Society of Clinical Oncology (ASCO) guidelines recommend the use of prophylactic G-CSF in patients at higher risk of chemotherapy-induced infections, including patients with a poor PS or comorbid illness [24]. Therefore, we suggest that the prophylactic use of G-CSF in this study was justified as the CI regimen used was near to the full-dose regimen even though only elderly patients with SCLC were studied.

As our study consisted of a heterogeneous patient population, including patients that had been previously treated, or over 75 years of age, three dose levels were used according to individual patient characteristics. Furthermore, stage was also different among the patients. Therefore, the limitation of this study was that it was neither considered phase I nor II study and was not designed based on the proper statistical methodology. However, at the time of study proposal, no prospective trial using carboplatin plus irinotecan regimen for elderly patients with SCLC was reported. Furthermore, we did not know whether this combination was feasible and effective for elderly SCLC patients. Therefore, dose levels were selected by patient characteristics and this study was designed as a prospective study to evaluate feasibility and efficacy for the elderly SCLC patients. For this reason, it may be difficult to mention on the efficacy of this treatment because of wide patient selection and uncommon study design. In terms of future trials using the CI regimen, level 1 or 2 appeared to be the appropriate dose level for previously untreated elderly patients with adequate organ function because majority of the patients were registered in level 1 and 2. However, phase I/II study using the CI regimen, which is based on the proper statistical method, is warranted for evaluating toxicity and efficacy in the chemo-naïve elderly SCLC patients with specific stage.

Recently, we reported a phase III trial that compared the CE regimen to a split doses of PE (SPE) regimen in elderly or poor-risk patients with ED-SCLC (JCOG 9702) [25]. Although the CE regimen led to pronounced but manageable thrombocytppenia, other toxicities, palliation scores, response rate, and overall survival rate were very similar between the two treatments. However, the CE regimen did not require hydration and could be given in an outpatient setting. Based on the results of this phase III study, many JCOG members prefer the CE regimen over the SPE regimen and consider it to be more suitable for use as a control treatment in future phase III trials.

Compared with the MST obtained for the JCOG 9702 trial (10.6 months for CE versus 9.8 months for SPE), the MST of 13.3 months for the CI regimen in the current study is promising, although the current study included both ED and LD patients as the same population and also included both treated and untreated patients. Furthermore, although 90–95% of the patients in the JCOG 9702 trial experienced grade 3 or 4 neutropenia [25], the toxicity of the current study was 50% and seemed to be generally mild. However, JCOG has also shown that IP is more effective than PE for treating non-elderly patients with ED-SCLC in a phase III trial [6]. Taking these findings together, we are now considering a comparative trial of CE versus CI in elderly patients with ED-SCLC.

In conclusion, the CI regimen was an effective and non-toxic regimen in elderly patients with SCLC, and should be evaluated in future phase III trials.

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ORIGINAL ARTICLE

Transbronchial needle aspiration cytology of subcarinal lymph nodes for the staging procedure in the diagnosis of lung cancer

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Transbronchial needle aspiration cytology of subcarinal lymph nodes for the staging procedure in the diagnosis of lung cancer
AONO H, OKAMOTO H, KUNIKANE H, NAGATOMO A, WATANABE K, NAGAI A. *Respirology* 2006; **11**: 782–785

Objective and background: The aim of this study was to improve the staging of lung cancer with or without lymphadenopathy on chest CT by using transbronchial aspiration cytology (TBAC).
Methods: TBAC of the subcarinal lymph nodes was performed on 153 consecutive patients with lung cancer, with or without subcarinal lymphadenopathy on chest CT.
Results: Thirty-four patients had enlargement of the subcarinal lymph nodes (>1 cm). Eighteen of these had TBAC confirmation of metastases. Another seven patients with no mediastinal involvement on CT were positive for metastases on TBAC. TBAC was the only way to confirm lung cancer in two patients. Therefore, routinely performed subcarinal TBAC contributed to an improved non-operative staging of the patients and diagnosis in 16% (25/153) of the patients with lung cancer. Forty-nine patients with NSCLC had surgical resection of the tumour. Surgical procedure revealed metastases to the subcarinal lymph nodes in three patients in whom the preoperative TBAC diagnosis was normal. No significant complications due to TBAC occurred in any of the patients.
Conclusion: TBAC of the subcarinal lymph nodes is a minimally invasive technique for staging of lung cancer and can provide useful information for the diagnosis of metastases to the subcarinal lymph nodes.

Key words: chest computed tomography, lung cancer, staging, subcarinal lymph node, transbronchial aspiration cytology.

INTRODUCTION

The efficacy of flexible bronchoscopy used in combination with transbronchial needle aspiration (TBNA) has been studied since the early 1980s. TBNA is also known as Wang needle aspiration, and can be performed safely with little morbidity.[1,2] TBNA is most frequently used for cytological diagnosis not only of the parenchymal nodules but also of the mediastinal lymph nodes. Shure and Fedullo reported that TBNA, when used to obtain diagnostic and staging information for mediastinal and subcarinal lymphadenopathy, showed a lower complication rate than mediastinoscopic examination.[3,4] TBNA has become a standard evaluation technique for suspected metastases involving the mediastinal nodes.

Transbronchial aspiration cytology (TBAC) of the subcarinal nodes was performed routinely so as to improve the staging procedure in lung cancer, with or without lymphadenopathy on chest CT. Cytological proof of metastases in the mediastinal lymph nodes and more accurate staging by TBAC.[5] Routinely performed TBAC for subcarinal lymph nodes and optional TBAC of other swollen mediastinal lymph nodes can result in a more correct staging and diagnosis in 25% of patients with lung cancer.[5] In the present study, we analyse how TBAC of subcarinal nodes using flexible bronchoscopy contributes to a

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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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ORIGINAL REPORT

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

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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.

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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 ≥ 20 years; adequate bone marrow function (leukocyte count ≥ 4,000/μL, absolute neutrophil count [ANC] ≥ 2,000/μL, platelet count ≥ 100,000/μL, and hemoglobin ≥ 9.0 g/dL) and hepatic function (AST and ALT ≤ 100 U/L, or ≤ 200 U/L in the presence of liver metastases; bilirubin level ≤ 1.5 mg/dL); ECG findings within the normal range, and a left ventricular ejection fraction ≥ 50%; arterial oxygen partial pressure ≥ 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 ≥ 1,500/μL, his or her platelet count had to be ≥ 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 neütropenia 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 × 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 ≥ 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

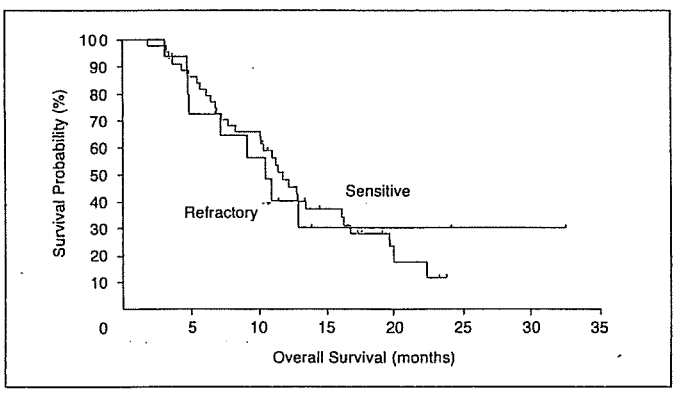

Fig 1. Median survival times in all patients with refractory or relapsed small-cell lung cancer were 10.3 months in the refractory group (n = 16) and 11.6 months in the sensitive group (n = 44), respectively ($P = .974$; log-rank test). The 1-year actuarial survival rate in patients with refractory disease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

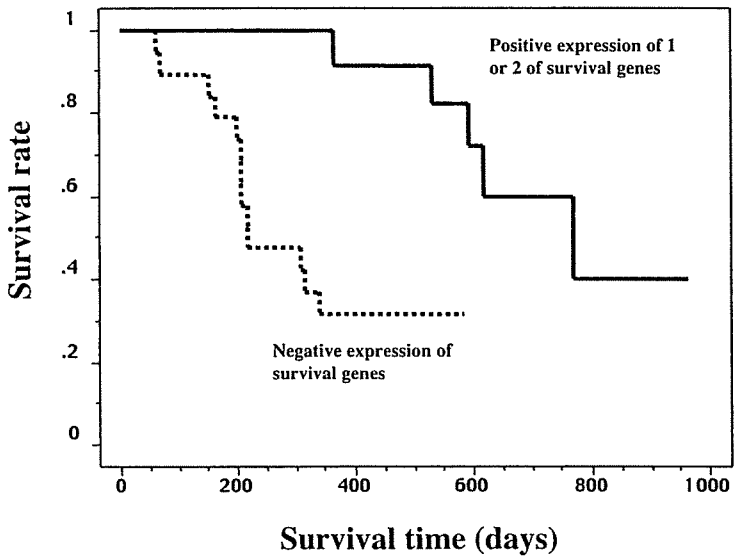

Figure 1. Survival curves constructed by the Kaplan-Meier method. The 12 of the 31 patients who showed positive expression of either the thyrotropin-releasing hormone receptor or alkylation repair genes had a significantly better chance of survival (log-rank, p = 0.0024; Wilcoxon, p = 0.0016)

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′

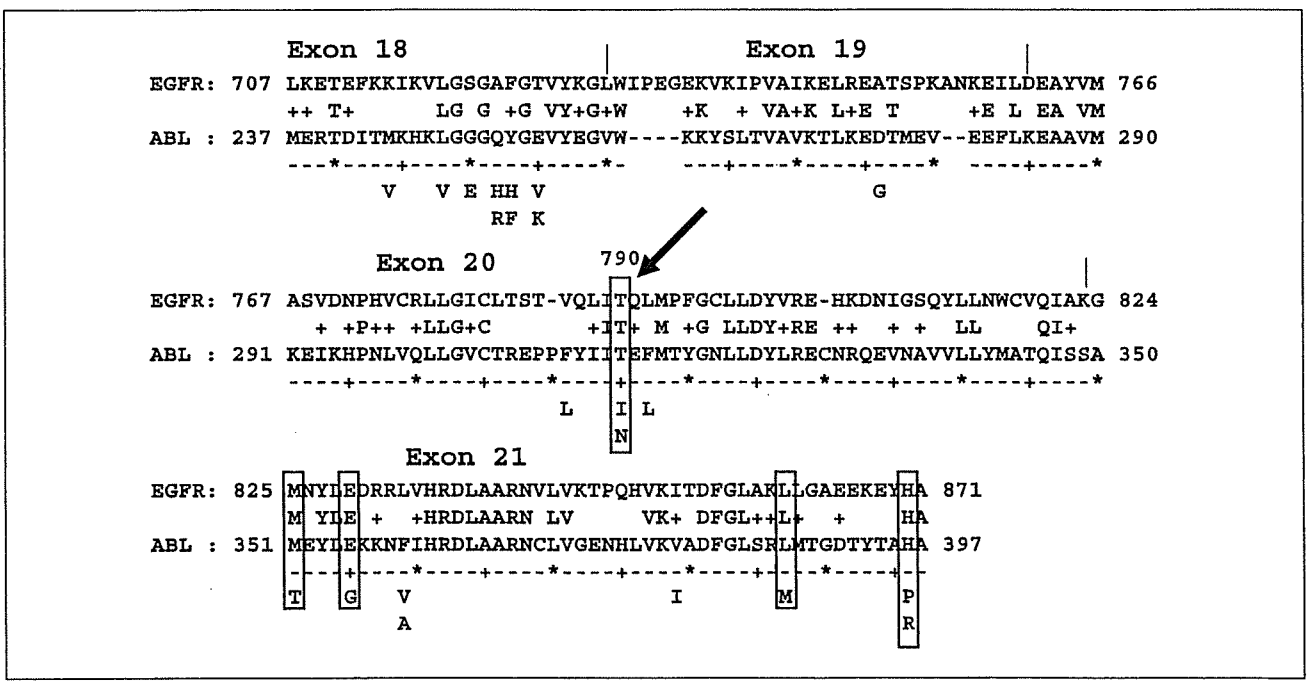

Fig. 1. Structural similarity between EGFR tyrosine kinase and ABL. This amino acid alignment was obtained using basic local alignment search tool, and both sequences were obtained from Genbank (accession nos.: EGFR, NM 005228; ABL, NM 005157). Top line, EGFR; bottom line, ABL. Vertical lines, boundaries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