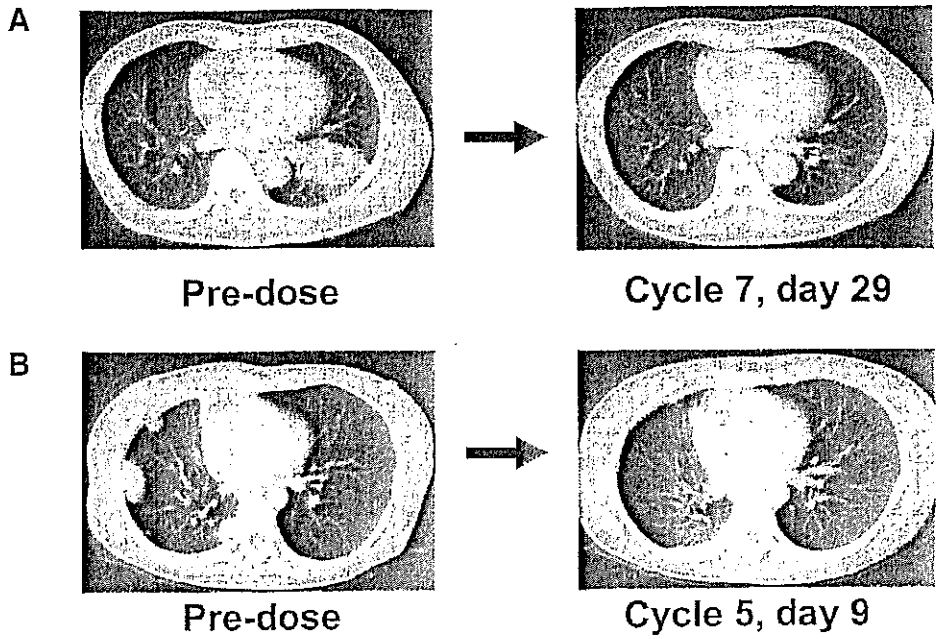

FIGURE 4. Representative CT scans before and after ZD6474 treatment in two NSCLC (adenocarcinoma) patients with partial responses. Baseline scans were performed within 4 weeks before the first dose. Male, 72 years (#301), initial ZD6474 dose = 200 mg. Female, 50 years (#406), initial ZD6474 dose = 300 mg.

of the vasodilator nitric oxide is downstream of VEGF-induced angiogenesis signaling,[24] inhibition of VEGFR-dependent signaling by ZD6474 may decrease nitric oxide production and lead to hypertension. Hypertension and elevated ALT levels were reported as DLTs in the 400-mg dose group during the period up to completion of cycle 2. As a result, this dose was considered to exceed the MTD.

Rash may be a consequence of EGFR inhibition, with the consideration that dose-dependent development of rash was reported in studies of other EGFR inhibitors, erlotinib[25] and gefitinib.[26,27] Because different types of rash, including erythema and photosensitivity, were observed in this study, it seems that the rash induced by ZD6474 may be more varied and systematic than was reported with those EGFR inhibitors.

Pharmacokinetic assessment in this study has confirmed that ZD6474 offers a convenient once-daily oral dosing schedule that is sufficient to achieve steady-state exposure. In this respect, the pharmacokinetic characteristics of ZD6474 in this Japanese study did not differ from those obtained in the U.S./Australian study.[21]

Although this study was primarily designed to assess safety and tolerability, secondary assessment of efficacy revealed that four out of nine patients with NSCLC exhibited a partial response to ZD6474 treatment at initial daily doses of 200 mg ($n = 3$) and 300 mg ($n = 1$). It is worth noting that partial tumor response was maintained in these patients (range 90–438 days) despite subsequent reductions in daily dose. This finding has prompted evaluation of ZD6474 in patients with NSCLC in phase II studies.[28–30] Although EGFR mutational status was not determined for any patients in the current study, a recent preclinical study showed that the antiproliferative effects of ZD6474 were augmented in an NSCLC cell line harboring EGFR containing a small in-frame deletion mutation.[31] Characteristics predicting response to gefitinib such as female gender, adenocarcinoma, nonsmoking status, Asian ethnicity, and EGFR mutations should be investigated in future studies.

Multiple signaling pathways contribute to tumor-related angiogenesis and tumor growth and metastasis. As such, novel therapies that target a single molecule or biochemical pathway may have less clinical efficacy than agents with more than one mode of action. Because ZD6474 is a selective inhibitor of VEGFR-2 and EGFR tyrosine kinase activity, this agent may be particularly beneficial in tumor types that display aberrant activity of both signaling pathways. However, the relative contribution of VEGFR-2 and EGFR tyrosine kinase inhibition to the clinical activity of ZD6474 in specific tumor types, as well as to the toxicity profile of ZD6474, remains to be determined.

In conclusion, these data indicate that ZD6474 at oral doses up to 300 mg/day was tolerated in Japanese patients with advanced tumors. A dose of 400 mg/day was considered to exceed the MTD, and doses of ≤300 mg/day were considered appropriate for evaluation in a further phase II study.[29]

Targeting multiple pathways in cancer may be necessary to provide sustained clinical benefit to patients, and ZD6474 has the potential to inhibit two key pathways in tumor growth by targeting VEGFR-dependent tumor angiogenesis and EGFR-dependent tumor cell proliferation and survival. Phase III development of ZD6474 in NSCLC has been initiated, and the clinical development program continues to investigate efficacy in other tumor types.

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EGFR mutation status in tumour-derived DNA from pleural effusion fluid is a practical basis for predicting the response to gefitinib

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Epidermal growth factor receptor (EGFR) mutations are strong determinants of tumour response to EGFR tyrosine kinase inhibitors in non-small-cell lung cancer (NSCLC). Pleural effusion is a common complication of lung cancer. In this study, we assessed the feasibility of detection of *EGFR* mutations in samples of pleural effusion fluid. We obtained 43 samples, which was the cell-free supernatant of pleural fluid, from Japanese NSCLC patients, and examined them for *EGFR* mutations. The *epidermal growth factor receptor* mutation status was determined by a direct sequencing method (exons 18–21 in *EGFR*). *EGFR* mutations were detected in 11 cases (E746_A750del in seven cases, E746_T751del insA in one case, L747_T751del in one case, and L858R in two cases). The *EGFR* mutations were observed more frequently in women and non-smokers. A comparison between the *EGFR* mutant status and the response to gefitinib in the 27 patients who received gefitinib revealed that all seven patients with partial response and one of the seven patients with stable disease had an *EGFR* mutation. No *EGFR* mutations were detected in the patients with progressive disease. The results suggest that DNA in pleural effusion fluid can be used to detect *EGFR* mutations and that the *EGFR* mutation status may be useful as a predictor of the response to gefitinib.
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Lung cancer is a major cause of cancer-related mortality worldwide and is expected to remain a major health problem for the foreseeable future (Parkin *et al*, 2005). Most patients have advanced disease at the time of diagnosis. The initial therapy for advanced non-small-cell lung cancer (NSCLC) is systemic chemotherapy with a two-drug combination regimen, which often includes a platinum agent, but the median survival of patients treated with such regimens has ranged from only 8 to 10 months. Little improvement in the efficacy of chemotherapy has been made in the last 20 years (Breathnach *et al*, 2001; Kelly *et al*, 2001; Schiller *et al*, 2002).

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Targeting epidermal growth factor receptor (EGFR) is an appealing strategy for the treatment of NSCLC, because EGFR has been found to be expressed, sometimes strongly, in NSCLC (Franklin *et al*, 2002). Gefitinib ('Iressa', AstraZeneca) is a small molecule and selective EGFR tyrosine kinase inhibitor that has shown antitumour activity in NSCLC patients as a single agent in phase II trials (Fukuoka *et al*, 2003). Adding gefitinib to chemotherapy in phase III studies of patients with untreated advanced NSCLC did not significantly improve the outcome over chemotherapy alone (Giaccone *et al*, 2004; Herbst *et al*, 2004), and a possible explanation for the failure to observe any added benefit in these trials is that the patients had not been screened or selected for their ability to derive any clinical benefit from an EGFR inhibitor.

An association between mutations in sites of *EGFR* tyrosine kinase in NSCLC and hyper-responsiveness to gefitinib has recently been reported (Lynch *et al*, 2004; Paez *et al*, 2004). The mutations consisted of small in-frame deletions or substitutions clustered around the ATP-binding site in exons 18, 19, and 21 of *EGFR*, and the mutations increased the affinity of the enzyme for ATP and gefitinib (Lynch *et al*, 2004). Some investigators subsequently found that *EGFR* mutations are one of the strong determinants of tumour response to EGFR tyrosine kinase inhibitors (Pao *et al*, 2004; Han *et al*, 2005). The investigators

used surgical tissue to detect the *EGFR* mutations in their studies, but most patients who require gefitinib therapy are diagnosed at an advanced stage of the disease and are inoperable. As it is often difficult to obtain a sufficient tumour sample from patients with inoperable NSCLC to detect *EGFR* mutations by direct sequencing, a method of detecting *EGFR* mutations in other specimens needed to be established.

Malignant pleural effusion is a common complication of lung cancer. It is present in approximately 15% of patients at the time of diagnosis (Pass *et al*, 2005) and in 10–50% of patients during the course of the disease (Fenton and David Richardson, 1995). In about half of NSCLC patients with a pleural effusion, the effusion fluid is cytologically positive at the first time examined, and ultimately most effusions are determined to be malignant. As pleural effusion fluid sampling is usually easy, non-invasive, and repeatable, we hypothesised that tumour-derived DNA in the pleural effusion fluid of NSCLC patients would be a source of useful information on the status of the *EGFR* gene and could allow prediction of the response to gefitinib. Some investigators have reported that pleural effusion fluid is a useful clinical specimen for searching for point mutations in oncogenes, such as *K-ras*, *rho A*, *p53*, and *FHIT* (Nakamoto *et al*, 2001; Lee *et al*, 2004). As the two trials were small, the results regarding the sensitivity and specificity of detection of the mutations in pleural effusion as a diagnostic method were unclear. Detection of *EGFR* mutations in pleural effusion fluid has been described in one case report, and the patient responded to gefitinib (Huang *et al*, 2005). The results in that patient encouraged us to hypothesise that the *EGFR* mutation status determined in pleural effusion fluid is useful for predicting the responsiveness to EGFR tyrosine kinase inhibitors.

In the present study, we attempted to detect *EGFR* mutations in pleural effusion fluid and to clarify the usefulness of their detection as a predictor of the response to gefitinib.

PATIENTS AND METHODS

Patients

The subjects were NSCLC patients who had a pleural effusion at the time of diagnosis. The diagnosis of NSCLC was based on the histological or cytological findings, and the histological type was determined according to the WHO criteria (Travis *et al*, 1999). Patients' records consisted of age, gender, smoking habit, histological type, and treatment. Smoking status was collected from the patients' records. Patients were divided into three groups according to their smoking status: never smokers (<100 cigarettes/lifetime), former smokers ($\geqslant 100$ cigarettes/lifetime, no smoking at present), and current smokers ($\geqslant 100$ cigarettes/lifetime). The response of the patients treated with gefitinib was evaluated every 4 or 8 weeks in accordance with the 'Response Evaluation Criteria in Solid Tumours (RECIST)' guidelines. (Therasse *et al*, 2000). Partial response (PR) and stable disease (SD) were confirmed by a sustained 4-week follow-up. This study was approved by the Institutional Review Board of the National Cancer Center Hospital and of Kanazawa University Hospital, and written informed consent was obtained from all participants. No research results were entered into the patient's records or released to the patient or the patient's physician.

Collection of pleural effusion fluid and DNA purification

The pleural effusion fluid was collected into heparinised tubes between 29 March 2005 and 30 January 2006. No particular collection method was used. A 2-ml sample of the fluid was centrifuged at 250 *g* for 10 min at room temperature, and the supernatant was collected and stored at −80°C until DNA extraction. DNA was extracted from 1 ml of the supernatant with a Qiamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the blood and body fluid spin protocol in the manufacturer's instructions, with the following protocol modifications. The same column was used repeatedly until the whole sample had been processed. The DNA obtained was eluted in 50 μl of sterile bi-distilled buffer, and the extracted DNA was stored at −20°C until used. The amounts of DNA extracted were estimated with spectrophotometry.

Polymerase chain reaction amplification and direct sequencing

Exons 18, 19, 20, and 21 of the *EGFR* gene were amplified by polymerase chain reaction (PCR). The primers were designed based on the report by Lynch *et al* (2004). Genomic PCR of 1 μl of template DNA was performed in 25 μl volumes containing 0.75 U of Ampli Taq Gold DNA polymerase (Perkin-Elmer, Roche Molecular Systems Inc., Branchburg, NJ, USA), 2.5 μl of PCR buffer, 0.8 μM dNTP, 0.5 μM of each primer, and different concentrations of $MgCl_2$, depending on the polymorphic marker. The first PCR analyses were performed in a volume of 25 μl by 25 cycles consisting of a denaturation step at 94°C for 45 s, a primer annealing step at 58°C for 30 s, and an elongation step at 72°C for 30 s. The final step at 72°C was extended for 10 min. Nested PCR was performed with 20 cycles under the same conditions as the first PCR. Sequencing of each sample was performed in duplicate with an ABI prism 310 (Applied Biosystems, Foster City, CA, USA). PCR products were sequenced in both sense and antisense directions. *Epidermal growth factor receptor* mutations detected in the initial round of sequencing were confirmed by subsequent rounds of independent PCR and sequencing reactions. Only specimens in which a mutation was identified in both rounds were recorded as mutation-positive. The sequences were compared with the GenBank-archived human sequence for *EGFR* (accession number: AY588246). The nucleic acid and protein coordinates used to name the mutations are based on NM_005228.3 and NP_005219.2, respectively.

Statistical analyses

This study was carried out as exploratory research for detecting *EGFR* mutations from pleural effusion fluid and clarifying the relationship between the mutation status and clinical manifestations. The number of enrolled patients was therefore not precalculated. Patient characteristics, including gender, tumour histology, and smoking habit were tabulated according to their mutation status. Fisher's exact test was used to test for associations between the presence of *EGFR* mutations and the patients' characteristics. The relationship between response to gefitinib and the mutation status was evaluated individually.

RESULTS

Patients and pleural effusion specimens

Forty-three patients were enrolled in this study (Table 1). Two hundred and sixty-two patients were seen with stage IIIB and IV at our institutions in the period of this study. Forty-three of the 262 patients were enrolled in this study. The enrolled patients were not all of the patients with pleural effusion because written informed consent was not obtained from any patients with pleural effusion. Their median age was 62 years (range, 39–82 years), and there were 21 females (53.8%) and 17 never smokers (43.6%). The histological and/or cytological diagnosis was adenocarcinoma in 39 patients, and squamous cell

Table 1 Patient characteristics and *EGFR* mutation status

	(n)	EGFR mutation (n)
No. of patients	43	11 (25.6%)
Age (years)		
Median	63	
Range	39–82	
Gender		
Male	22	4 (18.2%)
Female	21	7 (33.3%)
Smoking habit		
Current	9	2 (22.2%)
Former	16	2 (12.5%)
Never	18	7 (38.9%)
Histology		
Adenocarcinoma	39	11 (28.2%)
Squamous cell carcinoma	1	0 (0%)
Large cell carcinoma	1	0 (0%)
Unclassified	2	0 (0%)
No. of patients treated with gefitinib	27	8 (29.6%)
PR	7	7 (14.3%)
SD	7	1 (0%)
PD	13	0 (0%)

EGFR = epidermal growth factor receptor; PD = progressive disease; PR = partial response; SD = stable disease.

carcinoma and large cell carcinoma in one each, and unclassified NSCLC in two patients. Non-small-cell lung cancer cells in the pleural effusion samples of 40 of the patients were identified cytologically. There were no malignant cells in the pleural effusion fluid of the other three patients. We have no data of the proportion of malignant cells and normal cells. Twenty-seven patients were treated with gefitinib (250 mg day^{-1}) and evaluated for a response. Eight of the 27 patients were treated with gefitinib as an initial treatment and the other 19 patients were treated with the agent as a second or third line. The others were treated with systematic chemotherapy, including a platinum agent. The results of the evaluation showed that seven of the 27 patients who received gefitinib therapy had a PR and seven had SD. The other 13 patients had progressive disease (PD). DNA was extracted from all 43 samples of pleural effusion fluid. Amounts of the DNA extracted were detectable from 27 samples at a concentration up to 144.0 ng ml^{-1}. Amounts from 16 samples were under the detectable limit.

Detection of *EGFR* mutations in pleural effusion fluid

Direct sequencing of PCR products in exons 18–21 of *EGFR* in the pleural effusion fluid of all patients allowed their mutation status to be determined. Heterozygous mutations were identified in 11 (25.6%) of the 43 patients (Table 1). Nine mutations were deletional mutations located in exon 19 (E746_A750del in seven, L746_T751del insA in one, L747_T751del in one), and two were substitution mutations located in exon 21 (L858R) (Table 2) (Figure 1). No mutations were detected in exon 18 or 20. The E746_A750 deletion and L858R substitution mutations were the most common (9 out of 11 mutations. 81.8%) and are well-known hotspot mutations described previously (Kosaka *et al*, 2004; Pao *et al*, 2004). No more than one mutation was identified per patient, and no EGFR mutations were detected in pleural effusion fluid that did not contain malignant cells.

Table 2 Site of mutations in exons 18–21 of *EGFR*

Nucleotide changes	Amino-acid changes	No. of patients
2481_2495del	E746_A750del	6
2482_2496del	E746_A750del	1
2483_2497del	E746_T753del insA	1
2486_2500del	L747_T751del	1
2819T > G	L858R	2

EGFR = epidermal growth factor receptor; del = deletion; ins = insertion. The numbering of the mutation sites was based on NM_005228.3 (nucleotide) and NP_005219.2 (amino acid).

Epidermal growth factor receptor mutation status and patients' characteristics

EGFR mutations were detected more frequently in the samples from females (7 out of 21, 33.3% of females, 4 out of 18, 22.2% of males; $P = 0.310$) and non-smokers (7 out of 17, 41.1% of non-smokers, 4 out of 22, 18.1% of current or former smokers; $P = 0.156$), although the differences were not statistically significant (Table 3). Of the 11 mutations, 63.6% were in women and 63.6% were in non-smokers. All of the patients with mutations had adenocarcinoma. No *EGFR* mutations were found in any of the patients with squamous carcinoma or large cell carcinoma. A comparison between the *EGFR* mutant status and the response to gefitinib showed that all seven patients with a PR and one of the seven patients with SD had an *EGFR* mutation. No *EGFR* mutations were detected in any of the patients with PD (Table 4). We have no response data from the 16 patients who had never treated with gefitinib, and we have not evaluated the relationship between the response to chemotherapy and the EGFR mutation status in pleural effusion fluid.

DISCUSSION

This is the first report of an analysis of the *EGFR* mutation status in DNA obtained from the pleural effusion fluid of a series of NSCLC patients and evaluation of the relationship between the mutation status and the clinical response to gefitinib. It is interesting that all patients who achieved a PR to gefitinib had the *EGFR* mutations. We hypothesised that the mutation status in DNA extracted from pleural effusion fluid would allow prediction of the clinical outcome of gefitinib therapy in NSCLC patients, and we therefore expected the pleural effusion fluid to be a practical source of DNA for detection of *EGFR* mutations. The sites of *EGFR* mutations found in this study are identical to these reported in previous studies (Kosaka *et al*, 2004; Pao *et al*, 2004). The main mutations found were in-frame deletions in exon 19 and the missense mutation L858R in exon 21. No patients had more than one mutation. It was possible to determine the mutation status of *EGFR* by using the DNA in only 1.0 ml of pleural effusion fluid, even though the concentration of the extracted DNA specimens was in most cases below the concentration detectable by spectrophotometry (data not shown). The results of the comparison between the mutation status and clinical manifestations in this study confirmed the finding in previous studies that *EGFR* mutations are frequently present in small sub-groups of NSCLC patients, such as females and never smokers, although the differences were not statistically significant. It is well known that *EGFR* mutations are frequently observed in adenocarcinomas. As 36 of the 39 patients (92.3%) enrolled in this study had adenocarcinoma, we could not evaluate differences in the frequency of the *EGFR* mutations according to the histological type. Pleural effusion occurs in lung carcinoma of all cell types, but

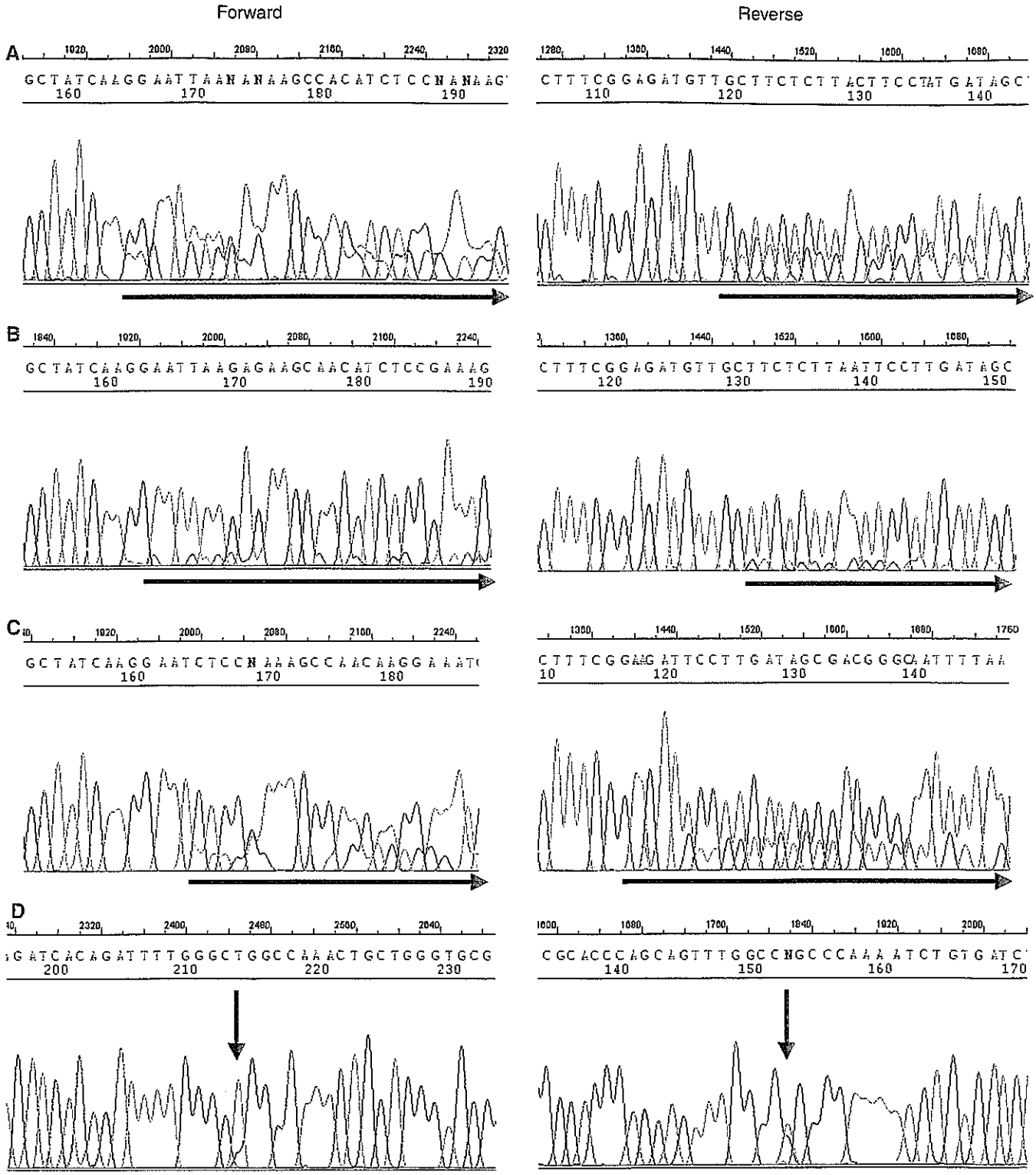

Figure 1 The wave figures of the nucleotide sequence of the *EGFR* gene with heterozygous mutations obtained by direct sequencing (see 'Patients and Methods') are shown. Horizontal arrows in both the sense and the antisense directions are shown to demonstrate the two breakpoints of the deletion. The patients in **A**, **B**, and **C** have inframe deletions in exon 19 (Figure **A**, E746_A750del; **B**, E746_T753del insA; **C**, L747_T751del; **D**, L858R). The double peaks (vertical arrows) represent the heterozygous missense mutations resulting in an amino acid substitution of L858R in exon 19 (Figure **D**).

appears to be most frequent in adenocarcinoma (Chernow and Shahn, 1997).

This study had several limitations. First, we could not compare the results of the *EGFR* mutation status in the pleural effusion fluid to the mutation status in tumour tissue. Forty of the 43 patients enrolled were cytologically diagnosed as having NSCLC from pleural effusion fluid specimens. As the DNA extracted from pleural effusion fluid consisted of DNA derived from both tumour cells and normal cells, the *EGFR* mutation status needs to be evaluated in a pair of DNA specimens from the tumour and pleural

Table 3 Frequency of *EGFR* mutations in DNA from the pleural effusion fluid of NSCLC patients according to (A) gender, (B) histology, (C) smoking habit, and (D) response to gefitinib

(A) Gender and *EGFR* mutation status

	EGFR mutation		
	+	−	
Female	7	14	
Male	4	18	$P=0.310$

(B) Histology and *EGFR* mutation status

	EGFR mutation		
	+	−	
Ad	11	28	
Non-Ad	0	4	$P=0.558$

(C) Smoking habit and *EGFR* mutation status

	EGFR mutation		
	+	−	
Never	7	11	
Current/former	4	21	$P=0.156$

Ad = adenocarcinoma; EGFR = epidermal growth factor receptor; + = mutation-positive; − = no mutations. (A)(B)(C): a total of 43 samples were evaluated.

Table 4 *EGFR* mutation status in patients who received gefitinib therapy

Age (years)	Gender	Smoking	Histology	EGFR mutation status	Response to gefitinib
62	F	Never	Ad	E747_P753insS	PR
58	F	Never	Ad	E746_A750del	PR
80	F	Never	Ad	E746_A750del	PR
61	M	Never	Ad	E746_A750del	PR
65	M	Former	Ad	E746_A750del	PR
60	M	Current	Ad	E746_A750del	PR
66	F	Never	Ad	E747_T750del	PR
76	F	Never	Ad	Wild	SD
57	F	Former	Ad	Wild	SD
40	F	Never	Ad	Wild	SD
72	F	Never	Ad	Wild	SD
58	F	Former	Ad	Wild	SD
66	F	Never	Ad	Wild	SD
65	F	Former	Ad	L858R	SD
39	F	Never	Ad	Wild	PD
69	M	Former	Ad	Wild	PD
72	F	Never	Ad	Wild	PD
74	F	Never	Ad	Wild	PD
67	M	Former	Ad	Wild	PD
62	M	Former	SCC	Wild	PD
59	F	Current	Ad	Wild	PD
77	M	Current	Ad	Wild	PD
82	F	Never	Ad	Wild	PD
66	F	Never	Ad	Wild	PD
56	M	Current	Ad	Wild	PD
61	M	Former	Ad	Wild	PD
65	M	Former	Ad	Wild	PD

Ad = adenocarcinoma; EGFR = epidermal growth factor receptor; F = female; M = male; NSCLC = unclassified NSCLC; PD = progressive disease; PR = partial response; SCC = squamous cell carcinoma; SD = stable disease.

effusion fluid to confirm the usefulness of the mutation status determined from pleural effusion fluid. However, it is sometimes difficult to obtain tumour samples from patients with advanced NSCLC, and even more so from patients diagnosed as having NSCLC using methods other than the histological examination of tumour tissue, such as on the basis of pleural effusion or sputum cytology. Second, direct sequencing may be not able to provide satisfactory results for detection of *EGFR* mutations in mixed samples of mutated and wild DNA. Although direct sequencing has generally been used to detect *EGFR* mutations in previous studies, detection of a mutation by this method requires at least 30% of the mutated DNA in a sample (Bosari *et al*, 1995; Fan *et al*, 2001). Lung cancers are very heterogeneous, and as normal cells, such as inflammatory cells or mesothelial cells, are contained in the pleural effusion fluid of lung cancer patients, in addition to tumour cells, a small amount of mutated DNA in pleural effusion fluid can be missed by direct sequencing. Unfortunately, we have no data at the present time on whether EGFR mutations were detectable in pleural effusion samples with either a few malignant cells, a small proportion of malignant cells with normal mesothelial cells, or cytologically negative samples. To establish a method for the detection of EGFR mutations from pleural effusion fluid, the mutation detectable proportion of malignant cells to normal cells in pleural fluid should be elucidated. We are planning an additional study using cytological examination to clarify the mutation detectable proportion as a next step. When pleural fluid is used as the material for detection of *EGFR* mutations, a patient with an *EGFR* mutation may be diagnosed as having wild-type *EGFR* because of the two limitations described above. Although we expected a high frequency of detection of *EGFR* mutations in this study because of the high proportion of adenocarcinomas (92.3%), we detected *EGFR* mutations in only 28.2% of the patients enrolled, a lower frequency than in two previous reports on Japanese NSCLC patients (Takano *et al*, 2005; Asano *et al*, 2006). Patients with false-negative results, meaning that no *EGFR* mutations were detected in a patient with an *EGFR* mutation, were not excluded from this study. Some investigators have tried to improve the sensitivity of detection of *EGFR* mutations in samples containing a mixture of tumour and normal cells. Wookey *et al* (2005) reported findings that the ARMS method was superior to the direct sequencing method and WAVE method for detecting *EGFR* mutations. Other groups have reported that LightCycler PCR assay (Sasaki *et al*, 2005), SSCP assay (Marchetti *et al*, 2005), and enriched PCR assay (Asano *et al*) are more sensitive than direct sequencing and are more rapid. A standardised method of detecting *EGFR* mutations needs to be established as soon as possible.

The final limitation in the present study is that it remains unclear whether there is any survival benefits associated with gefitinib therapy in those patients enrolled with *EGFR* mutations. The relationship between the *EGFR* mutation status determined in pleural effusion fluid and the gefitinib response in a portion of the patients enrolled supports the pleural effusion fluid *EGFR* mutation status as useful for predicting the response to gefitinib. The relationship between the *EGFR* mutation status determined in the pleural effusion fluid and the gefitinib response in the remaining patients and the survival benefit of gefitinib therapy in the patients with *EGFR* mutations are currently being evaluated, and confirmation of the results is expected in the very near future.

In conclusion, our results suggest that the DNA in pleural effusion fluid can be used to detect *EGFR* mutations and that the *EGFR* mutation status determined may be useful as a predictive factor of response to gefitinib.

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Knowledge of Efficacy of Treatments in Lung Cancer Is Not Enough, Their Clinical Effectiveness Should Also Be Known

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The benefits established in efficacy trials, usually randomized, controlled trials conducted under highly controlled circumstances with maximized internal validity, can frequently not be demonstrated in clinical practice at the community level. Effectiveness trials are tools to evaluate the applicability of a treatment in a wider setting with maximized external validity, to observe uncommon adverse events, and to identify factors influencing the main outcomes and risks. Important areas in relation to lung cancer treatment that will benefit from effectiveness trials include gefitinib monotherapy and bevacizumab therapy combined with cytotoxic chemotherapy for advanced non-small cell lung cancer. These therapies were found to produce life-threatening nonhematologic toxicity at a high incidence of up to 5%; however, the risk factors for these toxicities have not yet been fully established. Effectiveness trials of adjuvant chemotherapy after surgery with long-term follow-up are also important to obtain reliable information as to secondary malignancy and noncancer-related deaths. Development of an infrastructure for effectiveness trials is crucial because of the necessity to deal with large numbers of patients, sometimes as many as 10,000 patients, from many hospitals. The extensive research time involved and the considerable cost of these trials may be reduced with the use of Internet resources. Effectiveness trials are a fundamental step toward bridging the gap between clinical research and clinical practice and effectively implementing new therapies in clinical practice.

Key Words: Efficacy, Effectiveness, Large-scale trials, Lung cancer, Treatment.

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The current paradigm in medical practice is "evidence-based medicine," which has been defined as the "conscientious, explicit and judicious use of current best evidence in making decisions about the care of individual patients."[1] Randomized, controlled trials (RCTs) are considered the best evidence of efficacy because they employ an experimental design that reduces bias and confounding. The tacit assumption is that the potential benefits of new therapies as shown in RCTs will also be observed in clinical practice. The benefits established in RCTs, however, have been scarcely demonstrated in clinical practice in the community. The response to and compliance with a treatment can be highly dependent on factors such as the patient characteristics, the methods of application of the treatment, and the treatment setting. RCTs are usually performed on a homogeneous study population from which clinically complex patients such as the elderly and infirm patients are generally excluded for the sake of study feasibility. Evidence from such highly selected populations, therefore, cannot easily be generalized to nonselected patients.[2,3]

SUBGROUP ANALYSES AND META-ANALYSES

Subgroup analyses are an approach to enable the most effective use of treatment in routine practice. These analyses may be useful to compare the treatment effects and the risk of adverse events between subgroups in relation to patient characteristics, leading to identification of subgroups of patients most likely to benefit.[4] In this case, the limitations are lack of power due to the smaller number of patients involved, the limits of nonrandomized comparison, and false-positive results from the multiplicity of subgroups, and, therefore, validating the results of such analysis is needed in future trials.[4] Meta-analyses of RCTs aim to integrate the effects of treatment across trials in such a way that they can be translated into practice. Comparing the outcomes of patient subgroups within a meta-analysis may be more useful than a subgroup analysis within a trial, although analyses of individual patient data from trials are necessary.[5] In addition, a meta-analysis has a better external validity than an RCT if the benefit of a treatment was shown on RCTs performed in different settings, in different patient populations, and in different areas of the world.[6]

These methods can evaluate heterogeneity of results from subgroups of patients registered in RCTs, but cannot evaluate patients excluded from these trials, such as patients with comorbidities. Thus, another type of large trial that includes these patients, called effectiveness trials, is needed to apply the treatment in the real world of clinical practice.

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EFFICACY AND EFFECTIVENESS TRIALS

Efficacy and effectiveness are terms that are rarely used correctly and are often interchanged.[7,8] Efficacy is the true biological effect of a treatment under the ideal conditions of an investigation, whereas effectiveness is the beneficial effect observed when the treatment is used in clinical practice in the community at large, which is influenced by many aspects, including the patient characteristics and the social health system. Efficacy trials, also called explanatory trials, are primarily developmental tools used to make inferences related to the treatment modality in question (Table 1).[9] The maximum potential benefits that can be derived from a treatment are estimated under ideal, highly controlled circumstances in clinical research settings, usually in RCTs, to establish a causal link between the treatment and the primary outcome with maximized internal validity. Efficacy trials are conducted in a homogeneous group of patients who are carefully selected based on strict eligibility criteria. The sample size is large enough to have adequate power to detect significant effects. Patients are randomly allocated to either the treatment under investigation or a control standard treatment to equalize the distribution of potential confounding factors. In efficacy trials, the treatment is delivered by highly skilled, rigorously trained, and closely supervised specialists, using standardized, manual-based protocols under close monitoring to ensure fidelity or delivery of treatment as intended in teaching hospitals.

On the other hand, effectiveness trials, which have been called pragmatic, large-scale, or public health trials, are tools to evaluate the applicability of a treatment in a wider setting, to observe uncommon adverse events, and to identify factors influencing the main outcomes and risks (Table 1).[9] To maximize the external validity, or generalizability, effectiveness trials are conducted under naturalistic circumstances in clinical practice settings. Heterogeneous patients selected based on nonstringent eligibility criteria receive the broadly defined treatment without close monitoring or supervision with corrective feedback. The range of the heterogeneity should be as wide as that seen in clinical practice. Inclusion of atypical patients and those with comorbidities will ensure that patients to whom the treatment will be given in the clinical setting will be represented. Large-scale trials may fail to detect a benefit in a population mixed with groups of patients that benefit from the treatment and other groups in which the treatment has no effect or is harmful. It is thus essential to study factors predictive of the treatment effect and to have enough power to perform them. It is very important to identify the population of patients that benefits from the treatment.

The use of stratification is only to improve the power of the analysis and to limit bias in the comparison of subgroups, but not to avoid imbalance in prognostic factors as they are balanced in large trials. The follow-up period is often longer in effectiveness trials.

Although the study design used is often still that of a RCT for these trials, single-arm cohort studies may also be equally, and even sometimes more, appropriate.

HYPOTHESIS AND STUDY DESIGN OF EFFECTIVENESS TRIALS

In contrast to efficacy trials, of which the RCT is widely accepted as the standard procedure, the nature of what constitutes sound effectiveness trials is much less clear, and a few study designs have been tried according to their purpose. The hypothesis to be examined in effectiveness trials is the reproducibility of the results of an efficacy trial conducted under a controlled environment in the clinical practice setting. To confirm a hypothesis verified in an efficacy trial that "Treatment A" is better than "Treatment B," an RCT design may also be required in the subsequent effectiveness trials. Several confounding factors should be stratified at randomization, and the sample size may need to be larger than that in the relevant efficacy trial to detect small significant differ-

TABLE 1. General Characteristics of Efficacy and Effectiveness Trials

Characteristics	Efficacy (Explanatory) Trials	Effectiveness (Pragmatic) Trials
Need	To understand a therapeutic process	To make clinical decisions
Purpose	To demonstrate the efficacy in as short a time aspossible	To assess risk, effectiveness, and cost-effectiveness: to identify influencing factors
Focus of inference	Internal validity	External validity, generalizability
Setting	Highly controlled and specificclinical research setting	Less controlled and representativeclinical practice setting
Design	RCTs	Cohort studies or RCTs
Treatment	Clearly defined, manual based	Broadly defined, easily adaptable to the practice setting
Eligibility criteria	Strict	Relaxed
Study population	Homogeneous	Heterogeneous
No. of patients	<1000	1000–10,000
Monitoring	Close supervision with corrective feedback	Not close
Data	Complex and detailed	Simple
Clinician	Rigorously trained	Variable level of training
Institute	Academic hospital	Community hospital

RCTs, randomized controlled trials. Adapted from Nash JM, McCrory D, Nicholson RA. Efficacy and effectiveness approaches in behavioral treatment trials. *Headache* 2005;45:507–512 and Piantadosi S . *Clinical Trials*. New York: John Wiley & Sons, Inc., 1997.

ences in a heterogeneous patient population. In contrast, to confirm the efficacy of "Treatment A," such as the response and survival obtained in an efficacy trial, a prospective, single-arm cohort design may be adequate for the subsequent effectiveness trial. Diversity in patient population and setting should be enhanced by using practice-oriented protocols to reduce barriers to participation to identify prognostic factors. The primary end point in these trials, for example, the 2-year survival rate, should be evaluated in subset groups of patients categorized by prognostic factors as well as in a whole population. Because of its higher potential for bias than RTCs, detailed description of the cohort constitution and of the patients excluded from it should be included.

A meta-analysis of large RCTs with long-term follow-up can be used to evaluate harmful effects, but are not optimal to detect rare toxicities. To study acute and late toxic effects, several designs are possible: prospective cohorts, health insurance/claim databases, and cancer registries. Prospective cohort studies of combination chemotherapy and combined modality therapy are good candidates for investigator-initiated trials. In the framework of the new drug development, the efficacy and effectiveness are evaluated mainly in phase III and IV trials, respectively. Phase IV trials are conducted after obtaining approval for the drug use to monitor the safety and effectiveness in the general population. Rare, but life-threatening adverse events of a drug (e.g., interstitial lung disease [ILD]) or a combination of drugs (e.g., combination of the antiviral agent sorivudine and oral fluorouracil analogues) may be identified in this phase.[10,11] The Ministry of Health, Labor, and Welfare of Japan recently approved some new drugs on the condition that their toxicity is prospectively surveyed in the clinical setting. These include leflunomide, a newly developed disease-modifying antirheumatic drug that exhibits anti-inflammatory, antiproliferative, and immunosuppressive effects, and oxaliplatin for colorectal cancer. According to a recent report of a prospective postmarketing surveillance, of 5506 patients receiving leflunomide between August of 2003 and July of 2005, 76 patients (1.4%) had suspected ILD and 25 died of it, whereas the incidence of ILD associated with leflunomide reported from outside Japan is only 0.02%.[12] This high frequency of ILD among Japanese patients was revealed only by an effectiveness trial.

EFFECTIVENESS TRIALS RELATED TO LUNG CANCER TREATMENT (TABLE 2)

Gefitinib is an orally available, selective epidermal growth factor receptor tyrosine kinase inhibitor that has been shown to exert antitumor activity in patients with previously treated advanced non-small cell lung cancer (NSCLC). The safety and tolerability of gefitinib have been established in four open-label, multicenter, phase I dose-escalation studies and two multicenter, randomized phase II studies. After this drug was marketed in Japan, however, an unexpectedly high incidence of ILD, as high as 5%, was noted in subjects treated with the drug.[10,13] A prospective survey of gefitinib toxicity in 3354 patients with NSCLC treated at 698 hospitals in Japan between June and December of 2003 showed that the incidence of ILD was 5.8% and the mortality was 2.5%. This study also disclosed risk factors for the development of ILD in the Japanese population, including preexisting pulmonary fibrosis, smoking history, and poor performance status.[14] This is an example of the importance of an effectiveness trial for lung cancer treatment.

Bevacizumab, a humanized monoclonal antibody that inhibits vascular endothelial growth factor, has been shown to improve survival when given together with chemotherapy in patients with advanced nonsquamous NSCLC. However, grade 3/4 bleeding from the primary site, central nervous system, gastrointestinal tract, and other organs was noted in 4.5% of patients receiving the drug in a phase III study.[15] These new types of treatment agents with previously uncommon life-threatening toxicity are also considered important areas for effectiveness trials.

Another subject for effectiveness trials may be chemoradiotherapy for locally advanced lung cancer because the superiority of the concurrent over the sequential approach

TABLE 2. Important Areas Related to Lung Cancer Treatment for Effectiveness Trials

Therapy	Subject Population	Toxicity	Incidence
New agent with life-threatening toxicity			
EGFR inhibitors	Advanced NSCLC	Pneumonitis	1%–5%
VEGF inhibitors	Advanced NSCLC	Bleeding	5%
Intensive therapy with life-threatening toxicity			
Chemoradiotherapy	Stage III NSCLC	Pneumonitis	1%–4%
	Limited SCLC	Septic shock	1%
Intensive chemotherapy	Extensive SCLC	Septic shock	1%–2%
Treatment that requires long-term follow-up			
Adjuvant chemotherapy	Stage IB–IIIA NSCLC	Secondary malignancy	Rare
PCI	Limited SCLC	Neurocognitive disturbance	Rare
Treatment for heterogeneous populations			
Chemotherapy for the elderly	Advanced SCLC, NSCLC	Depends on general condition	

EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; PCI, prophylactic cranial irradiation; SCLC, small-cell lung cancer; VEGF, vascular endothelial growth factor.

was demonstrated only in patients in good general condition.[16,17] How widely applicable concurrent chemoradiotherapy is in the general patient population remains unknown. In addition, evaluation of late toxicities, including secondary malignancies related to smoking and treatment, and neurocognitive disturbance associated with prophylactic cranial irradiation has become more important as more long-term survivors are expected among these patients.[18,19]

Large-scale RCTs in patients with completely resected stage I–IIIA NSCLC aimed to confirm the effect of cisplatin-based adjuvant chemotherapy suggested by the meta-analysis in 1995.[20,21] Only effectiveness trials with long-term follow-up give reliable information as to secondary malignancy and noncancer related deaths in these patients.

Treatment of elderly patients with lung cancer is also an important field of effectiveness trials because many of these patients have comorbidity and decreased organ function, and, consequently, their general condition varies greatly from one patient to another.[22] There is a debate between those who promote age-unspecified large-scale trials with an analysis of the treatment effect according to age as a covariate and those who promote series of trials limited to an elderly population.[23,24] The outcome of the former trials can be generalized only to a small segment of the elderly population who meet the eligibility criteria of trials designed for younger patients, whereas the outcome of the latter trials depends greatly on the definition of the eligibility criteria. Confirmation of effectiveness will be needed in the both types of trials.

INFRASTRUCTURE OF EFFECTIVENESS TRIALS

Development of the appropriate infrastructure for effectiveness trials, which are conducted using a large number of patients, sometimes as many as 10,000 patients, is an urgent task. A central operations office and data coordinating center can handle many aspects of multi-institutional trials, including the recruitment of study institutions, randomization of patients, data collection, data analysis, and quality control. Clinical trials performed in an area with a cancer registry may cost less if collecting the events through a cancer registry without specific follow-up. The difficulty may be more linked to the construction of a network of general hospitals participating actively in clinical research.

The extensive research time and considerable cost of these processes can be reduced with the use of Internet resources.[25] In addition, a study Web site may facilitate communication among the trial personnel. A study Web site may also be used for the following tasks: providing information to potential participants, study subjects, and investigators; listing contact information; and centralizing data handling for patient registration, randomization, and data collection. A news section of the Web site can provide a progress report concerning the trial status and advertise upcoming meetings. A "Frequently Asked Questions" section can provide investigators with answers to common questions regarding the study protocol, and a download page can be a means of distributing study materials (protocol, case report forms, informed consent forms) to participating study centers.[25]

The electronic signature capture technology and electronic data capture system have been developed by several companies, including Fujitsu and Hitachi in Japan. An Internet clinical trial supporting system is now provided in Japan by commercial information technology service providers and the University Hospital Medical Information Network, a cooperative organization for national medical schools in Japan, sponsored by the Ministry of Education, Culture, Science, Sports, and Technology of Japan.[26]

Quality control and quality assurance of clinical trials become more difficult but more important as the numbers of participating hospitals, contributors, and patients grow. Careful study planning, use of information technology for data management, and efficient auditing are critical for effectiveness trials.[27,28] In addition, high-quality study conduct begins with the proper training of all personnel involved in the study.

TRAINING OF CLINICIANS

Efficacy trials are usually conducted by highly trained and experienced clinicians in academic institutes, including university-affiliated hospitals, cancer center hospitals, and central city general hospitals. When clinicians with varying academic backgrounds and levels of training are expected to implement new treatments in routine clinical practice at city hospitals, effective training of these clinicians is essential to bridge the gap between the research and practice environments.[29] However, passive dissemination of information, including via guidelines and didactic lectures, is generally ineffective in altering practices, irrespective of how important the issue or how valid the new treatment might be. Instead, it would seem necessary to use specific strategies to ensure improvements in common clinical practice, including the use of computerized decision support systems, educational outreach visits, and interactive educational meetings that include discussions of practice.[29] Opportunities for these should be provided to clinicians who participate in effectiveness trials.

CONCLUSION

Despite the considerable effort expended on efficacy trials, relatively little attention has been paid to ensure that the potential benefits of a new therapy are reproduced in routine clinical situations. Effectiveness trials are an important step toward bridging this gap and effectively implementing new therapies established in efficacy trials in clinical practice.

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High sensitivity detection of *epidermal growth factor receptor* mutations in the pleural effusion of non-small cell lung cancer patients

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Epidermal growth factor receptor (EGFR) mutations are a strong determinant of tumor response to gefitinib in non-small cell lung cancer (NSCLC). We attempted to elucidate the feasibility of *EGFR* mutation detection in cells of pleural effusion fluid. We obtained 24 samples of pleural effusion fluid from NSCLC patients. The pleural effusion fluid was centrifuged, and the cellular components obtained were used for detection. *EGFR* mutation status was determined by a direct sequencing method (exons 18–21) and by the Scorpion Amplified Refractory Mutation System (ARMS) method. *EGFR* mutations were detected in eight cases. Three mutations were detected by both methods, and the other five mutations were detected by Scorpion ARMS alone. The mutations were detected by both methods in all four partial responders among the seven patients who received gefitinib therapy. Direct sequencing detected the mutations in only two of four cases with partial response. These results suggest that the DNA in pleural effusion fluid can be used to detect EGFR mutations. The Scorpion *ARMS* method appears to be more sensitive for detecting *EGFR* mutations than the direct sequencing method. (*Cancer Sci* 2006; 97: 642–648)

Lung cancer is a major cause of cancer-related mortality worldwide and is expected to remain a major health problem for the foreseeable future.[1] Targeting the epidermal growth factor receptor (EGFR) is one appealing strategy for the treatment of non-small cell lung cancer (NSCLC), because EGFR has been found to be expressed, sometimes strongly, in NSCLC.[2] Mutations of *EGFR* tyrosine kinase in NSCLC and hyper-responsiveness to gefitinib, a selective EGFR tyrosine kinase inhibitor, have been reported.[3,4] These mutations consist of small in-frame deletions or substitutions clustered around the ATP-binding site in exons 18, 19 and 21 of *EGFR*, and increase the affinity of the enzyme for ATP and gefitinib. Some investigators have subsequently found that *EGFR* mutations are a strong determinant of tumor response to EGFR tyrosine kinase inhibitor.[5–7] Approximately 90% of the NSCLC-associated EGFR mutations in two reports consisted of two major *EGFR* mutations (E746_A750del in exon 19 and L858R in exon 21).[5,8] These investigators used surgical tissue to detect the *EGFR* mutations in their trials. As it is often difficult to obtain a tumor sample from patients with inoperable NSCLC, a method of detecting mutant *EGFR*, especially the two major mutations, in other specimens needs to be established.

Malignant pleural effusion is a common complication of lung cancer and is present in approximately 15% of lung cancer patients[9] and in 10–50% of patients at the time of diagnosis.[10] Approximately one-half of NSCLC patients with pleural effusion are initially positive cytologically, and most of the effusions are ultimately determined to be malignant. As sampling of pleural effusion fluid is usually easy, non-invasive and repeatable, we hypothesized that tumor cells in the pleural effusion fluid of NSCLC patients are a source of useful information on the status of the *EGFR* gene with regard to gefitinib response.

Genomic polymerase chain reaction (PCR) and the direct sequencing method have been used widely to detect *EGFR* mutations. It is well known that fusion between normal cells and tumor cells prevents detection of mutations in tumor cells by the direct sequencing method. Therefore it is necessary to enhance sensitivity for detection of *EGFR* mutations in a mixture of normal and tumor cells. We hypothesized that Scorpion Amplified Refractory Mutation System (ARMS) technology enhances sensitivity for detecting *EGFR* mutations. Scorpion primers are used in a fluorescence-based method for specific detection of PCR products.[11] A 'scorpion' consists of a specific probe sequence held in a hairpin loop configuration by complementary stem sequences on the 5′ and 3′ sides of the probe. A scorpion can be used in combination with ARMS to enable detection of single-base mutations.[11,12] The ARMS method was used for allele discrimination, and additional mismatches were introduced near the 3′ terminus of the primers to enhance specificity. A previous study showed that the ARMS method is superior to the direct sequencing method and the WAVE® (Transgenomic Inc., Cambridge, MA, USA) method for the detection of *EGFR* mutations.[13]

In the present study we attempted to detect major *EGFR* mutations in pleural effusion, and to find out whether the Scorpion ARMS method enhances sensitivity for detection of EGFR mutations in mixtures of DNA from normal cells and tumor cells.

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Patients and Methods

Patients

We studied NSCLC patients who had a pleural effusion at the time of diagnosis. The diagnosis of NSCLC was based on histological or cytological findings. This study was approved by the Institutional Review Boards of the National Cancer Center Hospital and Kanazawa University Hospital, and written informed consent was obtained from all participants. The patient record consisted of age, sex, smoking habit, histological type of NSCLC and treatment. The response of the patients treated with gefitinib was evaluated in accordance with the 'Response Evaluation Criteria in Solid Tumors (RECIST)' guidelines.[14] No research results were entered into the patient's record or released to the patient or their physician.

Collection of pleural effusion fluid and cell separation

The pleural effusion fluid was collected from patients in heparinized tubes between 29 March and 25 November 2005. No particular collection method was used. Pleural effusion fluid (1 mL) was centrifuged at 250 *g* for 10 min, and the cell pellet was stored at −80°C until use.

DNA extraction

DNA was extracted from the stored cell pellets using a Qiamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the protocol for tissue samples in the manufacturer's instructions. The DNA obtained was eluted in 50 μL of sterile bidistilled buffer, and the concentration and purity of the extracted DNA were assessed by spectrophotometry. The extracted DNA was stored at −20°C until use.

PCR amplification and direct sequencing

Exons 18, 19, 20 and 21 of the *EGFR* gene were amplified by PCR. The primers were designed based on a report by Lynch *et al.*[3] Genomic PCR of 20 ng of template DNA was carried out in 25-μL volumes containing 0.75 IU of Ampli *Taq* Gold DNA polymerase (Roche Molecular Systems, Branchburg, NJ, USA), 2.5 μL of PCR buffer, 0.8 μM dNTP, 0.5 μM of each primer, and different concentrations of $MgCl_2$, depending on the polymorphic marker. The first PCR analyses were carried out in a volume of 25 μL for 25 cycles, consisting of a denaturation step at 94°C for 45 s, a primer annealing step at 58°C for 30 s, and an elongation step at 72°C for 30 s. The final step at 72°C was extended for 10 min. Nested PCR was carried out for 20 cycles under the same conditions as the first PCR. Sequencing of each sample was carried out in duplicate using an ABI prism 310 (Applied Biosystems, Foster City, CA, USA). The sequences were compared with the GenBank-archived human sequence for EGFR (accession number AY588246).

Scorpion ARMS for the detection of E746_A750del and L858R

We used an EGFR Scorpion™ Kit (DxS, Manchester, UK), which combines the two technologies ARMS and Scorpion, to detect mutations in real-time PCR reactions. All reactions were carried out in 25-μL volumes with 1 μL of template DNA, 7.5 μL of reaction buffer mix, 0.6 μL of primer mix and 0.1 μL of *Taq* polymerase. Real-time PCR was carried out using SmartCycler® II (Cepheid, Sunnyvale, CA, USA) under the following conditions: initial denaturation at 95°C for 10 min, 50 cycles of 95°C for 30 s, and 62°C for 60 s with fluorescence reading (set to FAM that allows optical excitation at 480 nm and measurement at 520 nm) at the end of each cycle. Data analysis was carried out using Cepheid SmartCycler software (version 1.2b). The threshold cycle (Ct) was defined as the cycle at the highest peak of the second derivative curve, which represents the point of maximum curvature of the growth curve. Positive results were defined as Ct 45 and maximum fluorescence intensity 50. Analysis of each sample was carried out in duplicate. The EGFR Scorpion Kit is intended for detection of the two major somatic mutations in *EGFR*. These mutations consist of an in-frame deletion in exon 19 (E746_A750del) and a point mutation in exon 21 (L858R). There are two types of E746_A750del, with starting points at nucleotide positions 2235 and 2236 (NM_005228). The assay can detect both types of E746_A750del. Other deletion patterns in exon 19 and other mutations in the tyrosine kinase domain of EGFR, which are also associated with sensitivity of lung cancers to gefitinib, can not be detected using this assay.

Experiments comparing the detection of E746_A750del in mixtures of wild-type and E746_A750del DNA by direct sequencing and Scorpion ARMS

We used the standard DNA included in the EGFR Scorpion Kit to confirm sensitivity for the detection of E746_A750del. The following DNA mixtures were prepared: 10, 100, 1000 and 10 000 pg E746_A750del DNA, and 10 000 pg wild-type DNA with 10, 100, 1000 and 10 000 pg E746_A750del DNA. These DNA mixtures were used to evaluate the sensitivity of direct sequencing and Scorpion ARMS. The results obtained using Scorpion ARMS were quantified using a standard curve generated by plotting the Ct against the log of the amount of DNA contained in the known standards. The linear correlation coefficient (R^2) values and the formulas for the slopes were calculated. To validate this assay we carried out the assay using plasmid DNA derived from the PCR products of A431 cells, which are known to contain wild-type DNA, PC-9 cells, which are known to contain E746_A750del, and 11-18 cells, which are known to contain L858R. The plasmid DNA was subcloned into a cloning Topo® vector (Invitrogen, Carlsbad, CA, USA). The experiments were carried out at a copy number of 10^7.

Results

Patients and pleural effusion specimens

Twenty-four patients with NSCLC were enrolled in the present study (Table 1). There were 11 women (45.8%) and 10 never-smokers (41.7%). The histological diagnosis was adenocarcinoma in 23 patients and unclassified NSCLC in the other patient. NSCLC was diagnosed cytologically in the pleural effusion samples in 22 of the patients. There were no malignant cells in the pleural effusion fluid of the other two patients. The age range was 39–82 years (median 62 years). Seven patients were treated with gefitinib (250 mg/day) and their response was evaluated. The volume of the pleural effusion fluid collected from the patients ranged from 30 to 280 mL. DNA from cell pellets was extracted for all 24 samples at concentrations ranging from 3.2 to 335.5 ng/μL.

Table 1. Patient characteristics and epidermal growth factor receptor mutation status

No.	Age (years)	Sex	Smoking history	Histology	Response to gefitinib	EGFR mutation	
						Direct sequencing	Scorpion ARMS
1	62	F	Never	ADC	PR	Wild type	E746_A750del
2	40	F	Never	ADC	SD	Wild type	Wild type
3	39	F	Never	ADC	PD	Wild type	Wild type
4	69	M	Former	ADC	–	Wild type	Wild type
5	72	F	Never	ADC	–	Wild type	Wild type
6	66	F	Never	ADC	–	Wild type	Wild type
7	56	M	Current	ADC	–	Wild type	Wild type
8	61	M	Former	ADC	–	Wild type	Wild type
9	65	M	Former	ADC	PD	Wild type	Wild type
10	80	F	Never	ADC	–	Wild type	E746_A750del
11	82	M	Current	NSCLC	–	Wild type	Wild type
12	57	F	Former	ADC	–	Wild type	Wild type
13	55	M	Former	ADC	–	Wild type	Wild type
14	67	M	Former	ADC	–	Wild type	Wild type
15	61	M	Never	ADC	PR	Wild type	E746_A750del
16	65	M	Former	ADC	PR	E746_A750del†	E746_A750del
17	65	F	Former	ADC	–	Wild type	L858R
18	48	F	Never	ADC	–	Wild type	L858R
19	61	M	Current	ADC	–	Wild type	Wild type
20	60	M	Current	ADC	PR	E746_A750del‡	E746_A750del
21	63	F	Never	ADC	–	E746_A750del‡	E746_A750del
22	54	M	Former	ADC	–	Wild type	Wild type
23	49	M	Current	ADC	–	Wild type	Wild type
24	66	F	Never	ADC	–	Wild type	Wild type

Type of mutation: †2236–2250del; ‡2235–2249del (NM_005228). –, Patient did not receive gefitinib; ADC, adenocarcinoma; NSCLC, non-small cell lung cancer; PD, progressive disease; PR, partial response; SD, stable disease.

Sensitivity of direct sequencing and EGFR Scorpion for detection of E746_A740del

Preliminary experiments were carried out to evaluate the sensitivities of direct sequencing and the EGFR Scorpion Kit. When direct sequencing was used to detect E746_A750del in the standard E746_A750del DNA samples (10–10 000 pg), the mutation was detected at amounts as low as 10 pg. When diluted standard E746_A750del DNA was mixed with standard wild-type DNA at ratios from 1:1 to 1:1000, E746_A750del was detected by direct sequencing at ratios as low as 1:10.

When E746_A750del DNA was detected with Scorpion ARMS, all curves for standard E746_A750del DNA (10–10 000 pg) and the primer set for detection of E746_A750del increased for up to 45 cycles (Fig. 1A). When wild-type standard DNA and distilled water were used as negative controls, the curves did not increase, and remained flat at 50 cycles (Fig. 1A). When diluted standard E746_A750del DNA was mixed with wild-type DNA in ratios from 1:1 to 1:1000, all curves that indicated the presence of E746_A750del increased for up to 45 cycles (Fig. 1B). Standard curves in the range of measured amounts in this study were linear with R^2 values of 0.997 and 0.987. Both slopes of the curves were almost parallel (Fig. 1C). The Ct of diluted standard E746_A750del DNA mixed with wild-type DNA was almost the same as for standard E746_A750del DNA. Although the peak fluorescence levels of diluted standard E746_A750del DNA mixed with wild-type DNA were lower than without the wild-type DNA standard, the presence of E746_A750del was clearly detected at the ratio of 1:1000. Curves of DNA containing E746_A750del at amounts up to 10 pg were unaffected by interfusion of wild-type DNA.

The signals of plasmid DNA derived from the PC-9 cells and 11-8 cells were detected at approximately the same Ct values (E746_A750del, 28.6; L858R, 29.2) and, as expected, when plasmid DNA derived from A431 was used, the curve did not increase and remained flat after 50 cycles (Fig. 1D,E).

Detection of *EGFR* mutations by direct sequencing

EGFR mutations in three of the 24 patients (12.5%) were detected by direct sequencing (Table 1). All three were heterozygous, and E746_A750del was detected in all three of them. Figure 2 shows the wave figures of the nucleotide sequence obtained by direct sequencing of part of exon 19 in two patients (patient no. 10, Fig. 2A; patient no. 21, Fig. 2C). The data for patient no. 10 was judged to represent wild-type *EGFR* (Fig. 2A). That of patient no. 21 showed a mixture of wild-type and 2235–2249del sequences (Fig. 2C).

Mutation analysis using the Scorpion ARMS method

EGFR mutation status in all samples was analyzed using the EGFR Scorpion Kit. As wild-type curves were detected in all patients, we concluded that no sample was too small to be detect by the Scorpion ARMS method and that it would be possible to determine the *EGFR* mutation status based on the results. Curves for an *EGFR* mutation were detected in eight of the 24 patients (33.3%; Table 1). In six of these eight patients, curves indicating the presence of a deletion mutation

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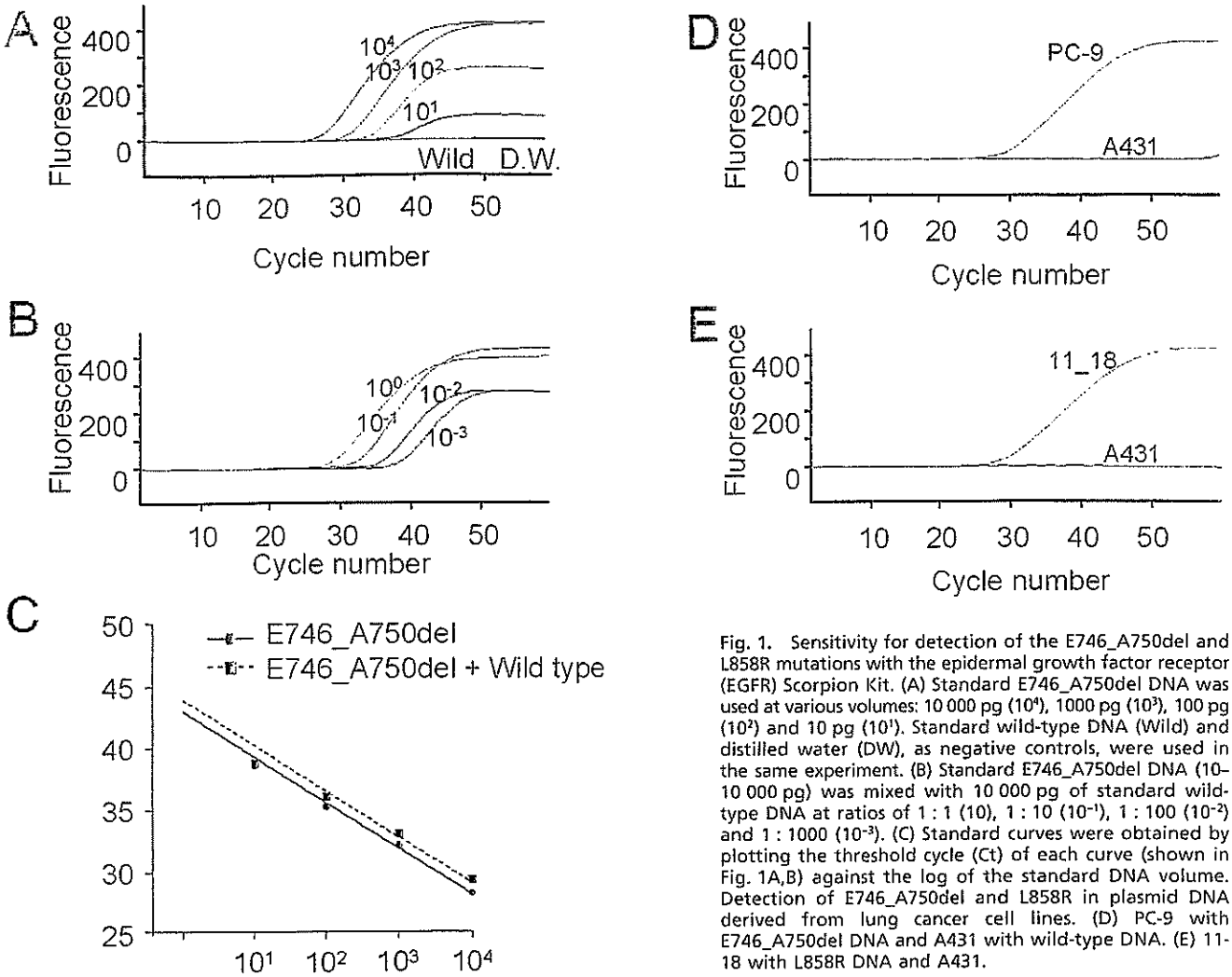

Fig. 1. Sensitivity for detection of the E746_A750del and L858R mutations with the epidermal growth factor receptor (EGFR) Scorpion Kit. (A) Standard E746_A750del DNA was used at various volumes: 10 000 pg (10^4), 1000 pg (10^3), 100 pg (10^2) and 10 pg (10^1). Standard wild-type DNA (Wild) and distilled water (DW), as negative controls, were used in the same experiment. (B) Standard E746_A750del DNA (10–10 000 pg) was mixed with 10 000 pg of standard wild-type DNA at ratios of 1 : 1 (10), 1 : 10 (10^{-1}), 1 : 100 (10^{-2}) and 1 : 1000 (10^{-3}). (C) Standard curves were obtained by plotting the threshold cycle (Ct) of each curve (shown in Fig. 1A,B) against the log of the standard DNA volume. Detection of E746_A750del and L858R in plasmid DNA derived from lung cancer cell lines. (D) PC-9 with E746_A750del DNA and A431 with wild-type DNA. (E) 11-18 with L858R DNA and A431.

in exon 19 were detected (Fig. 2B,D), and curves for the other two patients indicated the presence of L858R in exon 21.

Comparison of detection of the two major mutations by the two methods

In the present study *EGFR* mutations were detected in eight patients. In three of them (nos 16, 20 and 21) the *EGFR* mutations were detected by both methods, whereas in the other five (nos 1, 10, 15, 17 and 18) they were detected by the Scorpion ARMS method alone. No patients were found to have *EGFR* mutations by direct sequencing alone. *EGFR* mutations were not detected using either direct sequencing or the Scorpion ARMS method in two samples that were not diagnosed cytologically as NSCLC.

EGFR mutation status and clinical manifestations

EGFR mutations were detected more frequently in the samples from women (5/11, 45.5% of women; 3/13, 23.1% of men) and non-smokers (5/10, 50.0% of non-smokers; 3/14, 21.4% of smokers) (Table 2). Four of the seven patients who received gefitinib therapy had a partial response, one had stable disease, and the other three patients had progressive disease. All four patients with a partial response had *EGFR* mutations (Table 3). Evaluation of mutation status by the direct sequencing method revealed mutations in two of the four patients with partial response, whereas Scorpion ARMS revealed mutations in all four patients with partial response. Mutation status determined by Scorpion ARMS was superior to mutation status determined

Table 2. Frequency of *epidermal growth factor receptor (EGFR)* mutations in pleural effusion from patients with non-small cell lung cancer according to sex and smoking history

Variable	Direct sequencing		Scorpion ARMS	
	+	−	+	−
Sex and *EGFR* mutant state				
Female	1	10	5	6
Male	2	11	3	10
Smoking history and *EGFR* mutant state				
Non-smoker	1	9	5	5
Smoker	2	13	3	11

+, Mutation positive; –, no mutation; ARMS, Amplified Refractory Mutation System.

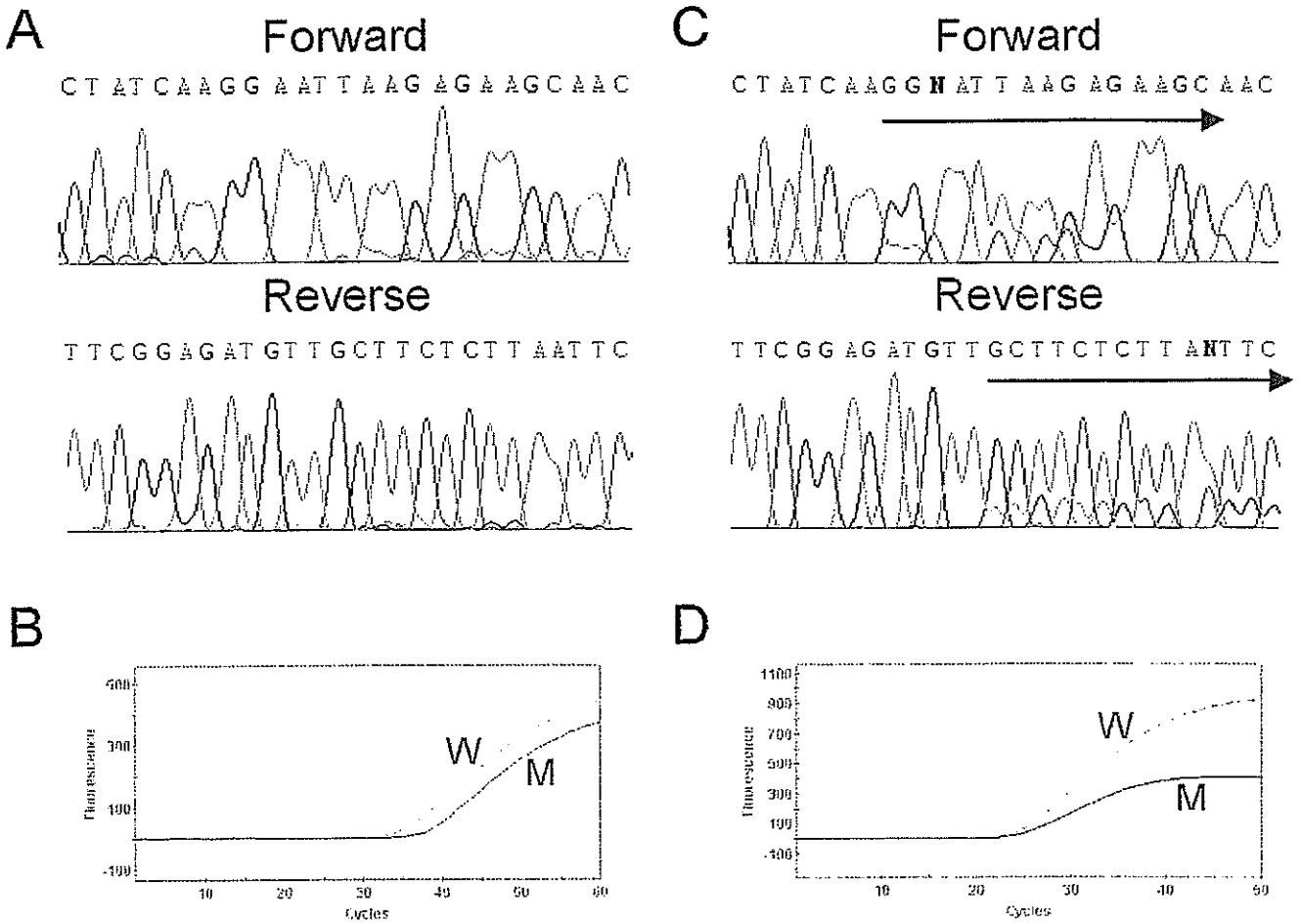

Fig. 2. Results of direct sequencing and the Scorpion Amplified Refractory Mutation System (ARMS) method in patient no. 10 (A,B) and patient no. 21 (C,D). (A) The wave figure represents wild-type *epidermal growth factor receptor (EGFR)*. (B) Two ascending curves, indicating that wild type and deletion mutation in exon 19 were detected. (C) The two waves start to overlap at the starting points of the arrows. These features mean that the nucleotide sequence of the *EGFR* gene in this patient has a heterozygous deletion. The deletion removed amino acids 746–750 (E746_A750del). (D) Two ascending curves, indicating that wild type and deletion mutation in exon 19 were detected.

Table 3. Frequency of *epidermal growth factor receptor (EGFR)* mutations in pleural effusion from patients with non-small cell lung cancer according to response to gefitinib

Variable	Direct sequencing		Scorpion ARMS	
	+	−	+	−
Partial response	2	2	4	0
Stable/progressive disease	0	3	0	3

The response to gefitinib was evaluated in all seven patients treated with gefitinib. +, Mutation positive; −, no mutation.

by direct sequencing for predicting responsiveness to gefitinib. No EGFR mutations were detected in patients with stable disease or progressive disease.

Discussion

The present study yielded two major findings. The first is that *EGFR* mutations, especially E746_A750 del and L858R, were detected in DNA from pleural effusion fluids, and the second is that the Scorpion ARMS method may be more sensitive for detecting *EGFR* mutations than the direct sequencing method. Patients with *EGFR* mutations may be misdiagnosed as not having any mutations if direct sequencing alone is used. Three patients were concluded to have mutations using both methods, but the other four patients were concluded to have mutations by the Scorpion ARMS method alone. As all four of these patients had partial responses to gefitinib, the results strongly suggest a correlation between mutation status and clinical responsiveness to gefitinib, although further clinical study is needed to make a definite conclusion. *EGFR* mutation status determined by the Scorpion ARMS method reflected responsiveness to gefitinib more accurately than direct sequencing.

Direct sequencing is currently the routine method used to detect *EGFR* mutations in tumor samples, and no standard method of detection of *EGFR* mutations in tumor specimens except surgical tissues has been established. The results of our small study lead us to conclude that the EGFR Scorpion Kit is superior to direct sequencing for detection of *EGFR*

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mutations, especially the two major mutations (deletion mutations in exon 19 and L858R), as predictive markers. As our preliminary experiment showed that the sensitivity of Scorpion ARMS for detection of *EGFR* mutations is superior to the sensitivity of direct sequencing when a mixture of wild-type and mutant DNA is used, we infer from these results that the differences in sensitivity for detection in the four patients with the mutations were attributable to the density of tumor cells in the pleural effusion fluid.

To our knowledge detection of *EGFR* mutations in pleural effusion fluid has been described in one case report where the patient responded to gefitinib.[15] Although our study did not confirm a correlation between mutation status and clinical responsiveness to EGFR tyrosine kinase inhibitors such as gefitinib, their results and our own in patients who received gefitinib therapy encourage us to conclude that *EGFR* mutation status determined in pleural effusion fluid may be useful for predicting responsiveness to EGFR tyrosine kinase inhibitors. The authors of the case report did not mention the possibility that normal cells may have prevented detection of *EGFR* mutations in tumor cells and that a patient with an *EGFR* mutation may be concluded not to have a mutation (false negative) as a result.

Some investigators have tried to increase the sensitivity of *EGFR* mutant detection. One attempt involved detection of *EGFR* mutations using a LightCycler PCR assay.[16] SSCP assay is more sensitive than direct sequencing and is a more rapid method.[17] Recently, two rapid and sensitive methods have been demonstrated: the peptide nucleic acid-locked nucleic acid PCR clamp method,[18] and the mutant-enriched PCR assay.[19] In these previous studies, *EGFR* mutations were detected in the presence of 1000-fold and 2000-fold wild-type *EGFR*, respectively. Although the minimum detectable mutation volumes were not evaluated, the sensitivity of these methods seems to be comparable with that of the Scorpion ARMS method, and the sensitivity of these assays seems to be sufficient for clinical use. The latter study used various clinical samples, including 20 samples of pleural fluid. We have shown a relationship between *EGFR* mutation status in pleural fluids and the gefitinib response in a portion of the enrolled patients. The relationship in the remaining patients is currently being evaluated, and confirmation is expected in the very near future. As the Scorpion ARMS method is simple and very fast, it may be suitable for mutation screening. However, one limitation of the EGFR Scorpion Kit is that it is only able to detect mutations targeted by the Scorpion primers. It is known that deletion mutations in exon 19 have many variations in deleted nucleotides and addition of point mutations. The Scorpion ARMS method could detect mutations targeted by primers designed in advance and is capable of detecting the specific mutation E746_A750del in exon 19. E747_P753del insS and L747_T751del are minor variations of deletion mutations in exon 19 and could not be detected using this method in another study (data not shown). All *EGFR* mutations are not at these two sites; some are clustered around the ATP-binding site in exons 18, 19 and 21.[3–8] Although approximately 90% of NSCLC-associated *EGFR* mutations consist of the two major EGFR mutations,[5,8] other mutations may be misdiagnosed as negative mutation results using the Scorpion ARMS method. Moreover, a secondary mutation, a substitution of methionine for threonine at position 790 (T790M), leads to gefitinib resistance in NSCLC patients who have *EGFR* mutations and are responsive to gefitinib.[20,21] These mutation states may also be critical factors for gefitinib therapy. Scorpion primers need to be designed to detect these mutations, and further study using these primers is required.

Our two initial aims, which were to detect two major EGFR mutations in pleural effusion fluid and to increase the sensitivity of detection of *EGFR* mutations in the mixtures of DNA from normal cells and tumor cells, were achieved in this study. As the next step, a prospective study of a large number of NSCLC patients with pleural effusion is likely to reveal a correlation between *EGFR* mutation state in pleural effusion fluids and clinical responsiveness to EGFR tyrosine kinase inhibitors, such as gefitinib.

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