksum test was applied. EGFR, epidermal growth factor receptor.

and 35% with benign non-specific diseases.[15] We showed previously that the lifestyle patterns of first-visit outpatients accords with those of a general population selected randomly from the general population of Nagoya City.[16] Therefore, non-cancer outpatients at ACCH can be regarded as appropriate controls for epidemiological studies. The study protocol was approved by the Institutional Ethics Review Board of Aichi Cancer Center Central Hospital, and all participants provided written informed consent to participate.

***EGFR* mutation analysis.** Approxmately 60% ($n = 268$) of tumor samples were analyzed by reverse transcription–polymerase chain reaction (RT-PCR) coupled with direct sequencing. When frozen tissue for RT-PCR was not available, the cases were examined with a DNA-based assay using formalin-fixed, paraffin-embedded tissue. Both methods have been described elsewhere.[10,17,18] Briefly, total RNA was isolated using an RNAeasy kit (Qiagen, Valencia, CA, USA), followed by RT-PCR with a OneStep RT-PCR kit (Qiagen). The purified PCR products were sequenced directly with an ABI PRISM 3100 (Applied Biosystems, Foster City, CA, USA). For DNA extracted from paraffin-embedded tissues ($n = 167$), we used the cycleave technique with the Smart Cycler system (SC-100; Cepheid, Sunnyvale, CA, USA) and fragment analysis with an ABI PRISM 3100 for detection of point mutations of codon 858 and gene deletions in exon 19, respectively, which together account for approximately 90% of mutations in the *EGFR* gene.[8]

Measurement of lifestyle exposure. All exposures were assessed from the self-administered questionnaire, as completed at first visit to ACCH before the diagnostic procedure was conducted. Subjects were questioned specifically about their lifestyle before the onset of the symptoms that occasioned their visit to ACCH.

Smoking status was divided into three categories: never, former and current. Former smokers were defined as those who quit smoking at least 1 year before the time of survey. Cumulative doses of smoking were evaluated as pack-years (PY), the product of the number of packets consumed in 1 day and the number of years of smoking. Age at starting smoking and years since quitting smoking were noted for ever smokers and former smokers, respectively. Past usage of hormone-replacement therapy (HRT) was also noted for 822 women in recent female subjects. An HRT history was defined as positive when subjects had received any type of hormonal therapy, including fertilization treatment, posto-ophorectomy and postmenopause.

Statistical analysis. We used STATA version 9 (STATA Corporation, College Station, TX, USA) for all analyses. The impact of smoking status, sex and reproductive factors was evaluated with odds ratios (OR) and their 95% confidence intervals (CI) in unconditional logistic regression models were adjusted for age. Differences in continuous variables between groups were evaluated using the Mann–Whitney test when appropriate. Trend tests were accomplished with scores in logistic regression models. We defined two-sided *P*-values of less than 0.05 as statistically significant.

Results

Baseline characteristics for controls and patients according to *EGFR* mutation status are shown in Table 1. Women were significantly more common in the $EGFR^{mut}$ group (62.5%) than in the $EGFR^{wt}$ (27.6%) or control (39.6%) groups. Current smokers were significantly common among the $EGFR^{wt}$ cases (51.6%) compared with $EGFR^{mut}$ cases (12.5%) and controls (27.0%). As for histopathology, adenocarcinoma was dominant in $EGFR^{mut}$ and relatively less common in $EGFR^{wt}$ than in $EGFR^{mut}$.

The OR for smoking-related characteristics are listed in Table 2. Ever smoking showed an increased risk for $EGFR^{wt}$ (OR, 4.05; 95% CI, 2.79–5.88; $P < 0.001$), but no significant risk change for $EGFR^{mut}$. This was observed consistently in both sexes. Cumulative exposure to smoking showed a linear increased risk for $EGFR^{wt}$ only (trend $P < 0.001$). The OR for PY 1–40 and more than 40 were 2.72 (1.79–4.14; $P < 0.001$) and 10.0 (6.33–15.8; $P < 0.001$), respectively. In contrast, no significant risk change was observed for $EGFR^{mut}$. Age at the start of smoking among ever smokers as well as years since quitting smoking among former smokers also demonstrated a strong correlation between $EGFR^{wt}$ cases and smoking. These results were not changed when cases were limited to adenocarcinoma only or when squamous-cell carcinoma cases were excluded.

To further examine the interaction between smoking and sex, two models were examined: (1) age, sex and smoking; and (2)

Table 2. Odds ratios (OR) for smoking-related characteristics for non-small-cell lung cancer cases with or without epidermal growth factor receptor (*EGFR*) mutation

Cases	$EGFR^{mut}$		$EGFR^{wt}$	
	OR (95% CI)†	*P*-value	OR (95% CI)†	*P*-value
Smoking status				
Never smoker	1.00 (Reference)		1.00 (Reference)	
Ever smoker	0.73 (0.46–1.14)	0.167	4.05 (2.79–5.88)	<0.001
Cumulative exposure to smoking				
0 pack-years	1.00 (Reference)		1.00 (Reference)	
1–40 pack-years	0.68 (0.42–1.12)	0.134	2.72 (1.79–4.14)	<0.001
>40 pack-years	0.79 (0.42–1.46)	0.45	10.0 (6.33–15.8)	<0.001
		Trend 0.303		Trend <0.001
Age at starting smoking (/10 years)‡	0.95 (0.54–1.67)	0.854	0.68 (0.47–0.98)	0.039
Years since quitting (/10 years)§	0.92 (0.62–1.39)	0.705	0.57 (0.43–0.76)	<0.001
Male smoking status				
Never smoker	1.00 (Reference)		1.00 (Reference)	
Ever smoker	0.69 (0.38–1.24)	0.215	8.75 (4.07–18.8)	<0.001
Male cumulative exposure to smoking				
0 pack-years	1.00 (Reference)		0 (Reference)	
1–40 pack-years	0.70 (0.36–1.35)	0.284	4.50 (2.02–9.99)	<0.001
>40 pack-years	0.73 (0.36–1.45)	0.366	14.8 (6.82–32.2)	<0.001
		Trend 0.382		Trend <0.001
Age at starting smoking (/10 years)‡	0.65 (0.22–1.89)	0.432	0.48 (0.28–0.83)	0.009
Years since quitting (/10 years)§	0.99 (0.65–1.52)	0.976	0.57 (0.43–0.77)	<0.001
Female smoking status				
Never smoker	1.00 (Reference)		1.00 (Reference)	
Ever smoker	0.81 (0.41–1.61)	0.55	2.36 (1.37–4.04)	0.002
Female cumulative exposure to smoking				
0 pack-years	1.00 (Reference)		1.00 (Reference)	
1–40 pack-years	0.61 (0.27–1.36)	0.225	1.87 (1.04–3.38)	0.038
>40 pack-years	3.30 (0.63–17.3)	0.158	14.8 (4.13–53.1)	<0.001
		Trend 0.729		Trend <0.001
Age at starting smoking (/10 years)‡	0.85 (0.39–1.85)	0.677	0.91 (0.52–1.59)	0.738
Years since quitting (/10 years)§	0.20 (0.02–1.92)	0.161	0.57 (0.17–1.87)	0.353

†Odds ratios were adjusted for age and sex in unconditional logistic regression models. ‡Analysis was limited to ever smokers. §Analysis was limited to former smokers. CI, confidence interval.

Table 3. Results of multivariable logistic regression models for the risk of non-small-cell lung cancer with or without epidermal growth factor receptor (*EGFR*) mutation

Varaiable	$EGFR^{mut}$		$EGFR^{wt}$	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Model 1				
Age (10 year increase)	1.15 (0.96–1.36)	0.121	0.94 (0.83–1.07)	0.355
Sex (male vs female)	2.19 (1.41–3.39)	<0.001	1.34 (0.94–1.92)	0.11
Smoking status (ever vs never)	0.73 (0.46–1.14)	0.167	4.05 (2.79–5.88)	<0.001
Model 2				
Age (10 years increase)	1.15 (0.97–1.37)	0.116	0.93 (0.81–1.05)	0.237
Sex (male vs female)	2.08 (1.22–3.56)	0.008	3.21 (1.45–7.13)	0.004
Smoking status (ever vs never)	0.68 (0.38–1.23)	0.204	8.80 (4.09–18.9)	<0.001
Interaction between sex and ever smoking	1.15 (0.47–2.85)	0.756	0.28 (0.11–0.71)	0.007

†Odds ratios (OR) were adjusted for age and sex in unconditional logistic regression models. ‡Analysis was limited to ever smokers. §Analysis was limited to former smokers. CI, confidence interval.

age, sex, smoking and interaction term between sex and smoking (Table 3). For $EGFR^{mut}$ cases, the first model demonstrated that smoking had no effect on susceptibility to $EGFR^{mut}$ and that female sex was the sole risk factor for $EGFR^{mut}$. The second model demonstrated no interaction between sex and smoking status for $EGFR^{mut}$. For $EGFR^{wt}$ cases, a significant negative interaction between female sex and smoking was demonstrated, indicating that the effect of smoking on the risk of $EGFR^{wt}$ was stronger among males. The OR for never-smoker females, ever-smoker males, and ever-smoker females relative to never-smoker males were 3.21 (1.45–7.13), 8.80 (4.10–18.9) and 7.90 ($3.21 \times 8.80 \times 0.28$; 3.28–19.0), respectively. Again, these results were observed consistently when cases were limited to adenocarcinoma only.

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Table 4. Impact of reproductive factors on non-small-cell lung cancer with or without epidermal growth factor receptor (*EGFR*) mutation among women

Factors	Control	Cases			
		$EGFR^{mut}$	Mutated OR (95% CI)[‡]	$EGFR^{wt}$	$EGFR^{wt}$ OR (95% CI)[‡]
Fertile life (years)					
Fertile years T1 (12–34)	336 (38.4%)	28 (28.9%)	1.00 (Reference)	38 (48.7%)	1.00 (Reference)
Fertile years T2 (35–37)	265 (30.3%)	29 (29.9%)	1.31 (0.76–2.26)	18 (23.1%)	0.64 (0.35–1.15)
Fertile years T3 (38–47)	240 (27.4%)	37 (38.1%)	1.85 (1.10–3.11)	18 (23.1%)	0.71 (0.39–1.27)
Unknown	34 (3.9%)	3 (3.1%)		4 (5.1%)	
			P for trend = 0.020		*P* for trend = 0.189
Age at menarche (years)					
16–24	329 (37.6%)	19 (19.6%)	1.00 (Reference)	17 (21.8%)	1.00 (Reference)
14–15	355 (40.6%)	51 (52.6%)	1.89 (1.15–3.12)	31 (39.7%)	1.06 (0.62–1.82)
11–13	164 (18.7%)	25 (25.8%)	1.52 (0.82–2.85)	27 (34.6%)	1.26 (0.67–2.38)
Unknown	27 (3.1%)	2 (2.1%)		3 (3.9%)	
			P for trend = 0.098		*P* for trend = 0.492
Menopause					
Premenopausal female	152 (17.4%)	11 (11.3%)	1.00 (Reference)	17 (21.8%)	1.00 (Reference)
Postmenopausal female	720 (82.3%)	86 (88.7%)	1.65 (0.86–3.17)	60 (76.9%)	0.75 (0.42–1.31)
Unknown	3 (0.3%)	0 (0.0%)		1 (1.3%)	
Age at menopause (years)[†]					
<45	78 (10.8%)	9 (10.5%)	1.00 (Reference)	11 (18.3%)	1.00 (Reference)
45–54	575 (79.9%)	64 (74.4%)	0.96 (0.46–2.02)	41 (68.3%)	0.51 (0.25–1.02)
55–60	67 (9.3%)	13 (15.1%)	1.68 (0.68–4.18)	8 (13.3%)	0.85 (0.32–2.23)
			P for trend = 0.234		*P* for trend = 0.573
Pregnancy[§]					
Ever	801 (91.5%)	91 (93.8%)	1.00 (Reference)	66 (84.6%)	1.00 (Reference)
Never	70 (8.0%)	5 (5.2%)	1.59 (0.63–4.04)	11 (14.1%)	0.52 (0.26–1.04)
Unknown	4 (0.5%)	1 (1.0%)		1 (1.3%)	
Age at first parity (years)[¶]					
<25 (median)	83 (10.4%)	5 (5.5%)	1.00 (Reference)	8 (12.1%)	1.00 (Reference)
≥25 (median)	689 (86.0%)	83 (91.2%)	2.00 (0.79–5.07)	57 (86.4%)	0.86 (0.40–1.86)
Unknown	29 (3.6%)	3 (3.3%)		1 (1.5%)	

[†]Adjusted for age in unconditional logistic regression. [‡]Women who experience menopause only. [§]Women only. [¶]Women who experienced pregnancy only. CI, confidence interval; OR, odds ratio.

As female sex was demonstrated as a risk factor for $EGFR^{mut}$ NSCLC, we examined the possible involvement of sex hormones in *EGFR* mutagenesis (Table 4). A longer total fertile life, defined as years between age at menarche and menopause (for premenopausal women, years between age at menarche and at enrolment), was significantly associated with $EGFR^{mut}$ NSCLC (trend $P = 0.020$). Compared with the group with the shortest fertile life, the OR for the intermediate and longest groups were 1.31 (0.76–2.26) and 1.85 (1.10–3.11). Although not significant, other indicators for reproductive history, such as age at menarche, menopausal status and pregnancy, showed an increased risk of $EGFR^{mut}$ with higher exposure to sex hormones. In contrast there was no significant risk change in $EGFR^{wt}$ NSCLC. Analyses for reproductive history were not modified when cases were limited to adenocarcinoma. In addition, exogenous hormonal exposure was assessed. HRT history, as a surrogate of exogenous hormonal exposure, was available for 822 subjects (685 controls, 61 $EGFR^{wt}$ and 76 $EGFR^{mut}$). Among them, the proportions of those with a positive history was 7.7% in controls, 6.6% in $EGFR^{wt}$ and 5.3% in $EGFR^{mut}$ ($P = 0.711$). From this data, exogenous hormonal exposure seemed not to have a strong impact on either $EGFR^{wt}$ or $EGFR^{wt}$.

Discussion

In the present case-control study, we demonstrated clearly that smoking is a significant risk factor for $EGFR^{wt}$ NSCLC and being female is also a risk factor for $EGFR^{wt}$ NSCLC but only among non-smokers. In contrast, female sex but not smoking is a significant risk factor for $EGFR^{mut}$ NSCLC. In addition, a longer fertile life, resulting in higher exposure to estrogen, is another risk factor for $EGFR^{mut}$ NSCLC. To our knowledge, this is the first study to assess the differential impact of smoking and sex on the risk of NSCLC with or without $EGFR^{mut}$.

Smoking was identified as a risk factor for only $EGFR^{wt}$ NSCLC not $EGFR^{mut}$ NSCLC after consideration of sex. This result is consistent with previous studies reporting that tobacco-related specific types of genetic alterations such as G-to-T transversion of *TP53* and *K-RAS* mutations are observed commonly in $EGFR^{wt}$ NSCLC.[10,11,19] No negative or positive association between smoking and $EGFR^{mut}$ suggests that a different carcinogenic pathway may exist in this category.

We identified that being female is a risk factor of $EGFR^{mut}$ NSCLC. This result motivated us to examine the possible involvement of endogenous sex hormone exposure in *EGFR* mutagenesis. Our findings regarding a longer fertile life support a positive association between endogenous estrogenic exposure in NSCLC with *EGFR* mutation. In contrast, an exogenous hormonal exposure, surrogated by HRT history, showed no clear association with the risk of NSCLC with or without *EGFR* mutation. Several epidemiological studies have shown associations between reproductive factors and NSCLC risk, but the direction of impact was not consistent.[20–29] Regarding HRT, a protective effect has been reported;[30] however, the results of our study contradict this. Female sex was also detected as a significant risk factor for *EGFR* NSCLC only among non-smokers, although there was no association with fertile years, which no impact on this category (data not shown). Several biological discussions have centered on the issue of EGFR and estrogen receptors and their role in carcinogenesis.[31–33] The results of

the present study add a new insight on the topic, although further epidemiological evaluation is needed.

The lack of association between smoking and *EGFR*^{mut} in the present study suggests a possible direction in epidemiological research as well as prevention. An increase in the incidence of adenocarcinoma has been observed in Japan.[34] Considering the higher probability of *EGFR* mutation in the Japanese population, one may assume that the overall incidence of *EGFR*^{mut} NSCLC is also increasing. Although smoking-related factors such as the increased use of filtered tobacco are regarded as risk factors for adenocarcinoma,[35] those cases prevented by antismoking action may be *EGFR*^{wt} NSCLC rather *EGFR*^{mut} NSCLC. Therefore, identification of other potential risk factors for *EGFR*^{mut} NSCLC as well as prevention strategies are required.

The strengths and weaknesses of the present study should be considered. The case-control design is the notable strength in its ability to draw causal inferences regarding smoking, sex and other exposures. Previous studies had a cross-sectional design that compared *EGFR*^{wt} NSCLC and *EGFR*^{mut} NSCLC, hampering the ability to draw causal inferences. Although information bias is always present with the case-control design, the data present were collected before diagnosis and patients with NSCLC had no information regarding their *EGFR* mutation status beforehand, so bias due to having the disease seems unlikely. Precision in the measurement of endogenous exposure to estrogen could be an issue. For example, exact age at menopause is difficult to obtain because menopause is a process that often requires a long time period. The hospital-based design is another limitation, but our control subjects tended to have attenuated OR with smoking for the risk of lung cancer compared with population-based controls,[36] which would suggest the underestimation of an association with smoking. We applied frequency matching for age and sex as a whole, therefore the assumption that sex is not a risk factor for NSCLC as a whole is required for valid estimation of the OR for sex in the subgroup analysis. Actually, this assumption was not warranted in the present study, so careful interpretation about sex in the subgroup analysis is therefore required. Finally, our study was limited to ethnic Japanese and might therefore not be extrapolatable to other population groups. Given that the contribution of an ethnic difference in the CA repeat polymorphism in the *EGFR* gene has been suggested in response to gefitinib,[37,38] this question requires further evaluation.

In conclusion, smoking or its cumulative dose did impact the risk of *EGFR*^{wt} NSCLC, but had no impact on the risk of *EGFR*^{mut} NSCLC. The significance of female sex is evident for *EGFR*^{mut} regardless of smoking status. The association between female non-smokers and *EGFR*^{wt} NSCLC risk, as well as that between sex hormone exposure and *EGFR*^{mut} NSCLC risk, requires further evaluation.

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Analysis of *Epidermal Growth Factor Receptor* Gene Mutation in Patients with Non-Small Cell Lung Cancer and Acquired Resistance to Gefitinib

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Abstract

Purpose: Non-small cell lung cancers carrying activating mutations in the gene for the epidermal growth factor receptor (EGFR) are highly sensitive to EGFR-specific tyrosine kinase inhibitors. However, most patients who initially respond subsequently experience disease progression while still on treatment. Part of this "acquired resistance" is attributable to a secondary mutation resulting in threonine to methionine at codon 790 (T790M) of EGFR.

Experimental Design: We sequenced exons 18 to 21 of the *EGFR* gene to look for secondary mutations in tumors with acquired resistance to gefitinib in 14 patients with adenocarcinomas. Subcloning or cycleave PCR was used in addition to normal sequencing to increase the sensitivity of the assay. We also looked for T790M in pretreatment samples from 52 patients who were treated with gefitinib. We also looked for secondary *KRAS* gene mutations because tumors with *KRAS* mutations are generally resistant to tyrosine kinase inhibitors.

Results: Seven of 14 tumors had a secondary T790M mutation. There were no other novel secondary mutations. We detected no T790M mutations in pretreatment specimens from available five tumors among these seven tumors. Patients with T790M tended to be women, never smokers, and carrying deletion mutations, but the T790M was not associated with the duration of gefitinib administration. None of the tumors had an acquired mutation in the *KRAS* gene.

Conclusions: A secondary T790M mutation of *EGFR* accounted for half the tumors with acquired resistance to gefitinib in Japanese patients. Other drug-resistant secondary mutations are uncommon in the *EGFR* gene.

Activating mutations in the gene for the epidermal growth factor receptor (EGFR) are present in a subset of pulmonary adenocarcinomas. Tumors with *EGFR* mutations are highly sensitive to gefitinib and erlotinib, small-molecule EGFR-specific tyrosine kinase inhibitors (1–3). These mutations occur in the tyrosine kinase domain of the *EGFR* gene. Deletion mutations in exon 19 and the substitution of leucine with arginine at codon 858 (L858R) account for ~90% of all these mutations (4). *EGFR* mutations are more prevalent in women, never smokers, patients of Asian ethnicity, and those with adenocarcinoma histology (4). These features are the same as those of patients whose tumors have elevated sensitivity to EGFR-specific tyrosine kinase inhibitors. The response rates of lung cancers with an *EGFR* mutation are as high as 80% (5). Responses are often dramatic, and several reports have shown that patients with *EGFR* mutations survive significantly longer after gefitinib treatment than patients without mutations (6). However, it is also common for patients to show disease progression after presenting with an initial marked response to EGFR-specific tyrosine kinase inhibitors. The mean duration of the initial response is about 3 to 7 months (7, 8).

Recently, it has been reported by two groups that a secondary threonine-to-methionine mutation at codon 790 (T790M) of the *EGFR* gene is related to the acquired resistance to gefitinib and erlotinib (9, 10). Crystal structure modeling has shown that residue T790 is located in the ATP-binding pocket of the catalytic region of EGFR, and it seems to be critical for the binding of erlotinib and gefitinib (9). Substitution of the threonine at codon 790 with a bulkier residue, such as methionine, would result in steric hindrance to the binding of these two drugs. A secondary T790M mutation has been identified in one tumor (9) and in three of six tumors (10) with acquired resistance to gefitinib.

Imatinib is a tyrosine kinase inhibitor specific for BCR-ABL, KIT, and platelet-derived growth factor A, which is used to treat

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chronic myelogenous leukemia (CML) and gastrointestinal stromal tumor. Analogous secondary mutations in the kinase domains of these genes are considered to constitute one of the mechanisms of acquired drug resistance (11–14). The structural similarity between ABL and EGFR tyrosine kinases is fairly high, and the most common mutation related to acquired resistance is a threonine-to-isoleucine mutation at codon 315 (T315I), corresponding to T790M in the *EGFR* gene (15). In CML, 20 to 30 other mutations of the *ABL* gene have been identified as responsible for acquired resistance to imatinib (12, 16–19), so secondary *EGFR* gene mutations other than T790M are possible (Fig. 1).

Secondary mutations of the *ABL* gene have also been detected in pretreatment samples from some CML patients, although the fraction of mutant cells was very low (16, 20). The existence of a similar mechanism is expected for non-small cell lung cancer. Furthermore, we and others have reported that the T790M mutation of the *EGFR* gene exists as a major mutation independently of gefitinib treatment, although instances are very rare (21, 22).

It has also been reported that *KRAS* mutations are associated with a lack of sensitivity to gefitinib and erlotinib (23, 24). Therefore, it is possible that acquired *KRAS* mutations are also associated with acquired resistance.

In this study, we looked for the T790M mutation and other secondary mutations of the *EGFR* gene in tumors from patients who showed disease progression after presenting with an initial response to EGFR-specific tyrosine kinase inhibitor treatment and in tumors before gefitinib treatment. We also looked for *KRAS* mutations in the same tumors.

Materials and Methods

Patients. Patients with non-small cell lung cancer who initially responded but subsequently experienced disease progression while on gefitinib treatment were defined as having "acquired resistance." A detailed definition of the effectiveness of gefitinib treatment was described in our previous study (25). Briefly, gefitinib treatment is judged to be effective when tumors show a decrease of at least a 30% in tumor diameter in imaging studies or when elevated carcinoembryonic antigen levels decrease to a level less than half the baseline level.

Fourteen tumor samples and 10 corresponding pretreatment tumor samples from eligible patients were obtained according to this definition at the time of diagnosis or treatment. The selection of patients depended only on whether a second tumor sample collected at the time of progression could be obtained. Appropriate approval from the institutional review board and the patients' written informed consent were obtained. Patient characteristics and details of the samples are shown in Table 1. All patients had adenocarcinomas, and the median duration of gefitinib treatment was 367 days (range, 69-921 days). We also analyzed the samples of 52 patients who had been treated with gefitinib for recurrent disease after they had undergone pulmonary resection. This cohort was part of our previous study, and their clinical details are described elsewhere (25).

Subcloning mutational analysis of the EGFR *gene.* Genomic DNA and total RNA (if possible) were extracted from each sample (Table 1). Exons 18 to 21 of the *EGFR* tyrosine kinase domain were amplified using PCR or reverse transcription-PCR (RT-PCR) methods. PCR for genomic DNA was done using AmpliTaq Gold (Applied Biosystems, Foster City, CA) and the following primers: exon 18, 5′-GAGGTGACCC-TTGTCTCTGTGT-3′ (forward) and 5′-CCCAAACACTCAGTGAAACAAA-3′ (reverse); exon 19, 5′-TGCCAGTTAACGTCTTCCTTCT-3′ (forward) and 5′-ATGTGGAGATGAGCAGGGTCTA-3′ (reverse); exon 20, 5′-TGAAACTC-AAGATCGCATTCAT-3′ (forward) and 5′-CATGGCAAACTCTTGCTATCC-3′

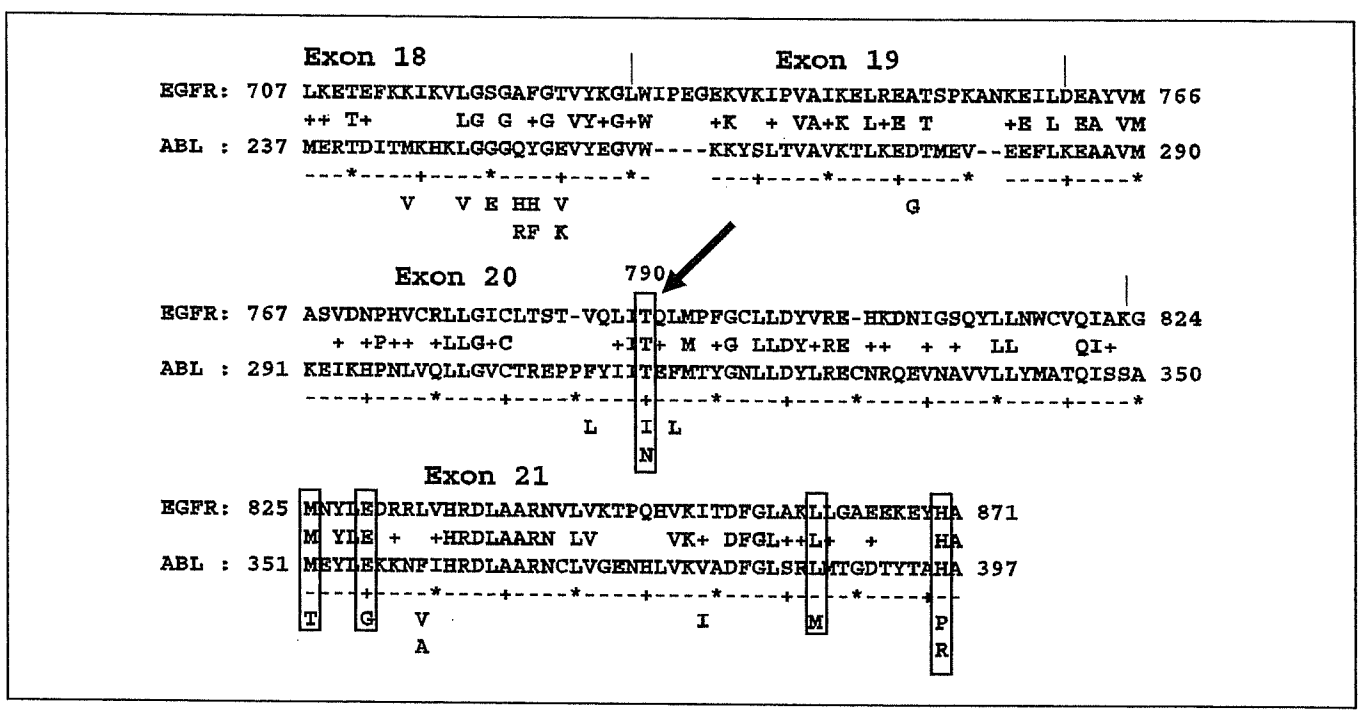

Fig. 1. Structural similarity between EGFR tyrosine kinase and ABL. This amino acid alignment was obtained using basic local alignment search tool, and both sequences were obtained from Genbank (accession nos.: EGFR, NM 005228; ABL, NM 005157). Top line, EGFR; bottom line, ABL. Vertical lines, boundaries between exons. Numbers at each end, codon numbers. Capital letters under the alignment, amino acid changes in ABL that have been reported as acquired imatinib resistance mutations. Square frames, qualifying codons as common codons in EGFR and ABL and as acquired resistance mutant codons in ABL. Arrow, location of codon 790 of EGFR and codon 315 of ABL.

Table 1. Patient characteristics and results of sequencing analysis

Patient no.	Sex	Smoking status	Prior treatment	Gefitinib response	Gefitinib treatment days	Analyzed specimen (state)	Nucleic acid	Activating mutation	T790M mutation	T790M (pre-gefitinib samples)
1	F	NS	S	E	642	LN (Fr)	RNA	Δ2	+	—
2	M	FS	S	E	368	PE (Al)	RNA	Δ3	–	—
3	M	NS	S	E	116	PE (Al)	RNA	Δ1	–	—
4	F	FS	CT	E	599	PE (CL)	RNA	Δ1	–	NA
5	F	NS	CRT	E	921	LU (Al)	RNA	Δ1	+	NA
6	F	NS	None	E	181	PE (Al)	RNA	Δ1	+	—
7	F	FS	CT	E	346	BO (Al)	RNA	Δ1	+	—
8	F	NS	S→CRT	E	623	LN (Al)	RNA	L858R	–	NA
9	M	FS	S	E	915	BR (Fr)	DNA	L858R*	–	—
10	M	FS	S→CRT	NE	69	PE (Al)	DNA	L858R	–	—
11	F	FS	None	E	560	LU (Fr)	RNA	L858R*	+	NA
12	F	NS	CT	E	239	PE (Al)	RNA	Δ1	+	—
13	F	NS	S	E	367	PE (Al)	RNA	L858R	–	—
14	F	NS	CRT	E	235	LN (Al)	RNA	Δ1	+	—

NOTE: Patients 1, 4, and 13 received gefitinib therapy twice. Pretreatment samples from patients 4, 5, 8, and 11 were not available. Patient 10 was defined as not evaluable according to our definition. However, this patient showed a 46% decrease in carcinoembryonic antigen and a marked reduction in pleural effusion on initial treatment before subsequent progression. Therefore, we regarded this case as eligible for this study.

Abbreviations: Al, alcohol fixed; BO, bone metastasis; BR, brain metastasis; CL, cell line; CRT, chemoradiotherapy; CT, chemotherapy; del, deletion; E, effective; F, female; Fr, frozen; FS, former smoker; ins, insertion; LN, lymph node; LU, lung tumor; M, male; NA, not available; NE, not evaluable; NS, never smoker; PE, pleural effusion; RT, radiotherapy; S, surgery; Δ1, del E746-A750; Δ2, del L747-P753 insS; Δ3, del L747-A750 insP.

*Patients 9 and 11 had another point mutation (L833V in patient 9 and R776H in patient 11).

(reverse); and exon 21, 5′-GAGCTTCTTCCCATGATGATCT-3′ (forward) and 5′-GAAAATGCTGGCTGACCTAAAG-3′ (reverse). The PCR conditions were as follows: 1 cycle of 95°C for 11 minutes, 45 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 40 seconds followed by 1 cycle of 72°C for 4 minutes.

RT-PCR for RNA was done with primers 5′-AGCTTGTGGAGCCTCTTACACC-3′ (forward 1) and 5′-TAAAATTGATTCCAATGCCATCC-3′ (reverse 1) in a one-step RT-PCR setup using Qiagen OneStep RT-PCR kits (Qiagen, Valencia, CA) as described previously (26). RT-PCR conditions were as follows: 1 cycle of 50°C for 30 minutes and 95°C for 15 minutes, 40 cycles of 94°C for 50 seconds, 62°C for 50 seconds, and 72°C for 1 minute followed by 1 cycle of 72°C for 10 minutes.

The PCR products were subcloned using TOPO TA Cloning kits (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Each clone was then directly amplified with the same primers using AmpliTaq Gold and cycle sequenced using BigDye Terminator v3.1/1.1 cycle sequencing kits (Applied Biosystems). Subcloning PCR conditions were as follows: 1 cycle of 95°C for 11 minutes, 45 cycles of 95°C for 50 seconds, 62°C for 50 seconds, and 72°C for 70 seconds followed by 1 cycle of 72°C for 4 minutes.

The sequencing reaction products were electrophoresed using an ABI PRISM 3100 system (Applied Biosystems). Both forward and reverse sequences were analyzed with basic local alignment search tool, and the chromatograms were analyzed by manual review.

Cycleave real-time PCR assay. Details of the cycleave real-time PCR assay have been described previously (27). Briefly, genomic DNA was extracted, and exon 20 of the *EGFR* gene was amplified by real-time quantitative PCR assay on a SmartCycler (TaKaRa, Gifu, Japan) using Cycleave PCR Core kits (TaKaRa) with a T790M-specific cycling probe and a wild-type cycling probe. As few as ~5% of tumor cell molecules could be detected in this assay.

Mutational analysis of the KRAS *gene.* A RT-PCR direct sequence assay was done for RNA, and a cycleave real-time PCR assay was done for DNA. *KRAS* primers for PCR were 5′-GGCCTGCTGAAAATGACTGA-3′ (forward 1) and 5′-TCTTGCTAAGTCCTGAGCCTGTT-3′ (reverse 3). Codon 12 cycling probes and a wild-type cycling probe were used in cycleave real-time PCR assays. Direct sequencing was used to identify codon 12, 13, and 61 mutations.

Results

Detection of secondary mutations in the EGFR *gene or the* KRAS *gene.* For the analysis of secondary mutations, we first amplified exons 18 to 21 of the *EGFR* gene, which include the region homologous to the region of the *ABL* gene that contains all the secondary mutations thus far reported to be responsible for imatinib resistance in CML. All 14 tumors with acquired resistance had activating mutations of the *EGFR* gene, either deletion mutations, including codons 746 to 750 (nine patients), or L858R (five patients). Seven tumors had a secondary T790M mutation (Table 1; Fig. 2).

When we sequenced corresponding tumor samples that had been obtained before gefitinib treatment, the same activating mutations were always present, whereas T790M was not detected in any of the available pretreatment samples (samples for patients 4, 5, 8, and 11 were not available).

Mutant bands for T790M in the sample from patient 7 were as strong as the wild-type bands, and the mutant bands were stronger than the wild-type bands in patient 12 (Fig. 2). However, in most cases, the T790M mutant bands were weaker than the wild-type bands.

Two tumors had another point mutation as well as L858R (L833V in patient 9 and R776H in patient 11). L833 corresponds to F359 of ABL, where a secondary mutation to valine or alanine has been reported in CML (Fig. 1; ref. 12). However, the pretreatment sample of patient 9 revealed that L833V existed before treatment in the same ratio as the L858R band. The ratios of L833V and L858R bands were unchanged

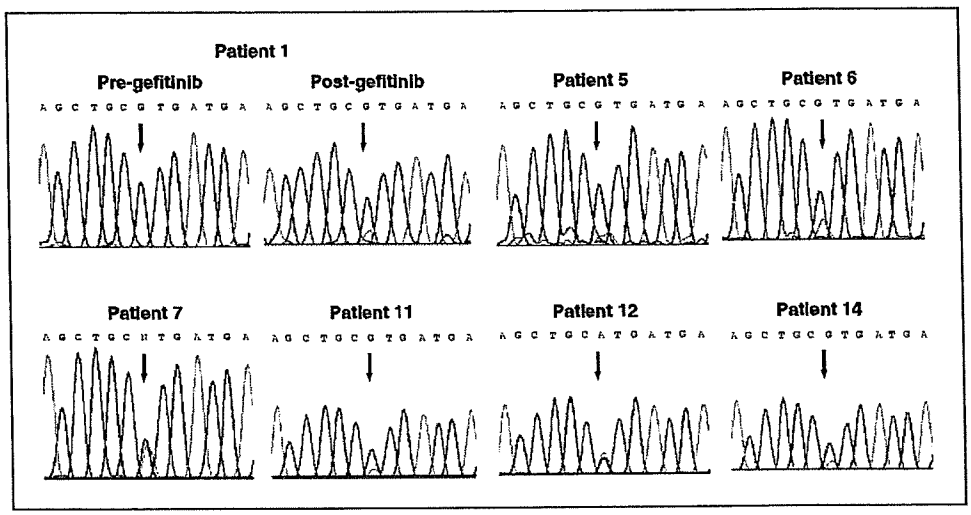

Fig. 2. Sequencing chromatograms for *EGFR* exon 20. SecondaryT790M mutations were observed in seven patients. Antisense strands of each chromatogram. Arrows, small peaks of the C→T substitution at nucleotide 2,369 (G→A on the antisense strand), which results in the T790M mutation. This substitution was observed only in posttreatment samples. T790M mutant bands were clearly detected on sequencing chromatograms, except in that of patient 5; in this patient, it was unclear because of artifacts.

before and after gefitinib treatment. Although the T790M mutant band was weaker than the L858R mutant band in patient 11, the intensity of the R776H mutant band was the same as that of the L858R mutant band and both mutations were heterozygous. We considered these point mutations to be primary mutations and not associated with "acquired" resistance.

To increase the sensitivity for the detection of T790M and other possible secondary mutations in the tyrosine kinase domain, each PCR product was subcloned and multiple subclones were amplified and sequenced directly. All the T790M mutations found by sequencing the noncloned PCR products were confirmed by this subcloning method, but no new T790M mutations were detected even when >50 clones were analyzed in samples from patients 2 and 3 (Table 2). Furthermore, we detected no secondary mutations in exons 18 to 21 other than T790M.

The T790M mutations were either present in clones with activating (or sensitizing) mutations or in other clones without activating mutations (Table 2). In three tumors (of patients 1, 5, and 14), T790M was present only in clones with activating mutations, whereas in the remaining four tumors (patients 6, 7, 11, and 12), T790M was present in both clones with and without activating mutations. No tumor carried the T790M mutation only in the wild-type clones. However, four of five T790M mutations were in clones without activating mutations in the tumor of patient 6.

We also looked for mutations in codon 12 (and codons 13 and 61 in RNA samples) in the *KRAS* gene. However, none of the samples from the tumors studied had *KRAS* mutations.

Relationship between T790M mutation and clinical and genetic features. T790M mutations were more frequent in women (women, 7 of 10; men, 0 of 4), who had never smoked (never smoker, 5 of 8; previous smoker, 2 of 6), and with deletion mutations (deletion, 6 of 9; L858R, 1 of 5). There was no difference in the incidence of T790M in the presence or absence of prior chemotherapy (with, 4 of 8; without, 3 of 6; Table 1).

We also compared the duration of gefitinib treatment, which is considered to correlate roughly with the time to progression, with the presence or absence of T790M. However, the median treatment times were almost identical (tumors with T790M, 346 days; tumors without T790M, 368 days; Fig. 3).

Analysis of corresponding tumor tissues before gefitinib treatment in patient 1. To determine whether rare T790M

Table 2. Analysis of acquired mutation using the subcloning method

Patient no.	Activating mutation	Total clones	Activating mutant clones		Wild-type clones	
			With T790M	Without T790M	With T790M	Without T790M
1	Δ2	21	8	10	0	3
2	Δ3	54	0	52	0	2
3	Δ1	51	0	50	0	1
4	Δ1	21	0	13	0	8
5	Δ1	51	3	39	0	9
6	Δ1	47	1	17	4	25
7	Δ1	20	4	5	1	10
8	L858R	18	0	14	0	4
9	L858R	20	0	14	0	6
10	L858R	20	0	5	0	15
11	L858R	21	5	10	1	5
12	Δ1	23	11	9	1	2
13	L858R	21	0	8	0	13
14	Δ1	19	7	8	0	4

mutant clones existed before gefitinib treatment, we analyzed the corresponding tumor tissues of patient 1, whose tissue after gefitinib treatment had a secondary T790M mutation. Tumor tissue was obtained at the time of operation. PCR products from the tumor before gefitinib treatment were subcloned, and 103 subclones were amplified and sequenced directly. However, at this sensitivity, we detected no clone carrying the T790M mutation. Among 103 clones, 92 (89%) had activating deletion mutations, suggesting that the mutant allele was amplified before gefitinib treatment. The incidence of clones with deletional mutations was similar (18 of 21, 85%) in a cervical lymph node taken after gefitinib resistance had developed.

To further explore of possible association of T790M with metastatic spread, we looked for the T790M mutation in hilar and mediastinal lymph nodes with metastases dissected at the time of surgery. Genomic DNA was extracted from lymph nodes from four stations (aortopulmonary, ascending aorta, main bronchus, and intrapulmonary) and analyzed using cycleave real-time PCR. However, we detected no T790M mutations.

Analysis of tumors for T790M before gefitinib treatment in 52 patients who were treated with gefitinib. The possible presence of T790M at a low frequency in tumors before gefitinib treatment might affect the tumor response or the time to progression after gefitinib treatment. In a previous study, we sequenced exons 18 to 23 of the *EGFR* genes of 52 patients who had been treated with gefitinib for recurrent disease after they had undergone pulmonary resection. None of them had the T790M mutation. Here, we used a cycleave real-time PCR assay, which is more accurate analysis than normal sequence, to investigate whether rare T790M mutant cells were present. However, we detected no T790M mutations in these 52 tumors.

Discussion

We studied 14 tumors with acquired resistance to gefitinib for secondary mutations occurring in the *EGFR* tyrosine kinase domain. Seven of the 14 tumors had a secondary T790M mutation, an incidence consistent with those of previous studies (9, 10). Whereas clones with activating mutations (deletion or L858R) might well have been eliminated by selection pressure during gefitinib treatment, those clones were always present in tumors that developed acquired resistance. In most cases, clones with the T790M mutation were not predominant.

The T790M mutations occur more frequently in women who had never smoked and who had a deletion-type mutation. Time to progression did not differ between tumors that acquired secondary T790M mutations and those that did not. However, these tendencies require careful interpretation because of the number of samples was small.

In a previous report, Kobayashi et al. (9) showed that the T790M mutation was observed with either wild-type or deletion mutation sequences, whereas Pao et al. (10) showed that both the T790M and L858R mutations were in the same allele. Our data showed that three samples had the T790M mutation only in the clones with activating mutation and four samples had the T790M mutation in the clones with and without activating mutation, whereas the most of T790M mutation was in the clones with activating mutation, except for

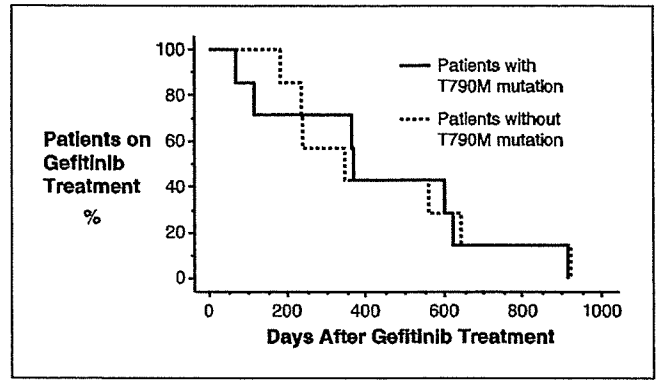

Fig. 3. Effect of the T790M mutation on the length of gefitinib treatment. The length of gefitinib treatment was considered to be roughly related to time to progression. Median treatment times were almost identical in both the presence and absence of the T790M mutation.

the samples of patient 6. It is possible that this could result from a PCR error or DNA repair error at the subcloning step. Bell et al. (28) have reported that artifactual PCR-generated allelic separation occurred with probability of ~30% in their analysis. However, it is also possible that the T790M mutation occurs in both alleles or that tumor heterogeneity exists.

In CML, 20 to 30 mutations in the *ABL* gene are responsible for acquired resistance to imatinib. Many types of mutations have been detected, and there are four distinguishable clusters (P-loop, T315, M351, and A-loop; ref. 29). Furthermore, secondary mutations in the ABL kinase domain are found in 50% to 90% of patients (29), many more than in patients with non-small cell lung cancer. We detected no novel mutations in the *EGFR* gene other than T790M. Two tumors had another point mutations together with L858R, L833V, or R776H. We considered these point mutations to be primary mutations and not associated with acquired resistance. However, these conclusions were based only on sequencing and subcloning methods, and we have no evidence of the functional effects of these mutations. There may be differences in the mechanisms of acquired resistance between non-small cell lung cancer and CML.

We previously reported that, in a series of 397 unselected patients with non-small cell lung cancer who had undergone surgery, 2 female patients with no history of smoking had L858R plus T790M mutations (21). Because these patients were not treated with gefitinib, T790M might well have conferred a growth advantage. These tumors were aggressive and later developed recurrent disease. One was treated with gefitinib but was refractory to treatment. A similar case was reported by another group (22). Inspired by this observation and because the secondary mutations related to imatinib resistance in CML were detected at low frequencies (0.01-0.9%) in pretreatment samples (16, 20), we attempted to detect minor clones with the T790M mutation in samples before gefitinib treatment. However, we could not detect the T790M mutation by assays that can detect mutant cells if there is about 1% to 5% at least. It remains unclear whether a more sensitive method would have detected rare clones with the T790M mutation in our samples.

Why tumors with T790M mutant cells acquire resistance to gefitinib despite the fact that mutant band for the T790M

mutation was almost always weaker than wild-type band remains unclear. It is possible that cells with the T790M mutation preexist at a very low frequency and gradually increase during gefitinib treatment by clonal selection as in cases of CML (16). It is also possible that amplification of the activating mutant allele occurs in resistant tumors and parts of them have the T790M mutation. Another possibility is that multiple coexisting mechanisms, including the T790M mutation, cause acquired resistance cooperatively or independently. A recent study suggested that increased internalization of ligand-bound EGFR is one of the mechanisms underlying acquired gefitinib resistance (30). It is also likely that *EGFR* gene amplification (31) by alteration of downstream molecules, such as AKT (32), might play a role in the acquisition of resistance to gefitinib.

Mutations in *KRAS* are associated with a lack of sensitivity to gefitinib and erlotinib (23). We looked for *KRAS* mutations because of the possibility that acquired *KRAS* mutations are associated with acquired resistance. There were no *KRAS* mutations in any tumor. The same finding has been reported in a previous study (10), suggesting that *KRAS* mutations are not associated with acquired resistance.

In conclusion, half of tumors with acquired resistance to gefitinib had secondary T790M mutations. No novel mutations in the *EGFR* gene were present in contrast to CML.

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A Rapid, Sensitive Assay to Detect EGFR Mutation in Small Biopsy Specimens from Lung Cancer

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It has been demonstrated that lung cancers, specifically a subset of pulmonary adenocarcinomas, with epidermal growth factor receptor (EGFR) mutation are highly sensitive to EGFR-targeted drugs. Therefore, a rapid, sensitive assay for mutation detection using routine pathological specimens is demanded in clinical practice to predict the response. We therefore developed a new assay for detecting EGFR mutation using only a paraffin section of a small biopsy specimen. The method was very sensitive, detecting as few as 5% cancer cells in a background of normal cells, the results usually being obtained within 4 hours. Furthermore, it was accurate, as shown by the high concordance with reverse transcriptase-polymerase chain reaction-coupled direct sequencing (186 of 195, 95%). The practical application of this assay to 29 cases treated with gefitinib resulted in a high prediction rate: 10 of the 11 responders were shown to be positive for the mutation, and all patients with progressive disease were negative. In addition, a mutation at codon 790, conferring gefitinib resistance, was successfully analyzed in a similar manner. In conclusion, the assay is a rapid, sensitive method using paraffin sections of biopsy specimens without a tumor cell-enrichment procedure and is quite useful to select a treatment of choice in clinical practice. *(J Mol Diagn 2006, 8:335–341; DOI: 10.2353/jmoldx.2006.050104)*

During the last decade, small molecules that inhibit receptor protein kinase activity have been developed.[1] Gefitinib is one such drug that targets epidermal growth factor receptor (EGFR) kinase. The EGFR, also known as HER1 or ErbB, is a 170-kd receptor tyrosine kinase (TK) that dimerizes and phosphorylates several tyrosine residues on the binding of several specific ligands.[2,3] These phosphorylated tyrosines serve as binding sites for several signal transducers that initiate multiple signaling pathways, resulting in cell proliferation, migration and metastasis, evasion of apoptosis, or angiogenesis, through Ras-Raf-MEK-ERK, phosphatidylinositol-3 kinase-AKT, and PAK-JNKK-JNK pathways. EGFR is expressed in more than 80% of non-small-cell lung cancers (NSCLCs), in addition to a wide range of epithelial cancers. However, clinical trials have shown significant variability in response to gefitinib: 10 to 20% of patients respond to gefitinib treatment, and in some patients, the response is dramatic, whereas the remaining patients show no response. Although further analysis has revealed some prevalence in responders, no definite determinant of the response has been established.

Recently, it has been reported that EGFR somatic mutation can be identified in a subset of pulmonary adenocarcinomas and that tumors with EGFR mutations are highly sensitive to gefitinib.[4,5] This correlation has subsequently been confirmed by our group and others,[6–9] and thus the development of a rapid and sensitive assay to predict gefitinib response by means of the presence or absence of the mutation is demanded clinically. Paraffin sections are a convenient source for such an assay in practice, but most studies using immunohistochemistry failed to predict the response.[10–12]

In this study, we introduce a practical approach using a rapid screening assay of EGFR mutation to predict gefitinib response. This method uses only a single paraffin section of a small biopsy specimen and does not require a tumor cell-enrichment procedure. The result is usually obtained within 4 hours and can be applied to a large number of samples.

Materials and Methods

Patients and Tissues

A series of 195 NSCLCs, in which the mutational status of the EGFR-TK domain with both reverse transcriptase-polymerase chain reaction (RT-PCR)-coupled direct sequencing and the new assay presented here was accessible, was used for this study. Some of the mutational results by RT-PCR-coupled direct sequencing have been

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reported previously.[13] DNA for the new assay was prepared from a section of tissue microarray blotted with 0.6-mm tissue cores of the 195 cases. To examine a correlation with the clinical response evaluated according to the guidelines of Response Evaluation Criteria in Solid Tumors (RECIST), a paraffin section of each biopsy specimen was examined for EGFR mutation in 29 patients treated with gefitinib because of the failure of first or second line therapy. To analyze the codon 790 mutation, which has been reported in association with acquired resistance to gefitinib treatment, four tissues were examined. One, reported as a rare case, was shown to have T790M, independent of gefitinib treatment.[13,14] The other three presented with a recurrent tumor after gefitinib treatment, and the recurrent tumor and corresponding initial tumor tissue were examined. Appropriate approval was obtained from the institutional review committee in addition to written informed consent from the patients.

Mutation Assay by RT-PCR-Coupled Direct Sequencing

Frozen tissue from the tumor specimens was grossly dissected to pass as many tumor cells as possible into the extraction solution (at least 25% of tumor cell content), followed by the extraction of total RNA with an RNeasy kit (Qiagen, Valencia, CA). For RT-PCR-coupled direct sequencing, the EGFR tyrosine kinase domain (exon 18 to 24) was amplified, and then the products were directly sequenced with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). The primer set used was described previously.[13]

DNA Extraction from Paraffin-Embedded Tissues

Tumor cell-rich area in a hematoxylin and eosin-stained section was marked under a microscope, and tissues were scratched from the area of another deparaffinized unstained section. Pieces of the scratched tissue were incubated with 1× PCR buffer containing 100 μg/ml proteinase K for 1 hour at 54°C. After heat inactivation with 95°C for 3 minutes, the solution was directly used for template DNA for the assay.

EGFR Mutation Detection

To detect the point mutations at codons 858 and 790 of the EGFR gene, we used the cycleave PCR technique. This technique is based on a chimeric DNA-RNA-DNA probe labeled with a fluorescent dye and quencher at each end. The RNA sequence of the probes corresponds to that of the wild type and point mutation labeled with FMA and ROX, respectively. When mutant molecules are present in the sample and PCR-amplified DNA generates a complete hybrid with the RNA portion of the mutant probe, RNase-H digests the probe at the RNA-DNA heteroduplex into two pieces, leading to a significant increase in fluorescence intensity by separation of the fluorescent dye from the quencher. The intensity of the wild-type probe served as an internal control for the assay. This assay was performed using a cycleave PCR core kit (TAKARA, Co., Ltd., Ohtsu, Japan), and sequences of the primer set and the probes were as follows: PCR forward primer for L858R, 5′-AGGAACGTACTGGTGAAAAC-3′; PCR reverse primer for L858R, 5′-TCCCTGGTGTCAGGAAAATG-3′; wild-type probe for L858R, 5′ FAM-CCA *U* CCCAAAAT-Eclipse 3′; probe for L858R mutation, 5′ FAM-CCC*G*CCCAAAAT-Eclipse 3′; PCR forward primer for T790M, 5′-ATCTGCCTCACCTCCAC-3′; PCR reverse primer for T790M, 5′-CAATATTGTCTTTGTGTTC-3′; wild-type probe for T790M, 5′ FAM-TGC*G*TGATGAG-Eclipse 3′; probe for T790M mutation, 5′ FAM-TGC*A*TGATGAG-Eclipse 3′ (italics represent RNA). Fluorescent signals were quantified with a Smart Cycler system (SC-100; Cepheid, Sunnyvale, CA).

To detect the deletion in exon 19 of the EGFR gene, common fragment analysis was used. Sample DNA was amplified with an FAM-labeled primer set as follows: forward, 5′ FAM-TCACAATTGCCAGTTAACGTCT-3′, and reverse, 5′-CAGCAAAGCAGAAACTCACATC-3′. PCR products were electrophoresed on an ABI PRISM 310. When a deletion mutation was present, PCR amplified the shorter segment of DNA, creating a new peak in an electropherogram.

Sensitivity Assay

In the preliminary examination, we prepared a mutation-positive control DNA, which contained exactly one-half each of wild-type and mutant molecules. According to the mixture ratio, the mutation-positive control DNA was mixed up with normal DNA, the concentration of which was equal to that of the mutation-positive control DNA. Therefore, 5% of tumor cells corresponded to 2.5% of mutant molecules in background of wild-type molecule. Using these mixtures of DNA, we examined the sensitivity of the assays (deletion of exon 19 and point mutation of L858R and T790M).

Statistical Analysis

The χ^2 test and Fisher's exact test for independence compared incidences of EGFR mutation, using SYSTAT software (SYSTAT Software Inc., Richmond, CA). A P value below 0.05 was considered statistically significant.

Results

Sensitivity of the New Assay

It is known that mutations in the EGFR tyrosine kinase domain are restricted to four exons, and the results of previous reports[4,5,8,9,13,15] revealed that the deletion in exon 19 and the point mutation of codon 858 in exon 21 covers about 90% of cases with EGFR-TK mutation. We therefore established assays using fragment analysis for

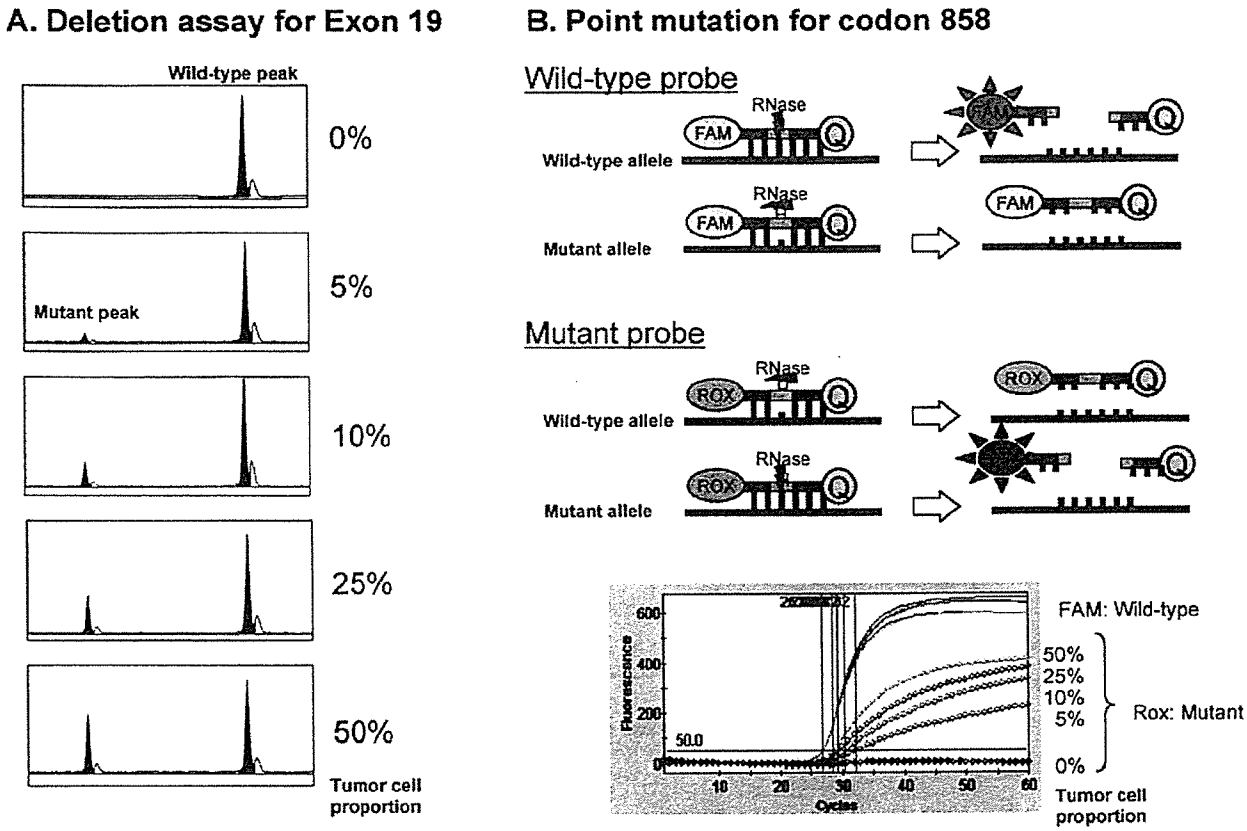


Figure 1. Sensitivity of the new assay. **A:** The sensitivity of the fragment analysis in the new assay. As few as 5% of tumor cells with the deletion could be detected. In the **top** of **B**, a brief explanation of the cycleave technology is displayed. Using this technique, as few as 5% of tumor cells with point mutation at codon 858 could be detected (**bottom** of **B**).

the deletion and cycleave real-time PCR for the point mutation of codon 858. The positive detection of mutated molecules makes this assay very sensitive, as shown in Figure 1. As few as ~5% of tumor cells could be detected in this assay.

Specificity of the New Assay and Concordance with Direct Sequencing

We evaluated the concordance of results between the new assay and conventional direct sequencing using 195 NSCLCs. The results are summarized in Table 1. Overall concordance was 186 of 195 (95%). When we excluded the seven evaluation cases, which were mutated in regions other than the targets of this assay, 99% of cases were concordant. In one case, mutation was only detected with the new assay, whereas one case was negative for mutation with the new assay but positive with direct sequencing. This disagreement resulted from the different tumor cell population in the samples examined. In the preliminary examination, at least 25% of tumor cells were required for detection of the gene mutation by direct sequencing (data not shown). Although tumor tissues in this analysis were dissected to contain more than 25% of tumor cells from most frozen sections, this case contained around 25% tumor cells, on the threshold of that detectable by the sequencing approach. In contrast, the paraffin section used for the new assay was rich in tumor cells. This difference in tumor cell content between frozen and paraffin sections may be the cause of the discrepancy. We confirmed this result by direct sequencing of the frozen section, using DNA microdissected with a laser capture microdissection system.

Practical Application for the Prediction of Gefitinib Response

To confirm whether the new assay is useful for the prediction of gefitinib response in clinical practice, we applied the assay to 29 gefitinib-treated cases whose response had been evaluated according to RECIST. A paraffin section of the large tumor tissue, which had been surgically resected a few years before relapse, was used in seven cases, whereas DNA was extracted from a paraffin section of transbronchial biopsy or computer tomography-assisted fine needle biopsy in 20 cases (Figure 2). Partial response was achieved in 11 cases; all but one were positive for the mutation, whereas five cases with progressive disease were negative with this assay (Table 2). EGFR mutation was detected in only 2 of 13 cases evaluated as stable disease. The correlation be-

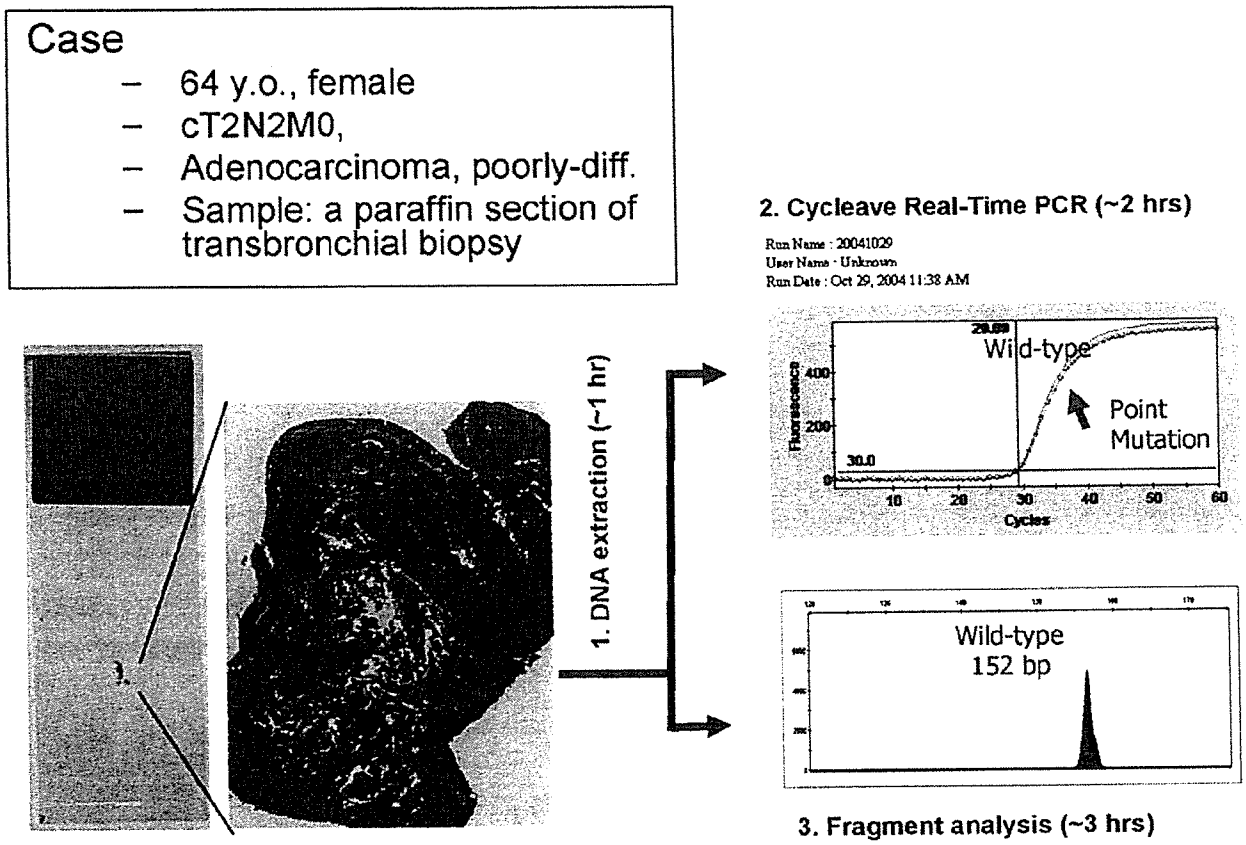


Figure 2. A representative result of the new assay. DNA was extracted from a paraffin section of the biopsy followed by simultaneous analysis using cycleave real-time PCR and fragment analysis. The entire procedure was completed within 4 hours. In this case, point mutation at codon 858 was detected, and the patient responded to gefitinib therapy.

tween EGFR mutation and gefitinib response was highly significant ($P = 0.0001$).

All of the 12 EGFR-mutated specimens were also examined by direct sequencing. In seven cases, identical results were obtained with both methods, whereas background noise prevented us from evaluating the results in the other five cases, all of which were small biopsy specimens. This may not indicate a lack of confirmation but rather suggests the superiority of this new assay, considering the good correlation of this result with clinical response and with the results obtained with direct sequencing using sufficient amounts of surgical tissue.

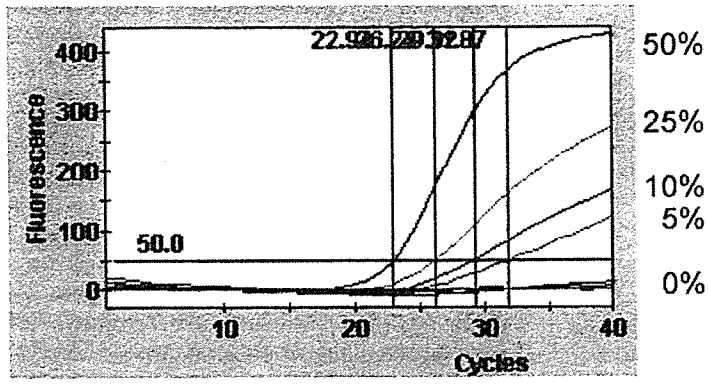
Detection of Mutation at Codon 790 Conferring Acquired Resistance to Gefitinib

Recently, it has been reported that a second mutation, at codon 790, was associated with acquired resistance to gefitinib.[16,17] On very rare occasions, the mutation was also detected independently of gefitinib treatment.[13,14] An assay for this mutation, using cycleave PCR, was similarly established (Table 3). In this assay, as few as 5% of tumor cells could be detected, as shown in Figure 3. A rare case, whose tumor was known to have T790M

Table 1. Comparison of Results between the Conventional and New Assays

	New assay		
Direct sequencing	Wild type	Mutation at codon 858	Deletion at exon 19
Wild type	116	1	0
Point mutation at codon 858	0	32	0
Deletion at exon 19	1	0	38
Point mutation at codon 719	3	0	0
Insertion at exon 20	3	0	0
Point mutation at codon 742	1	0	0

A. Sensitivity analysis

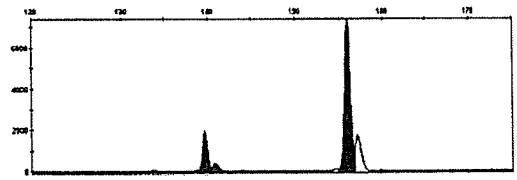


B. Representative case of acquired second mutation

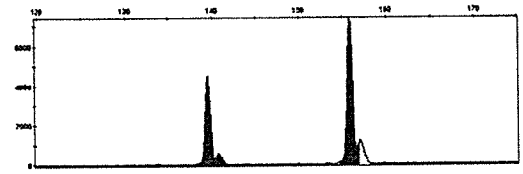
Primary tumor before treatment

Recurrent tumor in pleural effusion

Exon 19, fragment analysis

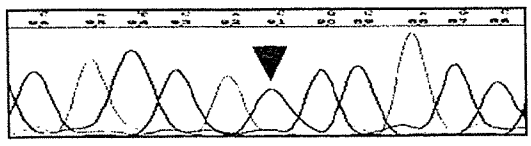


15bp-deleted

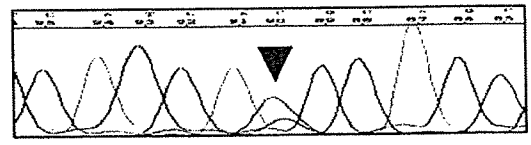


15bp-deleted

Exon 20, PCR-direct sequencing

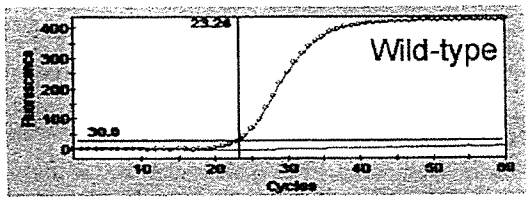


Wild-type



T790M

T790M cycleave PCR



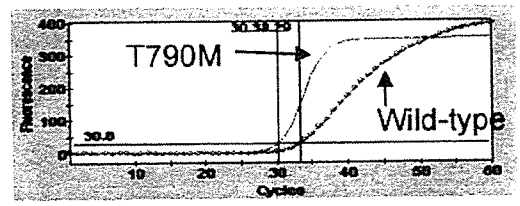


Figure 3. Detection of acquired mutation at codon 790. **A:** The sensitivity of this cycleave assay for T790M mutation. As few as 5% of tumor cells with T790M mutation could be detected. A representative result of acquired mutation at codon 790 after gefitinib treatment is displayed in **B** (Table 3, case 2). In contrast to the 15-bp deletion in exon 19 of the EGFR gene in both primary and recurrent tumors, T790M was detected only in the recurrent tumor, suggesting acquired mutation after gefitinib treatment. The result of the cycleave method was more obvious than that with direct sequencing.