more accurate staging by proving whether N2 disease, according to International Union Against Cancer (UICC) staging,[6] exists or not.

METHODS

Patients

Transbronchial aspiration cytology was performed on 153 consecutive patients with suspected lung cancer during initial diagnostic bronchofibrescopy over an 18-month period. All patients had histological or cytological confirmation of lung cancer after flexible bronchoscopy. Twenty-six patients had small cell lung cancer (SCLC) and 127 had non-small cell lung cancer (NSCLC).

Equipment

The flexible bronchoscope used in the present study was an Olympus (Tokyo, Japan) 1P10 type. The disposable cytology needle used for TBAC was an Olympus 21-gauge, with a length of 15 mm.

Procedure of bronchoscopic examination

As pre-medication, the patients received a 4% solution of nebulized lidocaine and the larynx was anaesthetized with a 2% solution of lidocaine. They were also administered an i.m. injection of atropine sulphate to reduce bronchial secretion. In all cases, a flexible bronchoscope was passed through an endotracheal tube. Prior to oral intubation, the patients were sedated with i.v. administration of diazepam and fentanyl citrate. During these procedures, patients were supplied with oxygen through an endotracheal tube, and fentanyl citrate was administered every 20 min. N-allylnoroxymorphone was given after the procedure was completed.

Transbronchial aspiration cytology was routinely performed on all patients who were suspected of having lung cancer. In order to avoid contamination, TBAC was performed before endobronchial observation and peripheral sampling. Triple punctures in each of the anterior, central and posterior portions of the carina were done to improve diagnostic accuracy with real time X-ray guidance. Once inserted, the needle was moved up and down while syringe suction was maintained.[7] Specimens were sprayed onto glass slides with a 20-mL syringe including air and fixed with 95% ethyl alcohol. We did not perform subcarinal TBAC on patients who had severe chronic pulmonary emphysema or enlargement of the left atrium of the heart, or who were on anticoagulant therapy.

RESULTS

The histological subtypes of the 153 patients enrolled in the study are listed in Table 1. The number of patients who had subcarinal node enlargement >1 cm in short axis diameter on CT (CT-positive) and who had cytological confirmation of metastases by TBAC (TBAC-positive) was 34 (nine SCLC and 25 NSCLC) and 25 (10 SCLC and 15 NSCLC), respectively (Table 2).

The relationship between the size of the subcarinal nodes and result of TBAC is shown in Table 3. Out of 34 CT-positive patients, 18 had confirmed metastases by TBAC. Patients with SCLC had increased TBAC-detection of metastases when they had enlargement

Table 1 Histology of lung cancer in 153 patients who had TBAC

SCLC	26
NSCLC	127
Adenocarcinoma	72
Squamous cell carcinoma	33
Large cell carcinoma	11
Others	11

NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; TBAC, transbronchial aspiration cytology.

Table 2 Number of patients who had enlargement of subcarinal nodes (CT-positive) and cytological confirmation of metastasis by TBAC (TBAC-positive)

	CT-positive	TBAC-positive
SCLC	9/26 (35%)	10/26 (38%)
NSCLC	25/127 (20%)	15/127 (12%)
Total	34/153 (22%)	25/153 (16%)

NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; TBAC, transbronchial aspiration cytology.

Table 3 Relationship between enlargement of the subcarinal nodes and result of TBAC

	CT-positive	CT-negative
SCLC ($n=26$)		
TBAC-positive	7	3
TBAC-negative	2	14
NSCLC ($n=127$)		
TBAC-positive	11	4
TBAC-negative	14	98
Total ($n=153$)		
TBAC-positive	18	7
TBAC-negative	16	112

CT-negative, patients without enlargement of the subcarinal nodes; CT-positive, patients with enlargement of the subcarinal nodes; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; TBAC, transbronchial aspiration cytology; TBAC-negative, patients who did not have confirmation of metastasis to the subcarinal nodes by TBAC; TBAC-positive, patients who had confirmation of metastasis to the subcarinal nodes by TBAC.

Table 4 Relationship between the site of primary tumour and CT findings or results of TBAC (n=153)

Primary site	No. patients	CT-positive	CT-negative	TBAC-positive	TBAC-negative
LUL	42	9	33	5	37
LLL	17	7	10	4	13
LMB	4	1	3	2	2
RUL	34	8	26	3	31
RML	11	3	8	3	8
RLL	35	3	32	4	31
RMB	1	1	0	1	0
Intermedius	5	2	3	2	3
Unknown	4	0	4	1	0
Total	153	34	119	25	128

LLL, left lower lobe; LMB, left main bronchus; LUL, left upper lobe; RLL, right lower lobe; RMB, right main bronchus; RML, right middle lobe; RUL, right upper lobe; TBAC, transbronchial aspiration cytology.

of the nodes (7/9) than ones with NSCLC (11/25). Out of 119 patients without enlargement of the subcarinal nodes (CT-negative), TBAC did not reveal metastases (TBLB-negative) in 112, but seven patients had confirmed metastases by TBAC. The lymphoid cells of TBAC samples were obtained in 112 (79%) of 153 cases.

Forty-nine patients with NSCLC had surgical resection of the tumour. There were no resected cases who were TBAC-positive. In our hospital, pathologically confirmed N2 disease was considered inoperable even though there was no enlargement of mediastinal lymph node on chest CT scan. Furthermore, during the study period, no clinical trials such as neoadjuvant chemotherapy followed by surgery or surgery after adjuvant chemotherapy were available for pathological confirmed N2 disease in our hospital. Therefore, seven patients with pathologically confirmed N2 were treated with radiotherapy with/without chemotherapy. The surgical procedure revealed metastases to the subcarinal nodes in three patients, although preoperative TBAC diagnosis did not show any metastases. All three p-N2 patients who had negative TBAC showed an absence of subcarinal lymph nodes swelling on preoperative chest CT scan. The other 46 patients who had negative subcarinal nodes biopsy by TBAC showed no metastases in resected specimens. The accuracy of TBAC for diagnosing metastases was 94% in the 49 patients. The relationship of the site of primary tumour and CT findings or results of TBAC is listed in Table 4. No exact correlation was observed between the site of primary tumour and the results of TBAC. Summary of the patients in which subcarinal TBAC contributed to the staging or diagnosis are as follows. Radiological N2 was positively confirmed by subcarinal TBAC in 18 patients. N2 was confirmed by subcarinal TBAC in the absence of subcarinal lymph nodes swelling in seven patients. Subcarinal TBAC was the only way to confirm lung cancer in two patients. Therefore, routinely performed subcarinal TBAC contributed to more correct staging and diagnosis in 16% of the patients with lung cancer. No severe complications occurred in any of the cases who received routinely performed subcarinal TBAC.

DISCUSSION

Accurate diagnosis of metastases to the mediastinal lymph nodes influences the treatment plan and prognosis of patients with lung cancer.[8] As approximately 30–40% of patients with lung cancer already have mediastinal metastases at the time of initial diagnosis,[9] and histological or cytological evaluation of metastases to the mediastinal nodes is essential.

Generally, diagnosis of metastases to the mediastinal lymph nodes is based upon imaging and histological information. Commonly used imaging equipment includes positron emission tomography (PET), magnetic resonance imaging and CT. In most clinical settings, contrast-enhanced CT is the investigation of choice, and the size of lymph nodes provides a standard for the diagnosis of metastases by CT.[9] However, micrometastases could be present in lymph nodes without node enlargement and equally enlarged nodes may be due entirely to inflammation.[10] The relationship between size of lymph nodes and presence of malignancy is highly variable. The diagnosis of mediastinal lymph node metastases by CT is based solely on size with the cut-off value being >1.0 cm on the short axis diameter. Mediastinoscopy, video-assisted thoracoscopic surgery and TBAC are used as invasive diagnostic procedures for the sampling of lymph node cells, but TBAC can be performed with relatively simple anaesthesia in a bronchoscopic examination.

Our study showed that TBAC confirmed metastases in 42% of cases with enlargement of the subcarinal nodes. This detection rate was lower than in previous reports, although a high detection (7/9) rate was achieved in patients with SCLC, consistent with previous reports.[7,8] One of the possible reasons for this low rate was that TBAC was performed only on subcarinal nodes, while TBAC was performed at multiple sites in other reports.[7,8] Accuracy of TBAC could not be assessed in the present study because metastases was not finally diagnosed in the TBAC-negative cases, and this is one of the study's limitations. Another limitation is that TBAC is a blind technique with guidance limited to a few endobronchial landmarks and mental reconstruction of the CT scan. We operated on 49

patients with NSCLC and subcarinal metastases was found in three patients by postoperative pathological assessment. The accuracy of TBAC was 94% in the operated patients, which showed the limit of TBAC in establishing a diagnosis. It is possible that the TBAC needle used in this study may not collect enough cells for assessment and would suggest our method might be less useful for identifying micrometastases of lymph nodes. Furthermore, lymphoid cells were obtained in only 112 (79%) of 153 cases. In other words, TBAC could not adequately sample the target lymph nodes in 21% of patients.

In operable cases, right upper lobe tumours might be more likely to spread to the paratracheal region than to the subcarinal region. However, as shown in Table 4, no exact correlation was observed between the site of primary tumour and the TBAC results. This may be due to the fact that more patients with advanced stage tumour were included and only 49 of 153 patients had surgery in our study.

Recent studies for the diagnosis of lung cancer have shown that the highest detection rate of metastases to lymph nodes is achieved by PET,[10] but the role of PET in the treatment plan remains controversial. Mediastinoscopy is usually the best choice for proof of metastases to mediastinal nodes, but it is unable to assess all lymph nodes. TBAC should be performed in combination with other diagnostic procedures. In order to improve the diagnosis by TBAC, TBAC under the guide of CT or endoscopic ultrasound has been developed,[10] although these procedures are still experimental. Metastases to the subcarinal nodes was demonstrated following TBAC in some patients without nodal enlargement. Few studies have been undertaken to assess the presence of metastases in mediastinal lymph nodes that are not enlarged, and TBAC may have diagnostic value in these cases. The potential contribution of the present study is to ask what a blind TBAC in normal sized nodes adds to preoperative staging. Of 119 patients with normal sized nodes there were seven with positive cytology on TBAC. Conversely there were three patients, which were not detected preoperatively in 49 operable patients. Based on the results of the present study, it might be difficult to recommend routine TBAC preoperatively. It was anticipated that analysis of the site of primary tumour might suggest which patients a clinician should have a blind TBAC but the data were not discriminatory as shown in Table 4.

Positron emission tomography is more accurate than CT for detecting mediastinal metastases. However, it should be noted that even PET scan frequently shows false-positive and false-negative in mediastinal staging in the range of 11–16%.[11] Because the detection rate of TBAC using our method was not very high, mediastinoscopy should still be considered the gold standard to confirm N2 disease. Toloza *et al.* reported a meta-analysis of invasive staging consisting of TBAC (TBNA), transtracheal needle aspiration, endoscopic ultrasound-guided needle aspiration and mediastinoscopy. They reported that TBAC has the worst sensitivity and negative predictive value among the invasive procedures.[9] However, considering that TBAC is an easy additional procedure during routine bronchofibreoscopy, the diagnostic yields of TBAC are comparable with other procedures. Furthermore, patients may avoid mediastinoscopy if TBAC is positive, therefore this is useful even if the yield is lower than mediastinoscopy

Transbronchial aspiration cytology of the subcarinal nodes is a minimally invasive technique for staging lung cancer. It can provide useful information for diagnosis of metastases to subcarinal nodes.

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Journal of Experimental Therapeutics and Oncology, Vol. 6, pp. 49-53
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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

Fumihiro Oshita[1], Akiko Sekiyama[2], Haruhiro Saito[1], Kouzo Yamada[1], Kazumasa Noda[1], Yohei Miyagi[2]

[1]Department of Thoracic Oncology, [2]Laboratory for Molecular Diagnostics, Kanagawa Cancer Center, Nakao 1-1-2, Asahi-ku, Yokohama 241-0815, Japan

Correspondence to: Fumihiro Oshita, M.D., Department of Thoracic Oncology, Kanagawa Cancer Center, Nakao 1-1-2, Asahi-ku, Yokohama 241-0815, Japan. Telephone: +81-45-391-5761. Fax: +81-45-361-4692. E-mail: foshita@kcch.jp

(Received August 25, 2006; revised September 6, 2006; accepted September 7, 2006)

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.

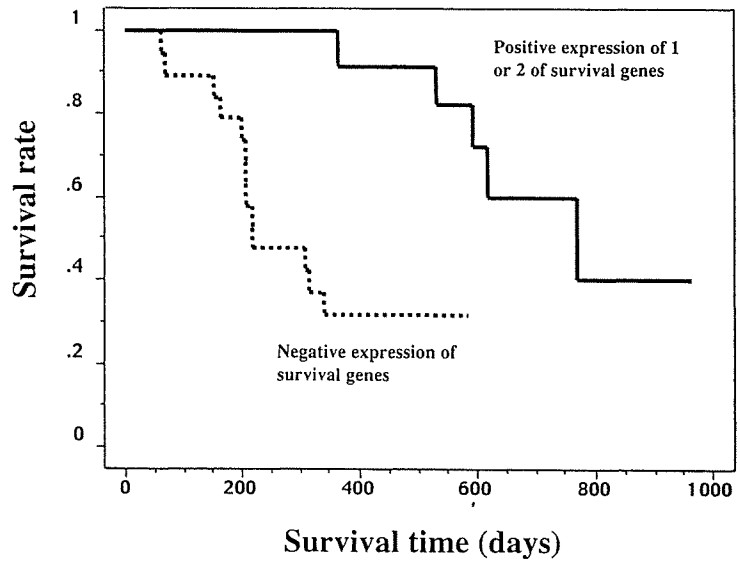

Figure 1. Survival curves constructed by the Kaplan-Meier method. The 12 of the 31 patients who showed positive expression of either the thyrotropin-releasing hormone receptor or alkylation repair genes had a significantly better chance of survival (log-rank, p = 0.0024; Wilcoxon, p = 0.0016)

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.

ACKNOWLEDGEMENTS

This work was supported in part by the Kanagawa Prefectural Hospitals Cancer Research Fund and Kanagawa Health Foundation.

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VOLUME 24 · NUMBER 34 · DECEMBER 1 2006

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Phase II Trial of Amrubicin for Treatment of Refractory or Relapsed Small-Cell Lung Cancer: Thoracic Oncology Research Group Study 0301

Sayaka Onoda, Noriyuki Masuda, Takashi Seto, Kenji Eguchi, Yuichi Takiguchi, Hiroshi Isobe, Hiroaki Okamoto, Takashi Ogura, Akira Yokoyama, Nobuhiko Seki, Yoshiko Asaka-Amano, Masao Harada, Akihiro Tagawa, Hiroshi Kunikane, Masanori Yokoba, Kazutsugu Uematsu, Takayuki Kuriyama, Yumi Kuroiwa, and Koshiro Watanabe

From the Department of Respiratory Medicine, Kitasato University School of Medicine, Sagamihara, Kanagawa; Department of Respirology, Tokai University School of Medicine, Isehara; Department of Respirology, Graduate School of Medicine, Chiba University, Chiba; Department of Pulmonary Disease, National Hospital Hokkaido Cancer Center, Sapporo; Department of Respirology, Yokohama Municipal Citizen's Hospital; Department of Respiratory Medicine, Kanagawa Cardiovascular & Respiratory Center, Yokohama; and Department of Internal Medicine, Niigata Cancer Center Hospital, Niigata, Japan.

Submitted July 26, 2006; accepted September 28, 2006.

Presented in part at the 42nd Annual Meeting of the American Society of Clinical Oncology, June 2-6, 2006, Atlanta, GA.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Address reprint requests to Noriyuki Masuda, MD, PhD, Department of Respiratory Medicine, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-0022, Japan; e-mail: masuda@med.kitasato-u.ac.jp.

0732-183X/06/2434-5448/$20.00

DOI: 10.1200/JCO.2006.08.4145

ABSTRACT

Purpose
This multicenter, phase II study was conducted to evaluate the activity of amrubicin, a topoisomerase II inhibitor, against refractory or relapsed small-cell lung cancer (SCLC).

Patients and Methods
SCLC patients with measurable disease who had been treated previously with at least one platinum-based chemotherapy regimen and had an Eastern Cooperative Oncology Group performance status of 0 to 2 were eligible. Two groups of patients were selected: patients who experienced first-line treatment failure less than 60 days from treatment discontinuation (refractory group), and patients who responded to first-line treatment and experienced disease progression ≥ 60 days after treatment discontinuation (sensitive group). Amrubicin was administered as a 5-minute daily intravenous injection at a dose of 40 mg/m^2 for 3 consecutive days, every 3 weeks.

Results
Between June 2003 and December 2004, 60 patients (16 refractory and 44 sensitive) were enrolled. The median number of treatment cycles was four (range, one to eight). Grade 3 or 4 hematologic toxicities comprised neutropenia (83%), thrombocytopenia (20%), and anemia (33%). Febrile neutropenia was observed in three patients (5%). Nonhematologic toxicities were mild. No treatment-related death was observed. The overall response rates were 50% (95% CI, 25% to 75%) in the refractory group, and 52% (95% CI, 37% to 68%) in the sensitive group. The progression-free survival, overall survival, and 1-year survival in the refractory group and the sensitive group were 2.6 and 4.2 months, 10.3 and 11.6 months, and 40% and 46%, respectively.

Conclusion
Amrubicin exhibits significant activity against SCLC, with predictable and manageable toxicities; this agent deserves to be studied more extensively in additional trials.

J Clin Oncol 24:5448-5453. © 2006 by American Society of Clinical Oncology

INTRODUCTION

Approximately 15% of all patients with lung cancer are diagnosed with small-cell lung cancer (SCLC). Unlike other types of lung cancer, SCLC is sensitive to chemotherapy or radiation therapy.[1] Nonetheless, after experiencing an apparently successful induction therapy, most patients experience relapse within 2 years because of the emergence of drug-resistant cancer cells during the induction therapy or the existence of such cells before chemotherapy. Therefore, long-term survival is quite uncommon, with less than 25% of patients with limited-stage, and 1% to 2% of patients with extensive-stage disease remaining alive at 5 years.[2-4] Furthermore, the results of second-line chemotherapy against SCLC are disappointing, with relatively low response rates, brief remissions, and a short survival time.[1,5] In particular, little progress has been made in the re-treatment of patients who experienced progression during first-line therapy or who failed to achieve a progression-free survival of more than 60 to 90 days. As a result, to control SCLC more efficiently, new drugs that are effective for patients who have failed to respond to standard treatment, and who may have multidrug-resistant tumors, are urgently needed.

Amrubicin, a totally synthetic 9-aminoanthracycline, is converted to an active metabolite, amrubicinol, through the reduction of its C-13 ketone group to a hydroxy group.[6] Despite the similarity of its chemical structure to that of a representative anthracycline, doxorubicin, the mode of action of amrubicin differs from that of doxorubicin.[7] Amrubicin and amrubicinol are inhibitors of DNA topoisomerase II, which exert cytotoxic effects by stabilizing a topoisomerase II-mediated cleavable complex, and are approximately 1/10 weaker than doxorubicin as a DNA intercalator. The in vitro cytotoxic activity of amrubicinol was 18 to 220 times more potent than that of its parent compound, amrubicin.[8] In preclinical studies, amrubicin showed a more potent antitumor activity than doxorubicin in several human tumor xenografts implanted in nude mice,[9] and caused almost no cardiotoxicity.[9,10] The response rates to amrubicin at a dose of 45 mg/m^2 on days 1 to 3 in chemotherapy-naive patients with stage III or IV non-SCLC and extensive-stage SCLC were 25% and 79% on an intent-to-treat analysis, respectively.[11,12] The major grade 3 or 4 toxicities were neutropenia (72.1%), leukopenia (52.5%), anemia (23.0%), thrombocytopenia (14.8%), anorexia (4.9%), and nausea/vomiting (4.9%) in a phase II trial.[13]

The high activity of amrubicin as a single agent in untreated patients with extensive disease (ED) SCLC led us to carry out this phase II trial, which was designed to determine the antitumor activity and toxicity of amrubicin in previously treated patients with SCLC.

PATIENTS AND METHODS

Patient Selection

Before participation in the present study, each patient was examined to ensure he or she met the following criteria: histologic or cytologic proof of SCLC; recurrent or refractory disease after one or two previous chemotherapy regimens (at least one platinum-containing regimen); measurable disease; no chemotherapy or chest radiotherapy within 4 weeks before entry (measurable disease outside the radiation field); life expectancy of at least 8 weeks; performance status of 2 or better according to the Eastern Cooperative Oncology Group scale; age $\geq$ 20 years; adequate bone marrow function (leukocyte count $\geq$ 4,000/μL, absolute neutrophil count [ANC] $\geq$ 2,000/μL, platelet count $\geq$ 100,000/μL, and hemoglobin $\geq$ 9.0 g/dL) and hepatic function (AST and ALT $\leq$ 100 U/L, or $\leq$ 200 U/L in the presence of liver metastases; bilirubin level $\leq$ 1.5 mg/dL); ECG findings within the normal range, and a left ventricular ejection fraction $\geq$ 50%; arterial oxygen partial pressure $\geq$ 60 torr; and the written informed consent of the patient. Patients were ineligible if they had serious infectious diseases or other severe complications (heart disease, pulmonary fibrosis/interstitial pneumonia, or uncontrollable diabetes); had massive pleural or pericardial effusion, or ascitic fluid; had symptomatic brain metastases; had active concurrent malignancies; were lactating or pregnant women or hoped to become pregnant; had a history of a drug allergy; or had other medical problems severe enough to prevent compliance with the protocol. Prior amrubicin chemotherapy was not allowed. Trial document approval was obtained in advance from the ethics committee or institutional review board of each hospital.

Treatment Schedule

Amrubicin was dissolved in 20 mL of normal saline, and administered intravenously as a 5-minute infusion at a dose of 40 mg/m^2/d on days 1 to 3 every 3 weeks. Patients with evidence of disease progression or who experienced intolerable toxicity, such as grade 2 or worse pneumonitis, were removed from the study. Before the next course could be started, the patient's ANC had to be $\geq$ 1,500/μL, his or her platelet count had to be $\geq$ 100,000/μL, and any nonhematologic toxicities should have been downgraded to at least grade 1. If more than 6 weeks passed from the time of the last treatment before these criteria were satisfied, the patient was removed from the study.

Granulocyte colony-stimulating factor (G-CSF) was permitted as a therapeutic intervention but was not mandatory as a prophylactic agent against neutropenia for hematologic toxicity.

Subsequent doses were modified based on hematologic and nonhematologic toxicities. If the leukocyte count was less than 1,000/μL for 4 days or longer, the ANC was less than 500/μL for 4 days or longer, the platelet count nadir was less than 20 $\times$ 10^3/μL, or grade 3 or worse nonhematologic toxicity was observed, the dose of amrubicin was reduced to 35 mg/m^2/d. The dose of amrubicin also was reduced to 35 mg/m^2/d in patients who developed grade 3 febrile neutropenia.

Evaluation

Patients were evaluated to determine the stage of disease at the time of disease progression or at the time of relapse by taking a complete medical history and performing a physical examination, chest radiograph, computed tomography of the chest and abdomen, and other staging procedures as indicated, including computed tomography of the head and a bone scintiscan. Limited disease (LD) was defined as that confined to one hemithorax, including bilateral mediastinal and bilateral supraclavicular nodes: any involvement beyond these confines was defined as ED. Primary refractory disease (refractory group) was defined as relapse during the first-line chemotherapy regimen or less than 60 days after completing the initial chemotherapy regimen, and sensitive disease (sensitive group) was defined as relapse $\geq$ 60 days after completion of the first-line chemotherapy. Before the first course, each patient was assessed using a CBC, including a differential count and a platelet count, and serum chemistry tests for renal and hepatic functions as well as electrolytes. The CBC and biochemistry tests were repeated at least once a week after this initial evaluation, whereas the other investigations were repeated at least every 6 weeks to evaluate the target lesions.

Adverse events were recorded and graded using the National Cancer Institute Common Toxicity Criteria, Version 2.0 grading system. After completing the chemotherapy regimen, each patient was restaged using all of the tests used during the initial work-up. The tumor response was classified in accordance with the Response Evaluation Criteria in Solid Tumors.[14] The duration of the response was defined as the number of days from the documentation of the response to the detection of disease progression. The eligibility, evaluability, and response of each patient were assessed by extramural reviewers. The duration of survival, determined as the number of days between the enrollment of protocol therapy and death, was censored at the time last known alive for patients who had not died.

Statistical Methods

Kaplan-Meier survival estimates were used to summarize the time-to-event variables.[15] These included time to response, response duration, progression-free survival, and survival. Time-to-event outcomes were compared using the log-rank test. Other statistical analyses were performed using the χ^2 test or Fisher's exact test, and $P < .05$ was considered to indicate statistical significance. The primary end point was the response rate, which determined the sample size. We chose a 40% response rate as a desirable target level and a 20% response rate as uninteresting in the sensitive group, with a power in excess of 80% and less than 2.5% type I error. For the refractory group, the sample size was planned using an adequate power to demonstrate that the overall response rate was greater than 5%. If the true overall response rate were assumed to be 25%, a sample size of 16 assessable patients would have a power of 80% based on a 5% α level (one-sided test) and an exact binomial distribution.

RESULTS

Between June 2003 and December 2004, 60 patients were enrolled onto this multicenter trial. Sixteen and 44 patients in the refractory and sensitive groups were eligible for the study, and assessable for toxicity, response, and survival. The characteristics of the 60 patients

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

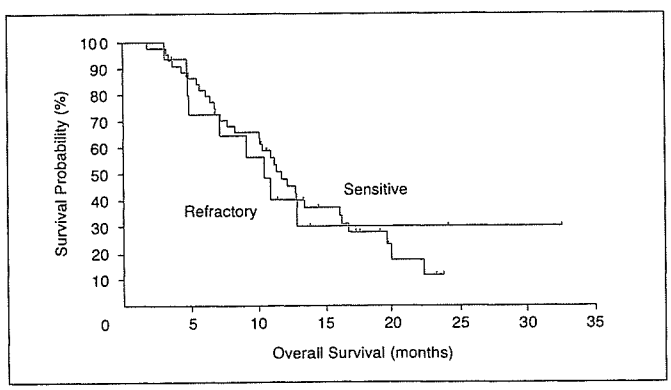

Fig 1. Median survival times in all patients with refractory or relapsed small-cell lung cancer were 10.3 months in the refractory group (n = 16) and 11.6 months in the sensitive group (n = 44), respectively ($P = .974$; log-rank test). The 1-year actuarial survival rate in patients with refractory disease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

We thank K. Ogawa, T. Okuda, M. Tomita, M. Matsushita, and K. Fujimaru for their help in data collection and statistical analysis; and K. Minato, MD, S. Tsuchiya, MD, and A. Yoshimura, MD, for an extramural review of this study.

Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

Author Contributions

Conception and design: Noriyuki Masuda, Koshiro Watanabe
Provision of study materials or patients: Sayaka Onoda, Noriyuki Masuda, Takashi Seto, Kenji Eguchi, Yuichi Takiguchi, Hiroshi Isobe, Hiroaki Okamoto, Takashi Ogura, Akira Yokoyama, Nobuhiko Seki, Yoshiko Asaka-Amano, Masao Harada, Akihiro Tagawa, Hiroshi Kunikane, Masanori Yokoba, Kazutsugu Uematsu, Takayuki Kuriyama, Yumi Kuroiwa
Collection and assembly of data: Sayaka Onoda, Noriyuki Masuda, Takashi Seto, Kenji Eguchi, Yuichi Takiguchi, Hiroshi Isobe, Hiroaki Okamoto, Takashi Ogura, Akira Yokoyama, Nobuhiko Seki, Yoshiko Asaka-Amano, Masao Harada, Akihiro Tagawa, Hiroshi Kunikane, Masanori Yokoba, Kazutsugu Uematsu, Takayuki Kuriyama, Yumi Kuroiwa
Data analysis and interpretation: Sayaka Onoda, Noriyuki Masuda, Takashi Seto, Koshiro Watanabe
Manuscript writing: Noriyuki Masuda
Final approval of manuscript: Noriyuki Masuda, Koshiro Watanabe

Journal of Molecular Diagnostics, Vol. 8, No. 3, July 2006

DOI: 10.2353/jmoldx.2006.050104

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

Yasushi Yatabe,* Toyoaki Hida,† Yoshitsugu Horio,† Takayuki Kosaka,‡ Takashi Takahashi,§ and Tetsuya Mitsudomi‡

From the Departments of Pathology and Molecular Diagnostics, Thoracic Oncology,† and Thoracic Surgery,§ Aichi Cancer Center Hospital, Nagoya; and the Division of Molecular Carcinogenesis,‡ Center for Neural Disease and Cancer, Nagoya University Graduate School of Medicine, Nagoya, Japan*

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

Accepted for publication March 9, 2006.

Address reprint requests to Yasushi Yatabe, M.D., Department of Pathology and Molecular Diagnostics, Aichi Cancer Center Hospital, Kanokoden, Chikusa-ku,. Nagoya 464-8681, Japan. E-mail: yyatabe@aichi-cc.jp.

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

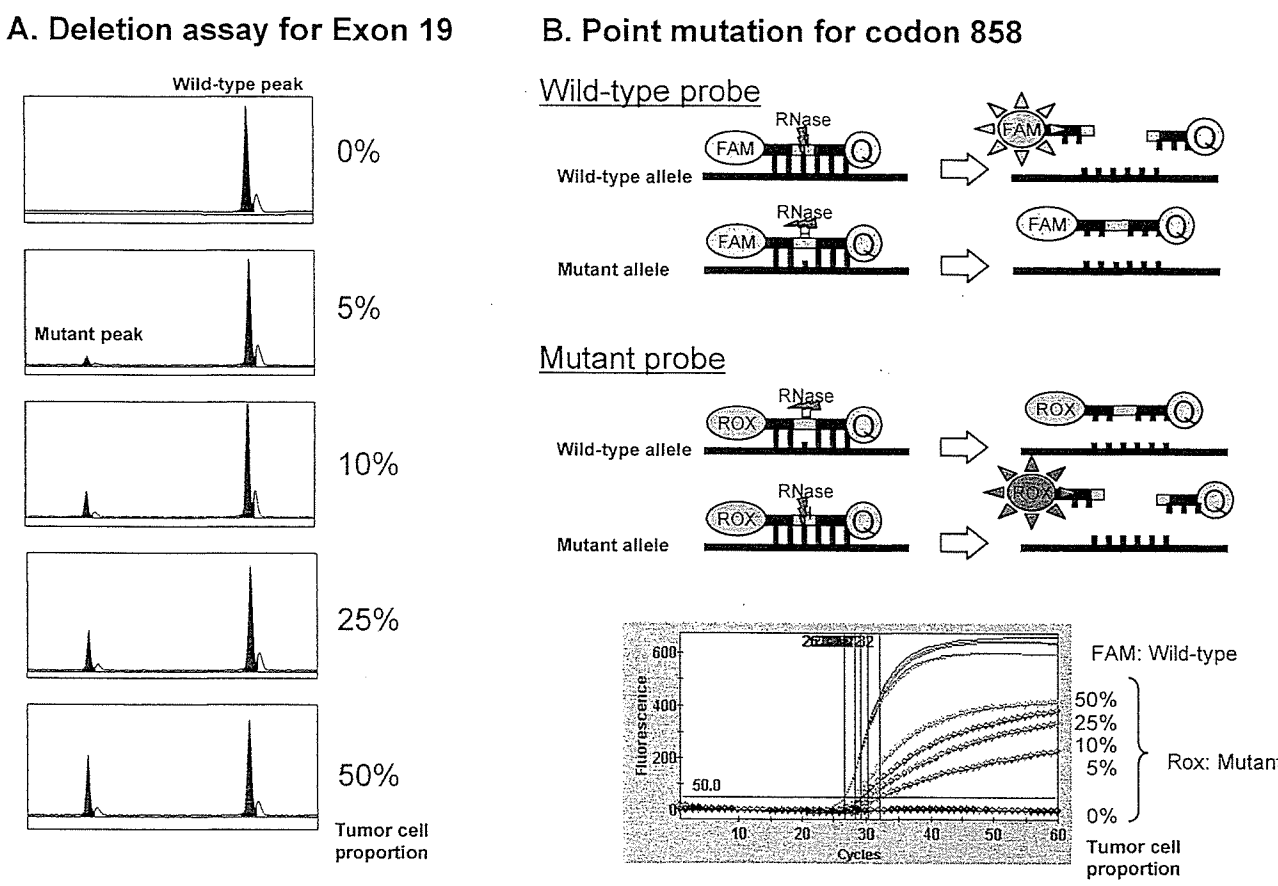

Figure 1. Sensitivity of the new assay. **A:** The sensitivity of the fragment analysis in the new assay. As few as 5% of tumor cells with the deletion could be detected. In the **top** of **B**, a brief explanation of the cycleave technology is displayed. Using this technique, as few as 5% of tumor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

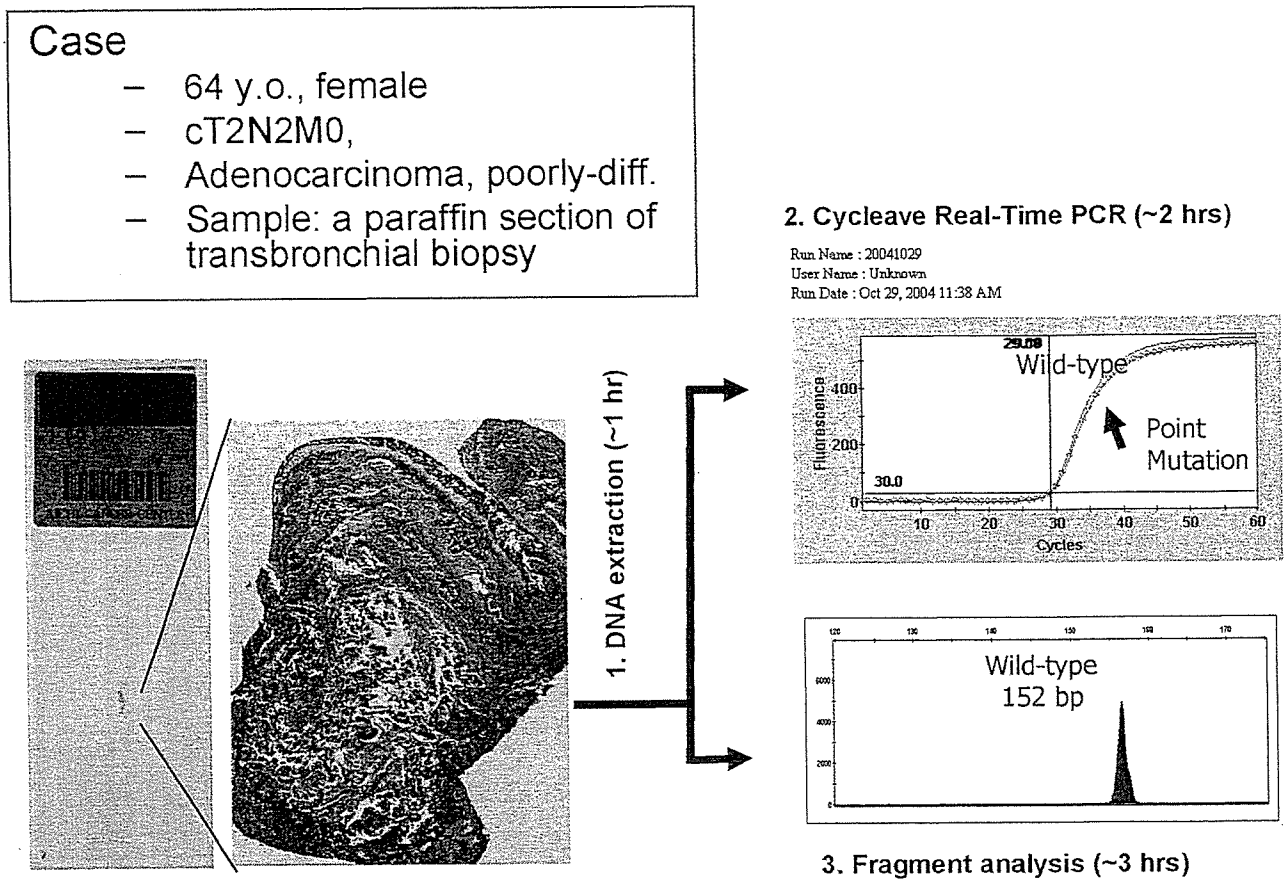

Figure 2. A representative result of the new assay. DNA was extracted from a paraffin section of the biopsy followed by simultaneous analysis using cycleave real-time PCR and fragment analysis. The entire procedure was completed within 4 hours. In this case, point mutation at codon 858 was detected,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

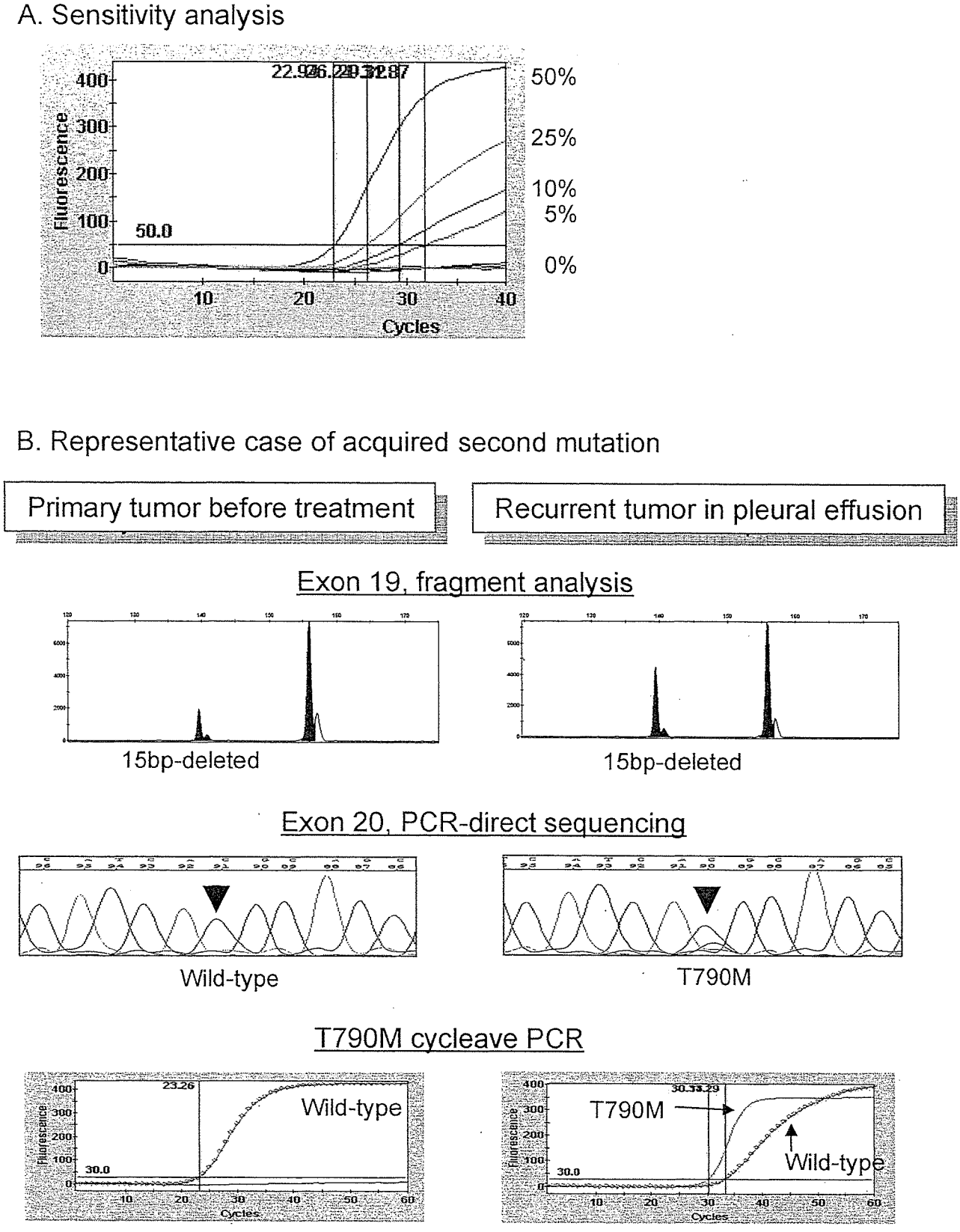

Figure 3. Detection of acquired mutation at codon 790. **A:** The sensitivity of this cycleave assay for T790M mutation. As few as 5% of tumor cells with T790M mutation could be detected. A representative result of acquired mutation at codon 790 after gefitinib treatment is displayed in **B** (Table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

Table 2. Practical Application of the New Assay

	Clinical response		
EGFR status	PD	NC	PR
Wild type	5	11	1
Mutated	0	2	10
Deletion in exon 19	0	2	5
Point mutation of codon 858	0	0	5

PD, progressive disease; NC, no change; PR, partial response.

mutation independently of gefitinib treatment,[16,17] was also positive in this assay. In one of the other three recurrent tumors, this assay clearly demonstrated the mutation (Figure 3), although it was often difficult to detect the mutated signal with direct sequencing of the PCR product.

Discussion

Paez et al[5] and Lynch et al[4] simultaneously published the result that somatic mutation of EGFR in lung adenocarcinoma predicts a clinical response to gefitinib. Erlotinib is another targeted small-molecule inhibitor of EGFR, and lung adenocarcinoma sensitive to erlotinib also harbored EGFR mutations. In addition, *in vitro* studies support the observation that EGFR mutations make tumor cells significantly sensitive to gefitinib[18] and erlotinib. This increased sensitivity may be explained by the "addiction to oncogene" hypothesis proposed by Weinstein.[19] Tumor cells with EGFR mutation are highly dependent on the activated EGFR pathway and are thus very susceptible to inhibition of this dependence. We have reported that patients with EGFR mutations survived longer than those without mutations after the initiation of gefitinib treatment.[7] Recently, failure to show a survival benefit in the IRESSA Survival Evaluation in Lung Cancer was announced. Gefitinib may not be effective enough to kill tumor cells that are not under a state of "addiction to EGFR mutation." Conversely, these findings suggest that selection of patients with EGFR-mutated tumors has the advantage of increasing the response rate of EGFR-targeted therapy. Furthermore, selection may also be efficient at preventing serious interstitial pneumonia occurring as a side effect.[20]

Although an assay using paraffin sections is very practical, immunohistochemical analysis of the tumors failed to predict the response. Currently, the microdissection of tumor cells and direct sequencing of PCR products is commonly used as a standard method. Regarding practical applications, the new assay reported here provides two benefits compared with the conventional method. First, microdissection is not necessary for the assay because a positive mutated signal makes this assay very sensitive. Second, this assay is rapid, does not require a purification step, and is usually completed within 4 hours: digestion with proteinase K for 1 hour, real-time PCR or regular PCR for 3 hours, and electrophoresis for 1 hour. In addition to paraffin sections, pleural effusion and specimens for fine needle aspiration cytology can be used. All three specimens of pleural effusion for the T790M cycleave assay were successfully analyzed, whereas direct sequencing occasionally resulted in an ambiguous result (Figure 3). The main targets for gefitinib or erlotinib therapy are recurrent and refractory tumors, and an assay using such specimens is therefore quite useful. However, the examination of limited regions of the EGFR gene appears to be a disadvantage of this study. Recent studies suggested that an insertion of exon 20 was shown to be resistant to EGFR inhibitors[25], whereas the gefitinib sensitivity of cells expressing the G719S mutant was significantly less than that of cells expressing the L858R mutant form[26]. Therefore, these results suggest that examination of the L858R mutation and deletion in exon 19 is reasonable, because these two mutations are likely to be a major target of the EGFR inhibitors.

A few approaches for the detection of EGFR mutation have been reported recently.[21–23] Comparing the assays, the advantage of the method presented here is its practical clinical use. Biopsy specimens frequently result in small, fragmented tissues containing only a few cancer cells. Using such biopsy specimens, the assay successfully demonstrated the EGFR mutations that correlate with gefitinib response, in contrast to failure of the direct sequencing of some biopsy specimens. Furthermore, the cycleave technique can be simultaneously applied for the detection of the K-ras mutation, which has been proposed to be an adverse prognostic marker for chemotherapy with erlotinib.[24]

In summary, we have introduced a new practical approach for the detection of EGFR mutations. This assay is very sensitive and useful for predicting gefitinib response. This rapid screening assay uses paraffin sections from biopsy without the need for a microdissection

Table 3. Detection of T790M Mutation Associated with Acquired Resistance to Gefitinib

Patient	Gefitinib treatment	Tissue examined	T790M mutation	Comments
Case 1*	No	Primary tumor	Yes	A rare case, harboring T790M mutation independent of gefitinib treatment
Case 2	Yes	Pleural effusion	Yes	15-bp deletion of exon 19 in the primary and recurrent cancers (Figure 3)
Case 3	Yes	Pleural effusion	No	9-bp deletion of exon 19 in the primary and recurrent cancers
Case 4	Yes	Pleural effusion	No	15-bp deletion of exon 19 in the primary and recurrent cancers

*The mutation of codon 790 in a primary cancer, which was demonstrated with RT-PCR direct sequencing, has been reported previously.