treated during this trial are listed in Table 1. Fourteen patients were women and 46 were men, and their median age was 67 years (range, 52 to 79 years). Eleven patients (18%) exhibited LD and 49 patients (82%) exhibited ED at the time of enrollment onto this study. All 60 patients had been pretreated using some form of topoisomerase inhibitor–based chemotherapeutic regimens: 24 patients had received prior topoisomerase I inhibitor (irinotecan or topotecan)–containing chemotherapy, 20 had had prior etoposide-containing chemotherapy, and 16 had received both topoisomerase I and II regimens (Table 2). Nineteen of these patients had received thoracic irradiation after or simultaneously with chemotherapy.

Response to Therapy and Survival

Among the 60 assessable patients, two patients (3%) achieved a complete response (CR) and 29 patients (48%) had a partial response (PR), for an overall response rate of 52% (95% CI, 38% to 65%; Table 2). Twelve patients had stable disease, and 17 had disease progression.

Table 1. Patient Characteristics

Characteristic	Sensitive Group	Refractory Group	Total
Total No. of patients	44	16	60
Sex			
Male	35	11	46
Female	9	5	14
Age, years			
Median	67	63	67
Range	52-79	52-76	52-79
Performance status (ECOG)			
0	23	5	28
1	20	8	28
2	1	3	4
Disease extent at relapse			
Limited disease	7	4	11
Extensive disease	37	12	49
Sites of metastases			
Adrenal gland	7	2	9
Lymph node	3	1	4
Lung	10	5	15
Bone	6	4	10
Brain	17	4	21
Liver	11	4	15
Skin	3	0	3
Other	5	0	5
Prior therapy			
Chemotherapy alone	28	12	40
Chemotherapy and chest irradiation	14	4	18
Chemotherapy and surgery	1	0	1
Chemotherapy, surgery, and irradiation	1	0	1
No. of prior chemotherapy regimens			
1	38	8	46
2	6	8	14
Response to prior chemotherapy			
CR	9	1	10
PR	35	8	43
SD or PD	0	7	7
Chemotherapy-free interval, days			
< 60	0	9	9
≥ 60	44	—	44

Abbreviations: ECOG, Eastern Cooperative Oncology Group; CR, complete response; PR, partial response; SD, stable disease; PD, progression of disease.

Seven (44%) PRs and one (6%) CR were found among refractory patients, with an overall response rate of 50% (95% CI, 25% to 75%). Of eight refractory patients who responded to amrubicin, six had responded to the prior treatment, but had a relapse less than 60 days after completing initial chemotherapy, and two had a relapse during prior treatment. Of five refractory patients who had progressed after second-line treatment, one patient attained a PR to amrubicin treatment. Twenty-two (50%) PRs and one (2%) CR were attained in sensitive patients, with an overall response rate of 52% (95% CI, 37% to 68%). No significant difference in the overall response rate was seen when the patients were analyzed according to sex, performance status (0 to 1 *v* 2), response to initial chemotherapy, or disease extent (LD *v* ED). Of 40 patients pretreated with topoisomerase I inhibitor–containing regimens, 21 patients (53%) achieved a PR. It is noteworthy that 17 PRs (47%) and two CRs (6%) were attained in 36 patients who had had prior etoposide-containing chemotherapy. Responses were usually observed at a median of 32 days (range, 15 to 91 days) after the start of amrubicin treatment and occurred at all sites, including the brain (six of 21). The median time to progression was 2.6 months in the refractory patients, and 4.2 months in the sensitive patients.

Of the 60 patients, 19 patients (32%) were still alive as of April 26, 2006. The median survival time from the enrollment of the protocol treatment for all patients was 11.2 months (sensitive group, 11.6 months; refractory group, 10.3 months; Fig 1). The 1-year actuarial survival rate in patients with sensitive disease was 45.5%, compared with 40.3% in the patients with refractory disease. The 1-year survival rate for all patients was 44.1% (95% CI, 30.6% to 56.8%).

Toxicity and Treatment Received

Four patients were removed from the study after the first cycle of treatment because of progressive disease. Therefore, 56 patients received multiple courses of treatment in successive cycles. A total of 224 courses (58 refractory and 166 sensitive) were administered; all of these courses were included in the toxicity analysis (median cycles per patient, four; range, one to eight). Reduction of the amrubicin dose was required in 42 (18.8%) of cycles only in the sensitive group. Consequently, it was possible to deliver the full doses of amrubicin treatment in 80.4% of the entire 224 cycles. Thirty-eight (63%) of 60 patients could receive the planned four cycles. The major reasons for early discontinuation of treatment were disease progression (14 patients), acute pneumonia (two patients), and patient refusal (two patients). Most of the episodes of severe leukopenia and/or thrombocytopenia were observed during cycle 1; dose modifications were made in subsequent cycles.

The most frequent toxicity was myelosuppression, which affected leukocytes primarily: grade 3 or 4 neutropenia was seen in 28% and 55% of patients, respectively (Table 3). G-CSF was administered in 134 (60%) of the 224 cycles that were administered; 42 patients (70%) received G-CSF. However, only three episodes of fever were observed during the period of neutropenia. Thrombocytopenia was relatively infrequent throughout the study: grade 3 and 4 toxicity occurred in 20% and 0% of the patients, respectively. Grade 3 or 4 anemia was reported in 20 patients (33%). Nonhematologic toxicity was generally mild. The most frequent grade 3 or 4 nonhematologic toxicities included anorexia (15%), asthenia (15%), hyponatremia (8%), and nausea (5%). No cardiotoxicity, except for one transient atrial fibrillation, was observed during this trial.

Table 2. Response to Amrubicin Monotherapy

Characteristic	No. of Patients	CR	PR	SD	PD	Response Rate (%)	P
Overall	60	2	29	12	17	52*	
Sex							
Male	46	0	23	10	13	50	.64
Female	14	2	6	2	4	57	
Performance status (ECOG)							
0/1	56	2	28	12	14	54	.35
2	4	0	1	0	3	25	
Disease extent							
Limited disease	11	2	2	3	4	36	.26
Extensive disease	49	0	27	9	13	55	
Sensitivity to prior CT							
Sensitive	44	1	22	10	11	52	.88
Refractory	16	1	7	2	6	50	
Prior treatment with topoisomerase inhibitor–based regimen							
Topo-I	24	0	12	5	7	50	.91
Topo-II	20	2	8	6	4	50	
Both	16	0	9	1	6	56	

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; CT, chemotherapy; ECOG, Eastern Cooperative Oncology Group; Topo-I, topoisomerase I inhibitor–containing regimen; Topo-II, topoisomerase II inhibitor–containing regimen.
*95% CI, 38% to 65%.

No evidence of cumulative leukopenia, anemia, or asthenia toxicity was seen during subsequent courses at two dose levels. No treatment-related deaths occurred during this trial.

DISCUSSION

Treatment options for patients who experience relapse remain limited. Recently, a multicenter randomized trial demonstrated that single-agent topotecan was at least as efficacious as the three-drug combination of cyclophosphamide, doxorubicin, and vincristine for the treatment of patients with sensitive disease.[16] Topotecan showed a response rate of 24% *v* 18% for cyclophosphamide, doxorubicin, and vincristine ($P = .28$), with improved symptom control. The median survivals were superimposable between two treatments (25 *v* 24.7 weeks). The results of the phase III trial have made topotecan the only drug approved by the US Food and Drug Administration for the single-agent management of patients with relapsed SCLC.

Several reports on single-agent activity for newer chemotherapeutic agents, including topoisomerase I inhibitors,[17-21] taxanes,[22] gemcitabine,[23] and vinorelbine,[24,25] in the second-line setting have been made. However, few single agents are capable of producing a

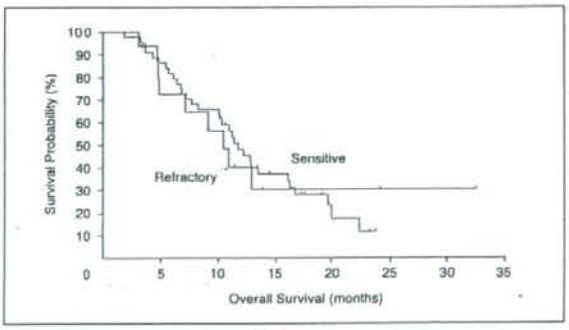

Fig 1. Median survival times in all patients with refractory or relapsed small-cell lung cancer were 10.3 months in the refractory group (n = 16) and 11.6 months in the sensitive group (n = 44), respectively ($P = .974$; log-rank test). The 1-year actuarial survival rate in patients with refractory disease was 40.3%, compared with 45.5% in the patients with sensitive relapse.

Table 3. Worst Toxicity by 60 Patients During Amrubicin Monotherapy

Toxicity	Grade 1	Grade 2	Grade 3	Grade 4	≥ Grade 3 No.	≥ Grade 3 %
Neutropenia	1	7	17	33	50	83.3
Leukopenia	4	12	30	12	42	70.0
Hemoglobin	15	24	17	3	20	33.3
Thrombocytopenia	21	14	12	0	12	20.0
Anorexia	22	8	8	1	9	15.0
Asthenia	24	11	6	3	9	15.0
Hyponatremia	21	0	5	0	5	8.3
Nausea	18	5	3	0	3	5.0
Febrile neutropenia	0	0	3	0	3	5.0
Hypokalemia	13	0	2	0	2	3.3
Fever	10	5	2	0	2	3.3
Pneumonia	0	0	2	0	2	3.3
Hypoalbuminemia	40	4	1	0	1	1.7
Elevated AST	20	0	1	0	1	1.7
Vomiting	7	3	0	1	1	1.7
Diarrhea	8	2	1	0	1	1.7
Constipation	3	1	1	0	1	1.7
Cognitive disturbance	0	0	1	0	1	1.7
Memory impairment	0	0	0	1	1	1.7
Atrial fibrillation	0	0	1	0	1	1.7
Infection with neutropenia	0	0	1	0	1	1.7

high incidence of response among patients with early relapse or disease progression during treatment. Smit et al[26] reported the results of phase II trial for paclitaxel given as a 3-hour infusion at a dose of 175 mg/m^2 every 3 weeks in patients refractory to cyclophosphamide, doxorubicin, and etoposide. Although the response rate of 29% was at the upper level of activity for any single agent in this setting, two early deaths and two toxicity-related deaths occurred in the trial, and the median survival time was a disappointingly short 100 days.

This phase II study demonstrated that amrubicin monotherapy is active against refractory or relapsed SCLC, as shown by the overall response rate of 52% (95% CI, 38% to 65%) in 60 patients (Table 2). Although the activity of second-line treatments usually depends on tumor responsiveness to first-line treatment, we could not find any difference in response rates between the two groups (the response rate of 50% [95% CI, 25% to 75%] for refractory disease, and 52% [95% CI, 37% to 68%] for sensitive relapse). This high response rate in chemotherapy-resistant patients is encouraging given the fact that response rates of less than 10% are usually attained for single-agent chemotherapy in patients with this disease category.[27] Furthermore, a promising similar survival outcome was obtained in the two groups (10.3 *v* 11.6 months in refractory and sensitive group, respectively; Fig 1). These results suggest that amrubicin may be a useful new addition to treatment strategies for chemotherapy-resistant patients. Obviously, however, more SCLC patients with refractory disease treated with amrubicin will be needed to determine the true response rate in this population, given that the number of patients in this study is too small to draw any valid conclusion about the ultimate clinical activity of this regimen.

DNA topoisomerase I and II are functionally related and are believed to act in concert in a variety of genetic processes.[28] Preclinical studies have demonstrated that resistance to camptothecin, a topoisomerase I inhibitor, is often accompanied by the upregulation of topoisomerase II, causing hypersensitivity to agents that target topoisomerase II.[29] This enhanced sensitivity (collateral sensitivity) may explain, in part, the high response rate observed in our patients, given that most of the patients had been heavily pretreated during topoisomerase I inhibitor (irinotecan or topotecan)–containing regimens. Furthermore, objective responses were documented in 19 of 36 patients who had been treated with etoposide, a potent topoisomerase II inhibitor, which suggests that there is some degree of non–cross resistance between amrubicin and etoposide.

The toxicity profile noted in this trial was predictable from that described previously for the phase I and II trials[12,13,30]; myelosuppression was the major toxic effect. All adverse effects were manageable. Because grade 3 or 4 neutropenia occurred in 85% of patients with no prior chemotherapy who were treated using the Japanese Ministry of Labor, Health and Welfare–approved dose level of 45 mg/m^2 per day for 3 days in a previous phase II trial,[12] a reduced dose of 40 mg/m^2 per day for 3 days was chosen in this trial in view of the chemotherapeutic and radiotherapeutic pretreatment. The low incidence of severe and clinically relevant bone marrow toxicity in our trial may be due to the use of this lower dose of amrubicin (Table 3). The incidence of a decrease in the left ventricular ejection fraction attributable to amrubicin was null, and this effect was never the cause of treatment discontinuation. The incorporation of amrubicin instead of doxorubicin in anthracycline-containing regimens might decrease the incidence of cardiotoxicity, thereby improving the therapeutic index of doxorubicin-containing regimens in future trials.

In conclusion, amrubicin is an active agent for the treatment of refractory or relapsed SCLC. The overall response rate of 50% and the overall survival time of 10.3 months in patients with refractory disease are noteworthy. Given the greater activity of single-agent amrubicin, additional studies in previously treated patients with SCLC are warranted, especially for the patients who are refractory to previous therapy, either as a single agent or in combination with cytotoxic agents or target-based agents.

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The authors indicated no potential conflicts of interest.

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Genome-wide cDNA microarray screening of genes related to the benefits of paclitaxel and irinotecan chemotherapy in patients with advanced non-small cell lung cancer

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Previous studies have demonstrated that not only the benefits but also the toxicities of chemotherapy can be predicted by cDNA microarray analysis of tumor specimens obtained before chemotherapy against non-small cell lung cancer (NSCLC). We conducted a study of cDNA microarray analysis to determine whether the gene expression in peripheral blood taken from patients prior to chemotherapy were correlated with the outcome of chemotherapy with paclitaxel (Pac) and irinotecan (CPT) against advanced NSCLC . Thirty-one patients with stage IIIB or IV NSCLC were treated with CPT at 60 mg/m^2 and Pac at 160 mg/m^2 every 2 weeks. Seventeen of 31 patients achieved PR and the overall RR was 54.8%. The median survival time was 426 days and the 1-year survival rate was 58.1%. The expression levels of 1176 genes were analyzed in 31 patients with the AtlasTM Human Cancer 1.2 Array. Stepwise multivariate analysis revealed that the genes encoding protein phosphatase, IL-1α and IgA were independent predictive factors for chemosensitivity. Stepwise regression analysis revealed that the thyrotropin-releasing hormone receptor and alkylation repair genes were independent prognostic factors. In conclusion, the expression of certain genes was able to predict the benefits of this Pac and CPT chemotherapy regimen.

Key words: microarray, paclitaxel, irinotecan, lung-cancer, gene

INTRODUCTION

Current chemotherapy regimens for metastatic non-small cell lung cancer (NSCLC) are not particularly effective, and the disease cannot be cured even with the most effective chemotherapy. Responders to chemotherapy may have a better prognosis than non-responders (1) and chemosensitivity is an important factor in deciding which patients should receive chemotherapy in such non-curative NSCLC. Previous study has demonstrated that not only the benefits but also the toxicities of chemotherapy can be predicted by cDNA microarray analysis of tumor specimens obtained before chemotherapy (2). The results suggest that the intrinsic genetic characteristics of individual patients will reflect the outcomes of chemotherapy and lead to the hypothesis that genetic analysis of non-malignant cells can also be used to predict the benefits and toxicities of chemotherapy.

Our previous phase I study of a paclitaxel (Pac) and irinotecan (CPT) combination led to a recommendation of Pac 160 mg/m^2 and CPT 60 mg/m^2 every 2 weeks for further study (3). This study also demonstrated an objective response rate of 58.3%, and a 1-year survival rate of 54.2%. Accordingly, we examined the correlations between gene expression in peripheral blood, which is easily available, and the benefits of the combination chemotherapy with Pac and CPT to display high activity against NSCLC.

Table 1. *Patient characteristics*

No. of patients		
Total		31
Age, years	Median	61
	Range	43 – 69
Gender	Male	20
	Female	11
Performance status	0	9
(ECOG)	1	22
Clinical stage	IIIB	5
	IV	26
Histology	Adenocarcinoma	24
	Others	7

PATIENTS AND METHODS

The Institutional Review Board of Kanagawa Cancer Center reviewed and approved this study prior to commencement.

Patients. Patients with histologically or cytologically confirmed NSCLC were registered. Eligibility criteria were: clinical stage IIIB or IV, age <70 years, Eastern Cooperative Oncology Group PS score ≤1. Patients who had received chemotherapy or radiotherapy were excluded from this study. Written informed consent was obtained from every patient.

Chemotherapy. All patients without disease progression were treated every 2 weeks for a total of four courses of chemotherapy. CPT was administered at a dose of 60 mg/m^2 on day 1. Pac was administered at a dose of 160 mg/m^2 on day 1. Premedication consisting of 20 mg dexamethasone and 50 mg ranitidine was infused. A 50 mg oral dose of diphenhydramine was also administered. Prophylactic G-CSF, 50 μg/m^2/day or 2 μg/kg/day, was administered subcutaneously on days 6 to 10. Patients were given a 5-HT$_3$ antagonist intravenously. Tumor response was evaluated according to RECIST criteria (4).

Blood samples, purification of RNA and cDNA microarray. Genomic DNA was obtained from peripheral blood mononuclear cells (PMNC) isolated from 10 ml of peripheral blood taken from patients prior to chemotherapy. The total RNA of each sample was isolated and treated with DNase I to avoid contamination by genomic DNA by using silica membrane affinity chromatography and a total RNA isolation kit (Macherey-Nagel GmbH & Co., KG, Germany). One hundred nanograms of the total RNA for each sample was reverse transcribed into cDNA. Each cDNA sample was subjected to microarray expression profiling with the BD Atlas™ Human Cancer 1.2 Array (Clontech) (2). Each labeled probe was then hybridized into a separate Atlas Array. The signal intensity for each spot, which corresponds to each gene examined, was determined with a STORM image analyzer (Amersham Bioscience, Picataway, NJ). The hybridization pattern and signal intensity were analyzed to determine changes in gene expression levels by using AtlasImage™ 2.01 software (Clontech Laboratory Inc., Japan).

Statistical methods. The association between gene expression and tumor regression during chemotherapy was tested with the Pearson correlation coefficient. To determine whether gene expression profiles were associated with differences in survival, Kaplan-Meier survival plots and log-rank tests were used. The influence of expression of each gene on chemotherapy outcomes was examined by stepwise multivariate regression analysis or cox proportional hazards model analysis. $P < 0.05$ was considered significant.

RESULTS

Between May 2002 and July 2004, 31 patients were registered in the study (Table 1). Twenty-seven patients received 4 to 6 cycles of chemotherapy, except for 4 patients who discontinued treatment in the first or second cycles because of disease progression in 3 patients and grade 2 pneumonitis in 1 patient. Seventeen of 31 patients achieved PR, 10 NC and 4 PD, and the overall RR was 54.8% in this study. The median survival time was 426 days and the 1-year survival rate was 58.1%.

The expression levels of 1176 genes in the peripheral blood cells of 31 patients were analyzed by cDNA microarray screening. Four housekeeping genes that were expressed in all 31 samples were used as controls for gene expression: ubiquitin, liver glyceraldehyde 3-phosphate dehydrogenase, 23-kDa highly basic protein, 60S ribosomal protein L13A and 40S ribosomal protein S9.

Stepwise multivariate analysis revealed that protein phosphatase with EF-hands-2 long form, IL-1α and IgA 1 heavy chain constant region + IgA2 heavy chain constant region were independent predictive factors for chemosensitivity ($p < 0.001$, Table 2). Of these genes, expression of protein phosphatase and IL-1α was positively, and expression of IgA was negatively, correlated with tumor regression rate. When we analyzed the relationship between gene expression levels and survival, the expressions of 10 genes were significantly correlated with survival times ($p < 0.01$). Stepwise regression analysis revealed that thyrotropin-releasing hormone receptor and alkylation repair genes were independent prognostic factors ($p < 0.01$, Table

Table 2. *Genes closely associated with sensitivity or survival in chmeotherapy.*

	Description	coefficient	P
Sensitivity	protein phosphatase with EF-hands-2 long form	-0.436	0.0134
	IL-1 alpha	-0.432	0.0145
	IgA 1 heavy chain constant region+ IgA 2 heavy chain constant region	0.463	0.008
Survival	thyrotropin-releasing hormone receptor	0.509	0.0029
	alkylation repair; alkB homologue	0.489	0.0046

Stepwise multivariate analysis for sensitivity and stepwise regression analysis for survival were used.

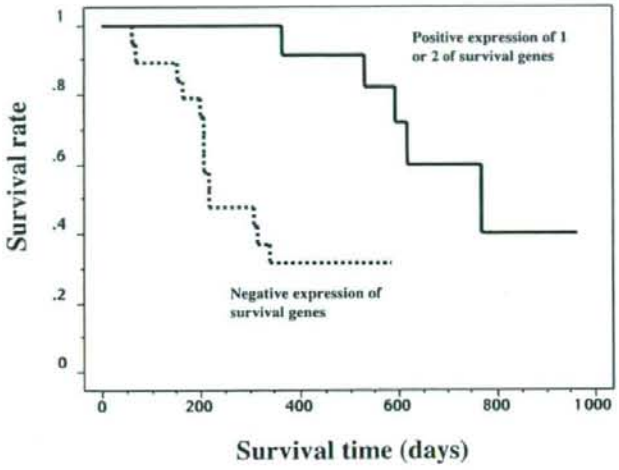

Figure 1. Survival curves constructed by the Kaplan-Meier method. The 12 of the 31 patients who showed positive expression of either the thyrotropin-releasing hormone receptor or alkylation repair genes had a significantly better chance of survival (log-rank, p = 0.0024; Wilcoxon, p = 0.0016)

2). The 12 of the 31 patients who showed positive expression of either thyrotropin-releasing hormone receptor or alkylation repair genes had a significantly better chance of survival (log-rank, p = 0.0024; Wilcoxon, p = 0.0016; Fig. 1). Cox proportional hazards model demonstrated that positive expression of these genes was only significantly dependent prognostic factor (p=0.0094, Table 3).

DISCUSSION

We previously reported that examination of tumor tissues revealed a number of genetic predictors not only of beneficial but also of toxic effects of cancer chemotherapy (2). The fact that genetic information from tumor cells can predict not only tumor susceptibility to chemotherapy but also toxicity suggests that certain genetic characteristics may be common to all somatic cells, irrespective of whether they are malignant or normal. To add support for this hypothesis, in this study we used peripheral blood cells as non-malignant normal cells for analysis of informative genetic factors that can predict the antitumor effects. Protein phosphatase, IL-1α and IgA were predictors of sensitivity to Pac and CPT combination chemotherapy. The adenoviral type 5 E1A protein has been shown to induce sensitization to apoptosis induced by different categories of anticancer drug. Up-regulation by E1A of the catalytic subunit of protein phosphatase 2A in human breast cancer cells was shown to enhance the activity of the phosphatase, which resulted in repression of Akt

Table 3. *Cox Proportional Hazards Model for Survival Analysis in paclitaxel and irinitecan treatment.*

		Hazard Ratio	95% CI	P
Gender		0.701	0.127-3.86	0.6833
	Female/Male			
Performance status		0.706	0.173-2.872	0.6264
	0/1			
Stage		0.247	0.030-2.024	0.1926
	IIIB/IV			
Hb		0.956	0.534-1.714	0.8803
Albumin		0.405	0.109-1.504	0.1770
LDH		1.002	0.997-1.006	0.4442
Survival gene		9.102	1.720-48.180	0.0094
	Negative/Positive			

activation in E1A-expressing cells (5). This up-regulation of protein phosphatase 2A might represent a novel mechanism for E1A-mediated sensitization to anticancer drug-induced apoptosis. IL-1α is a cytokine with many activities central to immune function and hematopoesis. This cytokine dramatically increases the sensitivity of osteosarcoma cells to etoposide when the two agents are used simultaneously (6). Thyrotropin-releasing hormone (TRH) receptor and alkylation repair genes were identified as independent prognostic factors. TRH plays a key role in the regulation of the thyroid axis. A number of changes in hormonal secretion patterns have been found in subjects with neoplastic disease. When mean nocturnal levels were compared, cortisol, TRH and growth factor levels were higher in patients with lung cancer than in normal controls (7). TRH and its receptor are also expressed in non-hypothalamic cells such as pancreatic cells, suggesting that TRH might play a biological role in an autocrine fashion (8). It is possible that a TRH-related autocrine system in normal cells may overcome the cachexia induced by lung cancer.

The development of cancer involves the concurrent disruption of regulation of expression of multiple genes. Therefore, DNA repair systems play an important role in tumor growth and patient survival. The acquisition of methylation of the DNA mismatch repair gene hMLH1 in plasma DNA after chemotherapy predicts poor survival for ovarian cancer patients (9), suggesting that depression of the repair system increases tumor growth and decreases patient survival time. It therefore appears reasonable that the present study showed that increased expression of alkylation repair genes is correlated with good survival.

We need to undertake prospective evaluations to determine whether the genes revealed in this study are truly important and potentially useful for predicting the beneficial of chemotherapy. Accumulation of such data could eventually allow chemotherapy to become "personalized", allowing the use of anticancer drugs that are effective in individual patients.

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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′

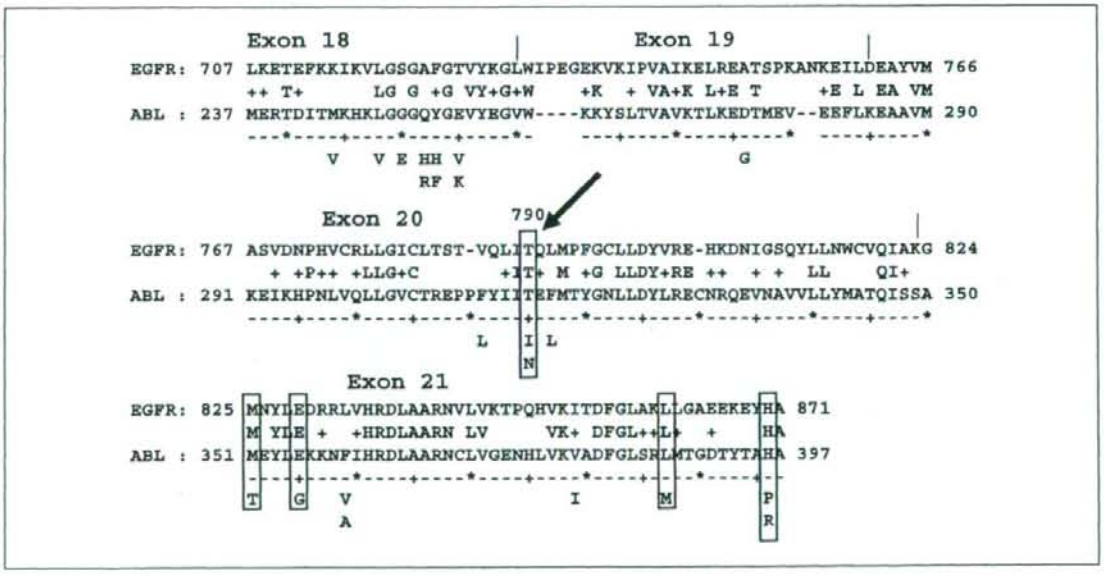

Fig. 1. Structural similarity between EGFR tyrosine kinase and ABL. This amino acid alignment was obtained using basic local alignment search tool, and both sequences were obtained from Genbank (accession nos.: EGFR, NM 005228; ABL, NM 005157). Top line, EGFR; bottom line, ABL. Vertical lines, boundaries 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′-AGCTTGTGGAGCCTCT-TACACC-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

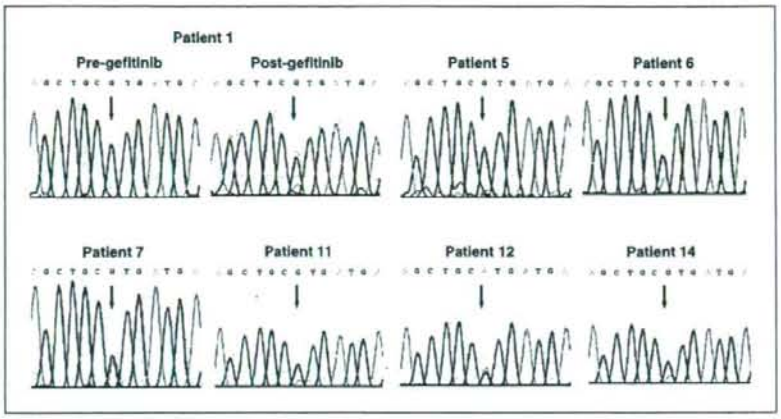

Fig. 2. Sequencing chromatograms for *EGFR* exon 20. Secondary T790M mutations were observed in seven patients. Antisense strands of each chromatogram. Arrows, small peaks of the C→T substitution at nucleotide 2,369 (G→A on the antisense strand), which results in the T790M mutation. This substitution 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

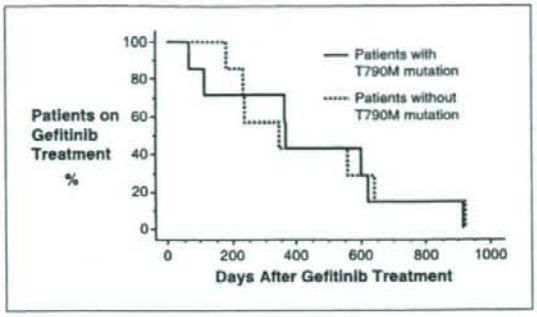

Fig. 3. Effect of the T790M mutation on the length of gefitinib treatment. The length of gefitinib treatment was considered to be roughly related to time to progression. Median treatment times were almost identical in both the presence and absence of the T790M mutation.

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

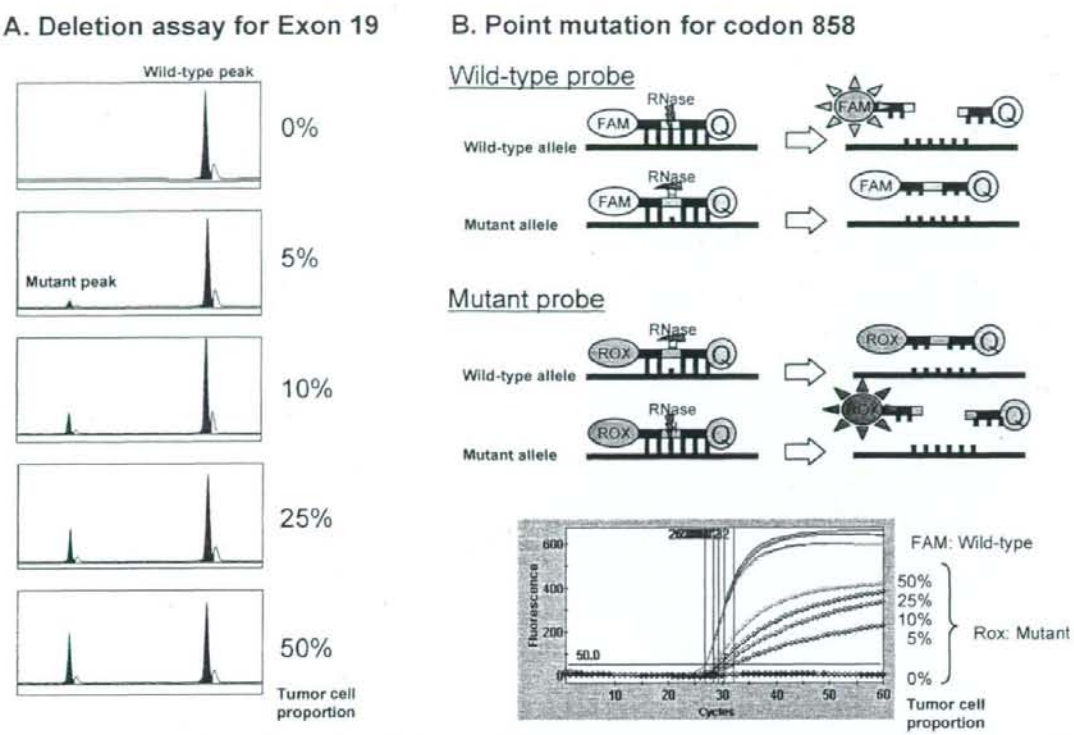


Figure 1. Sensitivity of the new assay. **A:** The sensitivity of the fragment analysis in the new assay. As few as 5% of tumor cells with the deletion could be detected. In the **top** of **B**, a brief explanation of the cycleave technology is displayed. Using this technique, as few as 5% of tumor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

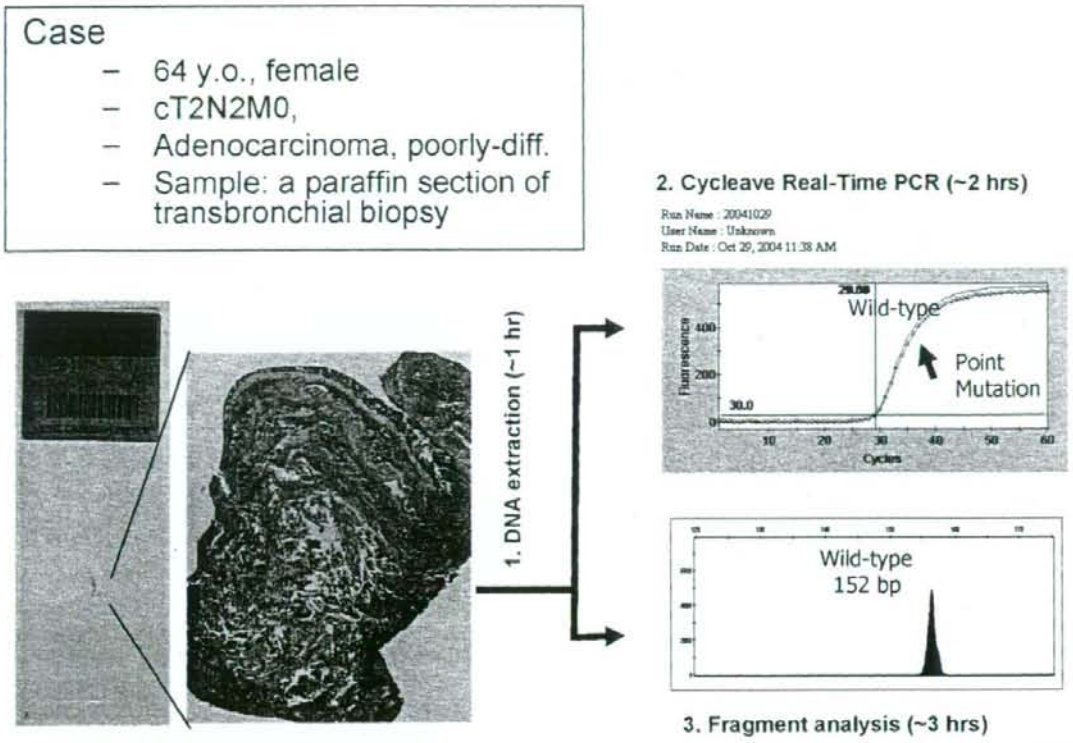


Figure 2. A representative result of the new assay. DNA was extracted from a paraffin section of the biopsy followed by simultaneous analysis using cycleave real-time PCR and fragment analysis. The entire procedure was completed within 4 hours. In this case, point mutation at codon 858 was detected,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

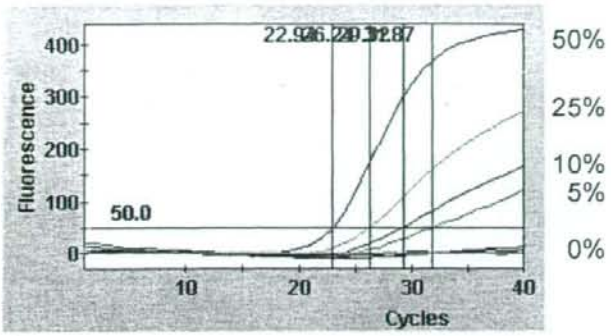
Detection of Mutation at Codon 790 Conferring Acquired Resistance to Gefitinib

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

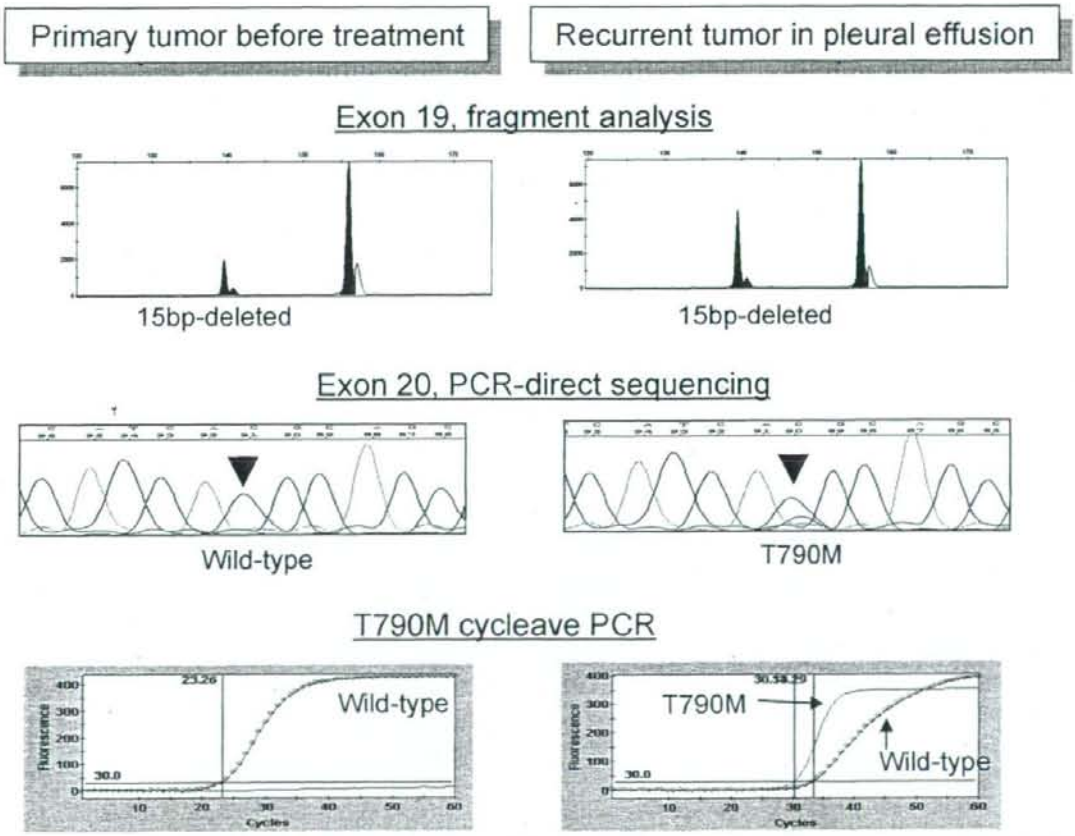


Figure 3. Detection of acquired mutation at codon 790. **A:** The sensitivity of this cycleave assay for T790M mutation. As few as 5% of tumor cells with T790M mutation could be detected. A representative result of acquired mutation at codon 790 after gefitinib treatment is displayed in **B** (Table 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.