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

Hideharu Kimura,[1,2] Yutaka Fujiwara,[3] Takashi Sone,[2] Hideo Kunitoh,[3] Tomohide Tamura,[3] Kazuo Kasahara[2] and Kazuto Nishio[1,4,5,6]

[1]Shien-Laboratory, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, [2]Respiratory Medicine, Kanazawa University Hospital, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8641, [3]Medical Oncology, National Cancer Center Hospital, Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045 and [4]Pharmacology Division and [5]Center for Medical Genomics, National Cancer Center Research Institute, Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045, Japan

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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.

[6]To whom correspondence should be addressed.
E-mail: knishio@gan2.res.ncc.go.jp

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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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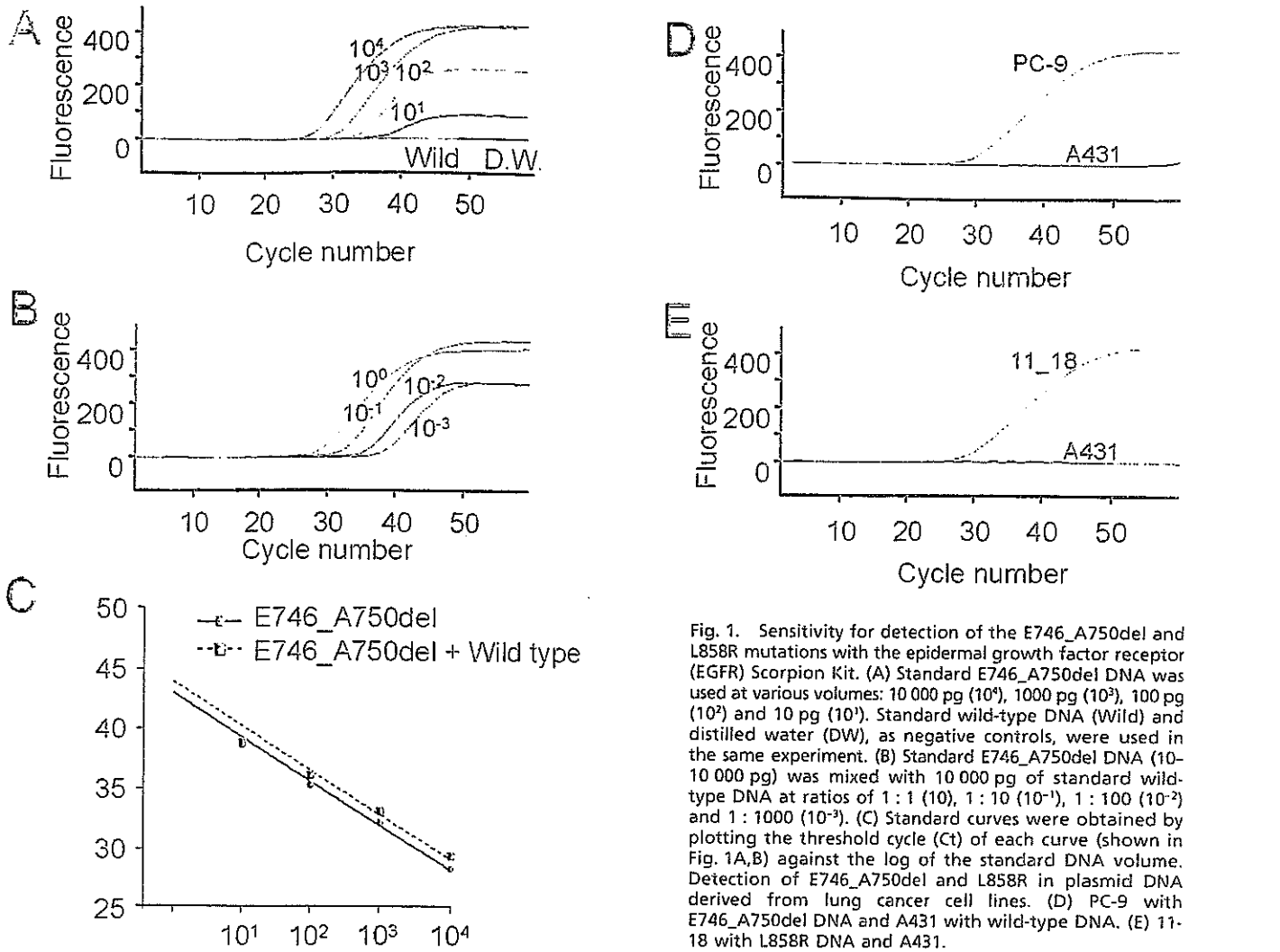

Fig. 1. Sensitivity for detection of the E746_A750del and L858R mutations with the epidermal growth factor receptor (EGFR) Scorpion Kit. (A) Standard E746_A750del DNA was used at various volumes: 10 000 pg (10^4), 1000 pg (10^3), 100 pg (10^2) and 10 pg (10^1). Standard wild-type DNA (Wild) and distilled 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.

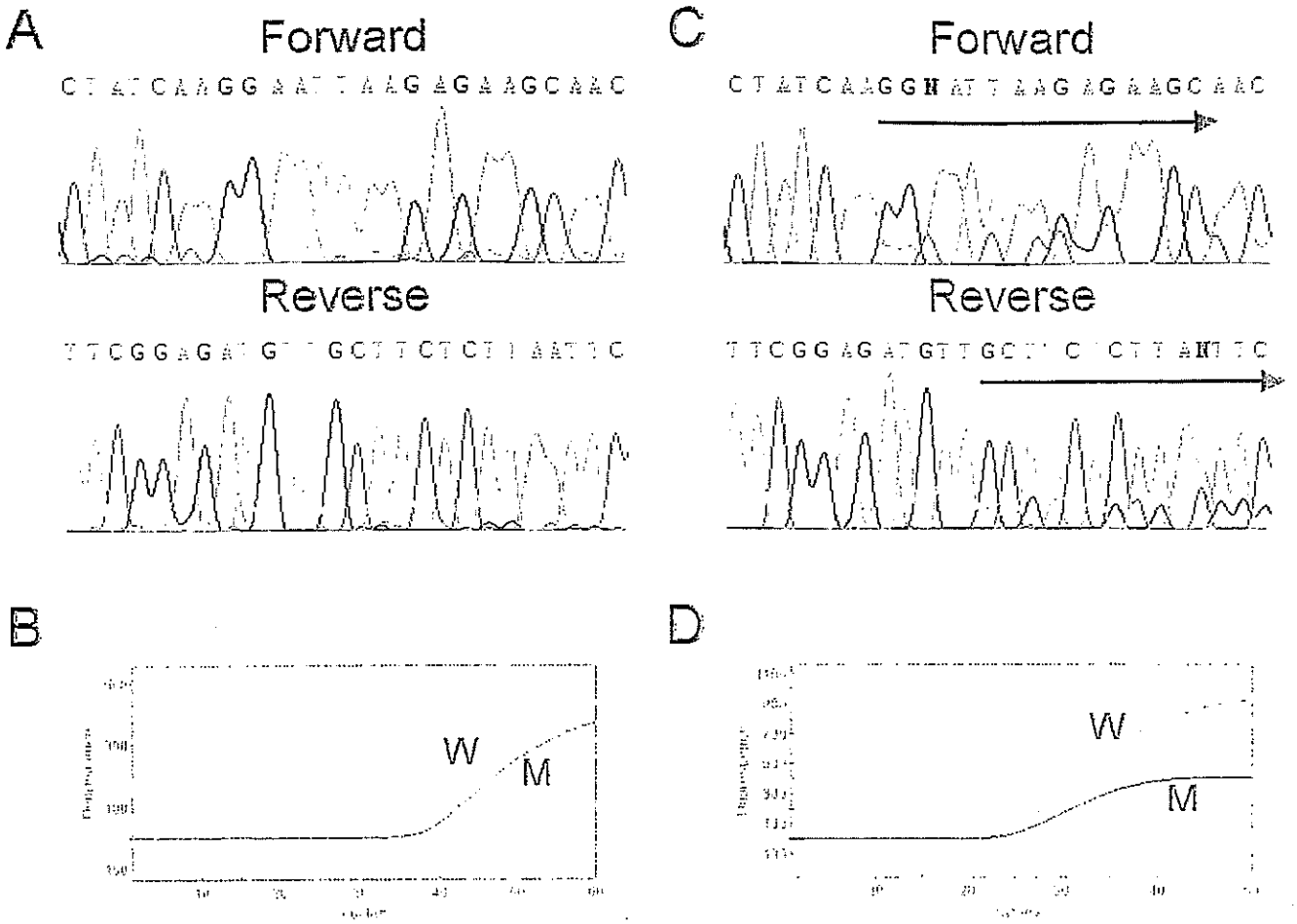

Fig. 2. Results of direct sequencing and the Scorpion Amplified Refractory Mutation System (ARMS) method in patient no. 10 (A,B) and patient no. 21 (C,D). (A) The wave figure represents wild-type *epidermal growth factor receptor (EGFR)*. (B) Two ascending curves, indicating that wild type and deletion 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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Detection of Epidermal Growth Factor Receptor Mutations in Serum as a Predictor of the Response to Gefitinib in Patients with Non–Small-Cell Lung Cancer

Hideharu Kimura,[1,4,5] Kazuo Kasahara,[5] Makoto Kawaishi,[1,2] Hideo Kunitoh,[2] Tomohide Tamura,[2] Brian Holloway,[6] and Kazuto Nishio[1,3,4]

Abstract Cases of non–small-cell lung cancer (NSCLC) carrying the somatic mutation of epidermal growth factor receptor (EGFR) have been shown to be hyperresponsive to the EGFR tyrosine kinase inhibitor gefitinib (IRESSA). If EGFR mutations can be observed in serum DNA, this could serve as a noninvasive source of information on the genotype of the original tumor cells that could influence treatment and the ability to predict patient response to gefitinib. Serum genomic DNA was obtained from Japanese patients with NSCLC before first-line gefitinib monotherapy. Scorpion Amplified Refractory Mutation System technology was used to detect EGFR mutations. Wild-type EGFR was detected in all of the 27 serum samples. EGFR mutations were detected in 13 of 27 (48.1%) patients and two major EGFR mutations were identified (E746_A750del and L858R). The EGFR mutations were seen significantly more frequently in patients with a partial response than in patients with stable disease or progressive disease ($P = 0.046$, Fisher's exact test). The median progression-free survival was significantly longer in patients with EGFR mutations than in patients without EGFR mutations (200 versus 46 days; $P = 0.005$, log-rank test). The median survival was 611 days in patients with EGFR mutations and 232 days in patients without EGFR mutations ($P > 0.05$). In pairs of tumor and serum samples obtained from 11 patients, the EGFR mutation status in the tumors was consistent with those in the serum of 8 of 11 (72.7%) of the paired samples. Thus, EGFR mutations were detectable using Scorpion Amplified Refractory Mutation System technology in serum DNA from patients with NSCLC. These results suggest that patients with EGFR mutations seem to have better outcomes with gefitinib treatment, in terms of progression-free survival, overall survival, and response, than those patients without EGFR mutations.

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 an appealing strategy for the treatment of non–small-cell lung cancer (NSCLC) as EGFR has been found to be expressed, sometimes strongly, in NSCLC tumors (2). Mutations of EGFR tyrosine kinase have been reported in NSCLC patients with dramatic responses to gefitinib (IRESSA), an EGFR tyrosine kinase inhibitor (3, 4). Studies have reported that EGFR mutations are strong determinants of tumor response to EGFR tyrosine kinase inhibitors (5–7). Approximately 30 mutations in exons 18 to 21 of EGFR were detected in a lung tumor specimen (3–8). The two most common NSCLC-associated EGFR mutations are the 15-bp nucleotide in-frame deletion in exon 19 (E746_A750del) and the point mutation replacing leucine with arginine at codon 858 in exon 21 (L858R; refs. 5, 8). These two mutations account for ~90% of all EGFR mutations and could explain the dramatic responders to gefitinib. Most EGFR mutations have been identified retrospectively from operative resected tumor samples. However, it is sometimes difficult to obtain tumor samples from patients with inoperable NSCLC in prospective studies; thus, it is necessary to establish a method to detect mutant EGFR, especially the two major mutations, from other more readily accessible patient samples.

Recently, PCR technology for the amplification of small amounts of DNA has made it possible to identify the same alterations, which are typically observed in DNA from resected or biopsied tumor cells, using serum samples from patients with various types of tumor, including NSCLC (9, 10). The detection of EGFR mutations in serum DNA may provide a noninvasive and repeatable source of genotypic information

Authors' Affiliations: [1]Shien-Lab, [2]Medical Oncology, National Cancer Center Hospital; [3]Pharmacology Division and [4]Center for Medical Genomics, National Cancer Center Research Institute, Tokyo, Japan; [5]Respiratory Medicine, Kanazawa University Hospital, Ishikawa, Japan; and [6]AstraZeneca, Alderley Park, Cheshire, United Kingdom

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Requests for reprints: Kazuto Nishio, Shien-Lab, Medical Oncology, National Cancer Center Hospital, Tsukiji 5-1-1, Chuo-ku, Tokyo, Japan. Phone: 81-3-3542-2511; Fax: 81-3-3542-1886; E-mail: knishio@gan2.res.ncc.go.jp.

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that could influence treatment and prognosis, especially in patients with NSCLC treated with gefitinib. However, it is well known that interfusion of normal cells with tumor cells prevents the detection of mutations in the tumor cells. Therefore, it is necessary to enhance the sensitivity of the detection of EGFR mutations from tumor-derived DNA mixed with normal cells.

Scorpion primers are used in a fluorescence-based method for the specific detection of PCR products (11). A Scorpion is a specific probe sequence that is held in a hairpin loop configuration by complementary stem sequences at the 5′ and 3′ ends of the probe. Scorpion can be used in combination with the Amplified Refractory Mutation System (ARMS) to enable the detection of single-base mutations (11, 12). ARMS technology is used for allele discrimination and additional mismatches are introduced near the 3′ terminus of the primers to enhance specificity. For the detection of known mutations, the Scorpion-ARMS method is highly sensitive and fast (13). Our hypothesis was that the ARMS and Scorpion methods could enhance the sensitivity of the detection of EGFR mutations from the wild type.

The aims of this study were to develop a highly sensitive assay for the detection of EGFR mutations in serum DNA, to compare the mutation status in serum to tumors from a subset of their patients, and to clarify the relationship between the EGFR mutation status in serum DNA and clinical manifestations, and in particular the responsiveness to gefitinib.

Materials and Methods

Patients and clinical trials. This study was carried out as a correlative study in a multicenter clinical phase II trial of gefitinib monotherapy at the Department of Respiratory Medicine, Kanzawa University Hospital; the Department of Internal Medicine, Kouseiren Takaoka Hospital; the Department of Internal Medicine, Shinminato Municipal Hospital; the Department of Internal Medicine, Fukuiken Saiseikai Hospital; the Department of Respiratory Medicine, Toyama City Hospital; the Department of Respiratory Medicine, Ishikawa Prefectoral Hospital; and the Department of Respiratory Medicine, Kanazawa Municipal Hospital. According to Simon's minimax design, our study, with a sample size of 25, had an 80% power to support the hypothesis that the true objective response rate was >30% and a 5% significance to deny the hypothesis that the true objective response rate was <10%. Assuming an inevaluability rate of <20%, we projected an accrual of 30 patients. The study was conducted with the approval of the appropriate ethical review boards based on the recommendations of the Declaration of Helsinki for biomedical research involving human subjects. Japanese patients with stage IIIB or IV histologically or cytologically proven chemotherapy-naïve NSCLC were enrolled in this trial. Gefitinib was orally given to all patients at a fixed dosage of 250 mg/d. Efficacy was assessed using the Response Evaluation Criteria in Solid Tumors guidelines (14). The analysis of the samples in this study was done blinded to the clinical outcome.

Blood sample collection and DNA extraction. Blood samples from the 27 patients with NSCLC were collected before the initiation of gefitinib administration. Separated serum was stocked at −80°C until use. Serum DNA was extracted and purified using a Qiamp Blood Kit (Qiagen, Hilden, Germany) with the following protocol modifications. One column was used repeatedly until the whole sample had been processed. The resulting DNA was eluted in 50 μL of sterile bidistilled buffer. The concentration and purity of the extracted DNA were determined by spectrophotometry. The extracted DNA was stocked at −20°C until use.

Scorpion ARMS primers for the detection of E746_A750del and L858R. We used an EGFR Scorpion Kit (DxS Ltd., Manchester, United Kingdom), which combined two technologies (i.e., ARMS and Scorpion) to detect mutations in real-time PCR reactions. Four kinds of scorpion primers for the detection of E746_A750del, L858R, and wild type in both exon 19 and exon 21 were designed and synthesized by DxS. The sequences of the scorpion primer for E746_A750del and L858R were based on the GenBank-archived human sequence for EGFR (accession no. AY588246). All reactions were done in 25-μL volumes using 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. All reagents are included in this kit. Real-time PCR was carried out using SmartCycler II (Cepheid, Sunnyvale, CA) under the following conditions: initial denaturation at 95°C for 10 minutes, 50 cycles of 95°C for 30 seconds, and 62°C for 60 seconds 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 done with Cepheid SmartCycler software (Ver. 1.2b). The cycle threshold (Ct) was defined as the cycle at the highest peak of the second derivative curve, which represented the point of maximum curvature of the growth curve. Both Ct and maximum fluorescence (Fl) were used for interpretation of the results. Positive results were defined as follows: Ct ≤45 and Fl ≥50. These analyses were done in duplicate for each sample and reviewed by two investigators blinded to any clinical information. To confirm the sensitivities for the detection of E746_A750del and L858R, we used the standard DNA that was included in the EGFR Scorpion Kit. Standard DNA with E746_A750del and L858R at a volume of 1, 10, 100, 1,000, or 10,000 pg and the mixture of standard DNA with wild type at 10,000 pg and standard DNA with E746_A750del and L858R at a volume of 1, 10, 100, 1,000 or 10,000 pg were used. For quantification, a standard curve was generated by plotting the cycle number of Ct against the log of the DNA volume of the known standards. The linear correlation coefficient (R^2) values and the formula of the slopes were calculated. DNA (10,000 pg) for the positive control was extracted from a Japanese human adenocarcinoma PC-9 cell line known to contain E746_A750del, a Japanese human adenocarcinoma 11_18 cell line known to contain L858R, and a human epidermoid carcinoma A431 cell line known to contain wild-type exon 19.

Tissue sample collection and DNA extraction. Tumor specimens were obtained on protocols approved by the Institutional Review Board. Twenty paraffin blocks of tumor material, obtained from 15 patients at the time of diagnoses (and before treatment), were collected retrospectively. Eleven tumor samples were collected from the primary cancer via transbronchial lung biopsy, one was resected intraoperatively, and nine were from metastatic sites (four from bone, three lymph nodes, one brain, and one colon). All specimens underwent histologic examination to confirm the diagnosis of NSCLC. DNA extraction from tumor samples was done using a DEXPAT kit (TaKaRa Biomedicals, Shiga, Japan).

PCR amplification and direct sequencing. Amplification and direct sequencing were done in duplicate for each sample obtained from serum and tissue specimens. PCR was done in 25-μL volumes using 15 μL of template DNA, 0.75 units of Ampli Taq Gold DNA polymerase (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, NJ), 2.5 μL of PCR buffer, 0.8 mmol/L deoxynucleotide triphosphate, 0.5 μmol/L of each primer, and different concentrations of $MgCl_2$, depending on the polymorphic marker. The sequences of primer sets and schedules of amplifications were followed as previously described (12). The amplification was done using a thermal cycler (Perkin-Elmer, Foster City, CA). Sequencing was done using an ABI prism 310 (Applied Biosystems, Foster City, CA). The sequences were compared with the GenBank-archived human sequence for EGFR (accession no. AY588246).

Statistical analysis. Fisher's exact test was used to assess the relationship between the presence of EGFR mutations in patients with NSCLC and different characteristics, including gender, tumor histology, and response to gefitinib. Regarding analyses of response to gefitinib, patients were categorized into the two groups: (*a*) partial response and (*b*) stable disease or progressive disease (Response Evaluation Criteria

in Solid Tumors criteria). We compared Kaplan-Meier curves for overall survival and progression-free survival using the standard log-rank test. Overall survival was defined as the time from the initiation of gefitinib administration to death from any cause; patients known to be still alive at the time of the analysis were censored at the time of their last follow-up. Progression-free survival was defined as the time from the initiation of gefitinib administration to first appearance of progressive disease or death from any cause; patients known to be alive and without progressive disease at the time of analysis were censored at the time of their last follow-up. $P = 0.05$ was considered statistically significant. The statistical analyses were done using the StatView software package version 5.0.

Results

Patients and extracted DNA from serum. Twenty-eight patients were enrolled between October 23, 2002 and August 3, 2003 (Table 1). All patients were evaluated for response and followed for progression-free survival and overall survival. Blood samples (2 mL) were collected from 27 of these patients before the initiation of gefitinib administration. These 27 patients represented a subset of that phase II study. Serum DNA was extracted in all 27 samples at a median concentration of 70.0 ng/mL (range, 0-1,720.0 ng/mL).

Sensitivity of the EGFR Scorpion. Preliminary experiments were done to evaluate the sensitivity of the EGFR Scorpion kit (Fig. 1A-C). All curves using E746_A750del and L858R standard DNA (volumes of 1-10,000 pg) increased 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 continued flat at 50 cycles (Fig. 1A and C). When diluted E746_A750del and L858R standard DNA were mixed with wild-type standard DNA at ratios from 10^0 to 10^{-5}, all curves that indicated the presence of E746_A750del and L858R increased up to 45 cycles (Fig. 1B and D). Standard curves in the range of measured volumes in this study were linear with r^2 values from 0.987 to 0.998. Both slopes of curves were almost parallel (Fig. 1E). The Ct of diluted mutant standard DNA mixed with wild-type DNA was close to that of mutant standard DNA alone. Although the peak fluorescence levels of diluted E746_A750del standard DNA mixed with wild-type DNA were lower than without wild-DNA standard, the presence of E746_A750del was clearly detected at ratios less than 10^{-4}. The peak fluorescence levels of diluted L858R standard DNA mixed with wild-type DNA were equivalent to those without wild-DNA standard. Curves of DNA with the mutations at an amount of up to 1 pg were unaffected by interfusion of DNA of wild-type EGFR. There were no significant differences between either the minimum detectable volume of the mutations or the minimum detectable ratio of wild type to the mutations.

In the cell-based experiments using genomic DNA of human cancer cell lines, the signal using DNA derived from the PC-9 cells was detected whereas the signal using DNA from the A431 cells was, as expected, not detected (Fig. 1D and E).

EGFR mutation status of serum DNA detected by EGFR scorpion. The E746_A750del or L858R status of serum DNA derived from 27 patients with NSCLC was examined. Wild-type exons 19 and 21 were detected from all serum samples. E746_A750del was detected in samples of 12 patients. L858R was detected in 1 patient (Table 2). In total, EGFR mutations were detected in 13 of 27 (48.1%) patients. The histologic subtypes of the original tumors are summarized in Table 3A in the 27 patients who were assessed for EGFR mutation in serum. Eleven of 23 (47.8%) cases of adenocarcinoma, one of two cases of squamous-cell carcinoma, and one of two cases of large-cell carcinoma were positive for EGFR mutations. An EGFR mutation was more frequently detected in the samples from female patients than those from males [7 of 10 (70%) versus 6 of 17 (35%); Table 3B].

EGFR mutation status in serum (EGFR Scorpion) and response to gefitinib. EGFR mutations were more frequently observed in the samples from the patients who showed a partial response (7 of 9 cases, 77.8%) than in samples from patients with stable disease or progressive disease (6 of 18 cases, 33.3%; $P = 0.046$, Fisher's exact test; Table 3C).

EGFR mutation status in serum (EGFR Scorpion) and effect on survival. Median progression-free survival and overall survival of all the patients treated with gefitinib were 98 and 306 days, respectively. Patients with EGFR mutations in serum showed a significantly longer median progression-free survival compared with the patients without EGFR mutations (200 versus 46 days, $P = 0.005$; Fig. 2A). The patients with EGFR mutations showed a longer median overall survival compared with the patients without EGFR mutations, although there was no statistical significance (611 versus 232 days, $P = 0.078$; Fig. 2B). These results suggest that patients who were serum EGFR mutation positive seem to have better outcomes with gefitinib treatment, in terms of progression-free survival, overall survival, and response, than those patients who were EGFR mutation negative.

EGFR mutation in serum analyzed by direct sequencing and in comparison with EGFR Scorpion. The deletional mutation (E746_A750del) was detected by direct sequencing in serum DNA extracted from 10 of 27 patients (37.0%). No point mutation in exons 18, 19, and 21 was detected in the PCR

Table 1. Patient characteristics

	(*n*)
No. patients	27
Age (y)	
Median	64
Range	44-87
Sex	
Male	17
Female	10
Performance status	
0	19
1	6
2	2
Stage	
IIIB	3
IV	24
Histology	
Adenocarcinoma	23
Squamous-cell carcinoma	2
Large-cell carcinoma	2
Response	
Partial response	9
Stable disease	8
Progressive disease	10

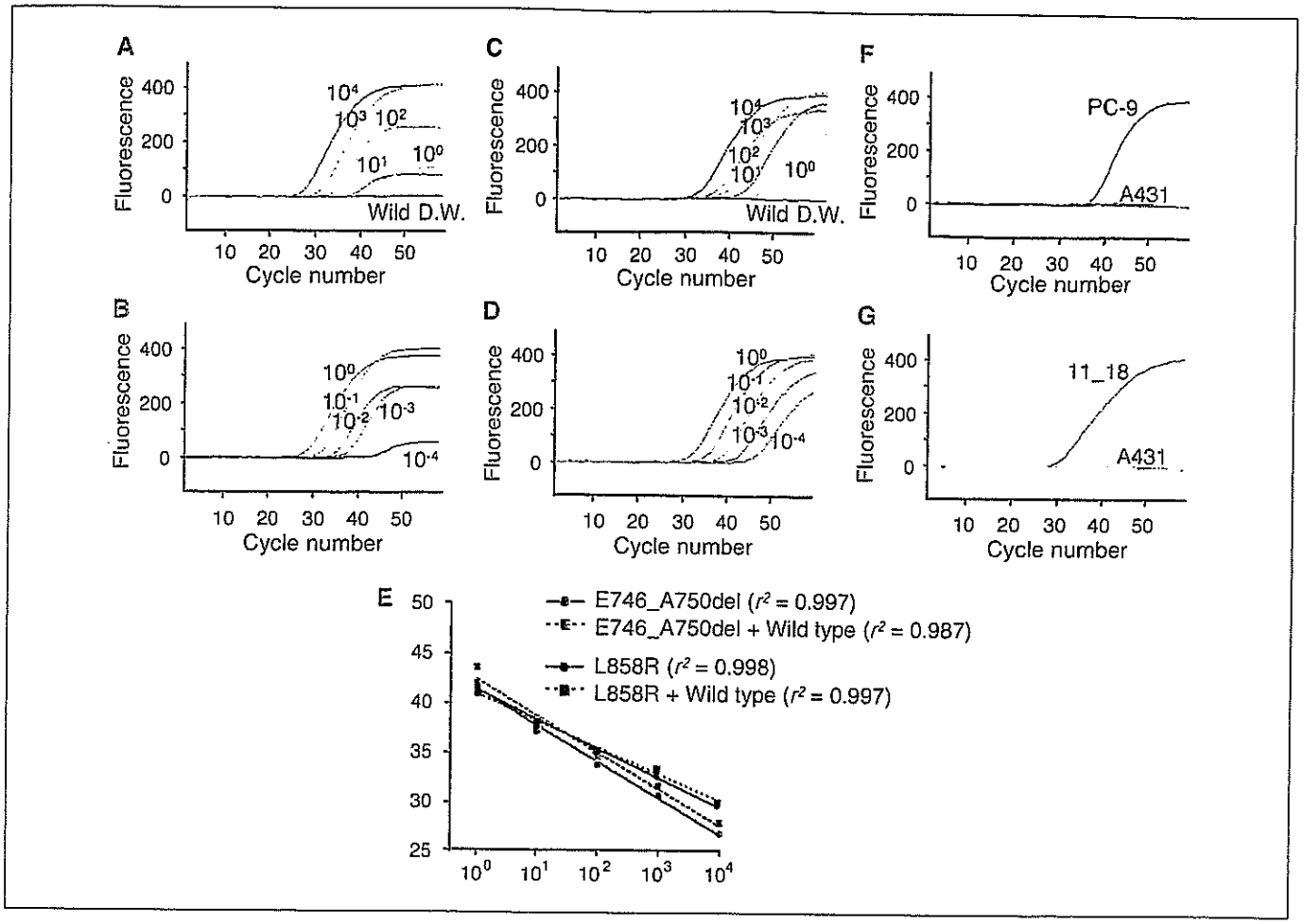

Fig. 1. Sensitivity of detection for mutations of E746_A750del and L858R using the EGFR Scorpion kit (*A* and *B*, E746_A750del; *C* and *D*, L858R). Standard DNA with E746_A750del (*A*) and L858R (*C*) were used at various volumes of 10,000 pg (10^4), 1,000 pg (10^3), 100 pg (10^2), 10 pg (10^1), and 1 pg (10^0). Standard DNA with wild-type (*Wild*) and distilled water (*D.W.*) were used as negative controls in the same experiment. Standard DNA with E746_A750del (*B*) and L858R (*D*) at concentrations from 1 to 10,000 pg were mixed with 10,000 pg of standard DNA with wild-type at a ratio of 1:1 (10^0), 1:10 (10^{-1}), 1:100 (10^{-2}), 1:1,000 (10^{-3}), and 1:10,000 (10^{-4}). *E*, standard curves were derived by plotting the Ct of each curve (shown in *A-D*) against the log of the standard DNA volume (*black lines*, E746_A750del; *blue lines*, L858R). *F*, PC-9 with E746_A750del and A431 with wild-type. *G*, 11_18 with L858R and A431.

products from serum samples. The serum EGFR status detected by direct sequence was not correlated statistically with histologic type, gender, response to gefitinib (Table 3), or survival (progression-free survival, P = 0.277; overall survival, P = 0.859). EGFR mutation status, as assessed by direct sequence, was consistent with those assessed by EGFR Scorpion in 15 of 27 (55.6%) of the paired samples. In four cases, EGFR mutation status (E746_A750del) was positive by direct sequence and negative by EGFR Scorpion. Eight cases were negative by direct sequence and positive by EGFR Scorpion. Thus, the sensitivity of EGFR Scorpion seems to be higher than that of direct sequencing due to the use of the specific primers for EGFR mutations in this kit.

EGFR mutations in tumors in comparison with those in serum. Twenty tumor samples were obtained from 15 patients retrospectively. Sequencing of EGFR exons 19 and 21 was done in samples from 12 of these under the same PCR conditions (Table 4; the other three samples were not evaluated because of low amplification of PCR products). EGFR mutations were detected in four cases (25.0%); three were the 15-bp deletion (E746_A750del) in exon 19 and one was the L858R point mutation in exon 21. Tumor histology of patients with EGFR mutations was adenocarcinoma in three and large-cell carcinoma in one. The responses to gefitinib in these four patients were two partial response, one stable disease, and one progressive disease.

Pairs of tumor samples and serum samples were obtained retrospectively from 11 patients (Table 4). The EGFR mutation status in the tumors was consistent with those in the serum of 8 of 11 (72.7%) of the paired samples. The E746_A750del mutation was positive in the tumor and negative in the serum in two patients, and the E746_A750del mutation was negative in the tumor and positive in the serum in one patient.

Discussion

Our findings have shown that EGFR mutations were detectable in serum samples obtained from patients with NSCLC and that the EGFR Scorpion kit consisting of ARMS and Scorpion technology is a useful method for detection of EGFR mutations. The EGFR mutation status in serum detected by the EGFR Scorpion was correlated statistically with responsiveness to, and the progression-free survival of, gefitinib treatment. Our finding supports the hypothesis that the EGFR

mutation status from serum DNA is useful to predict the responsiveness to gefitinib.

The mutation rate observed in our study seems to be relatively high (48%) although we have detected only two major mutations. EGFR mutations have been detected at a higher frequency in lung tumors from female patients, those with adenocarcinoma histology, nonsmokers, and patients of Asian origin (6, 8). However, previous reports show that the mutation rate of EGFR in operative samples of Japanese patients was from 26% to 59% (4, 6, 15, 16). The EGFR mutation rate in our study is equivalent to that observed in these reports. It can be speculated that the high sensitivity and specificity of the EGFR Scorpion allowed us to detect the EGFR mutations even in serum. Another possible reason is the high number of patients with adenocarcinoma in our study (23 of 27, 85.2%). Previous studies have shown that very few patients with nonadenocarcinoma, including squamous cell carcinomas and large-cell carcinomas, have EGFR mutations (3–8). Our results were in line with the previous studies and showed that no patients with squamous cell carcinoma or large-cell carcinoma had the mutations.

Table 2. Patients' characteristics and EGFR mutant status detected from serum DNA using the EGFR ARMS-Scorpion method

Response	Gender	Histology	Exon 19		Exon 21	
			Wild	E746_A750del	Wild	L858R
PR	M	Ad	+	–	+	+
PR	F	Ad	+	+	+	–
PR	M	Ad	+	–	+	–
PR	F	Ad	+	+	+	–
PR	M	Ad	+	+	+	–
PR	F	Ad	+	–	+	–
PR	M	Ad	+	+	+	–
PR	F	Ad	+	+	+	–
PR	F	Ad	+	+	+	–
SD	M	Large	+	–	+	–
SD	F	Ad	+	+	+	–
SD	M	Ad	+	–	+	–
SD	F	Ad	+	–	+	–
SD	F	Ad	+	+	+	–
SD	M	Ad	+	–	+	–
SD	F	Ad	+	+	+	–
SD	M	Scc	+	+	+	–
PD	F	Scc	+	–	+	–
PD	M	Ad	+	–	+	–
PD	M	Ad	+	–	+	–
PD	M	Large	+	+	+	–
PD	M	Ad	+	–	+	–
PD	M	Ad	+	–	+	–
PD	M	Ad	+	–	+	–
PD	M	Ad	+	–	+	–
PD	M	Ad	+	+	+	–
PD	M	Ad	+	–	+	–

Abbreviations: SD, stable disease; PD, progressive disease; PR, partial response; M, male; F, female; Ad, adenocarcinoma; Large, large-cell carcinoma; Scc, squamous-cell carcinoma; +, curve detected by SmartCycler; –, curve not detected by SmartCycler.

Table 3. Frequency of EGFR mutations in serum DNA from patients with NSCLC according to histology (A), gender (B), and response to gefitinib (C)

	EGFR Scorpion kit			Direct sequence		
	+	–		+	–	
(A) Histology and EGFR mutant states						
Ad	11	12		8	15	
Non-Ad	2	2	$P > 0.999$	2	2	$P > 0.999$
(B) Gender and EGFR mutant states						
Female	7	3		5	5	
Male	6	11	$P = 0.120$	5	12	$P = 0.415$
(C) Response to gefitinib and EGFR mutant states						
PR	7	2		4	5	
SD/PD	6	12	$P = 0.046$	6	12	$P = 0.683$

NOTE: A total of 27 samples were obtained from 28 patients before treatment.

We identified 12 deletion mutations and a single point mutation (L858R). Previous reports have shown that the frequency of detection of E746_A750del is almost equivalent to that of L858R (15, 16). It seems that the rate of detection of L858R in our study was very low compared with the rate of E746_A750del. The sensitivity for detection of L858R using the Scorpion ARMS method is very high and equivalent to that of E746_A750del. We thus consider that it is unlikely that the low-frequency L858R mutation could be due to assay-related false-negative findings. On the other hand, it also seems unlikely that either sampling method or the patients' eligibility criteria are biased toward the high rate of E746_A750del. Therefore, we have not been able to clarify the moot point. Further analyses in much larger groups of patients will be necessary to clarify the frequency of the major two mutations in serum DNA. Unfortunately, parallel tumor tissue investigations were done only on a small subset of the participating patients. Furthermore, findings in the serum were divergent from those obtained from the primary tissue in 3 of 11 patients from whom the paired samples were obtained. Therefore, this study is at best hypothesis-forming and will require follow-up analysis in much larger groups of patients.

Some investigators reported that mutations in the EGFR tyrosine kinase domain enhanced responsiveness to the EGFR tyrosine kinase inhibitors gefitinib and erlotinib, and seemed to be associated with the prolonged survival of the patients who received these drugs (7, 8). In a placebo controlled study showing a survival advantage for NSCLC patients who received erlotinib, Tsao et al. (17) showed that the presence of an EGFR mutation might increase responsiveness to erlotinib, but was not indicative of a survival benefit, and concluded that EGFR mutation analysis was not necessary to identify patients in whom treatment with EGFR inhibitors was appropriate. Our results are not in line with their conclusions. In their study, the rate of mutation analysis was low and 107 of 731 patients

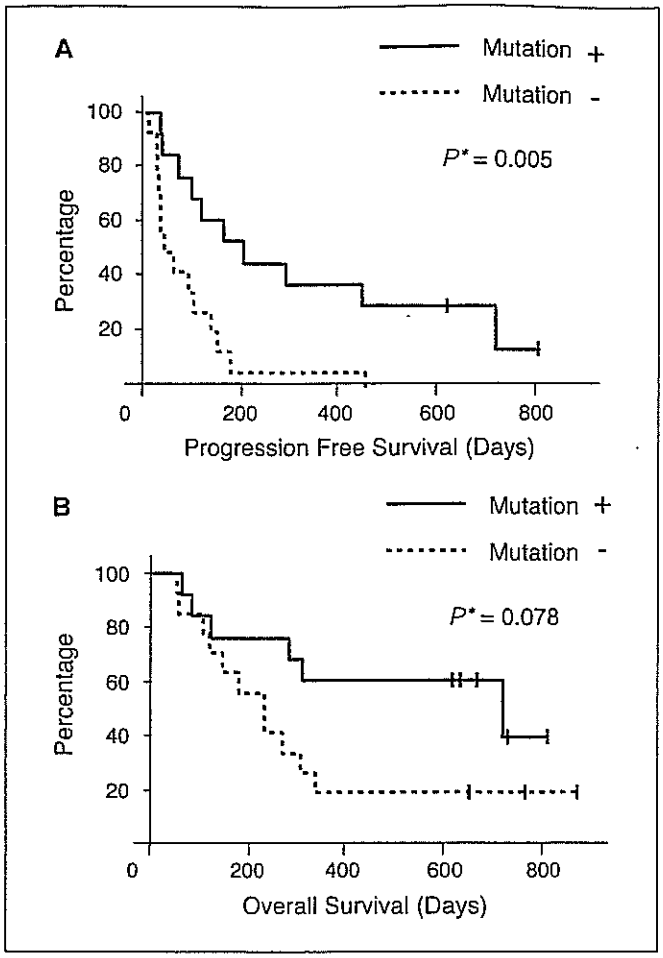

Fig. 2. Progression-free survival (*A*) and overall survival (*B*) with respect to the EGFR mutation status of NSCLC. *, log-rank test.

enrolled in their study were successfully analyzed for EGFR mutation. Sensitivity for detecting EGFR mutation in their study might be unstable as interfusion of normal cells in tumor cells decreases the sensitivity for detecting tumor-derived mutations using direct sequencing. They propose that additional processes (such as microdissection) to enrich tumor cell DNA might increase the rate of detection of new mutations; however, it seems that their results are insufficiently robust to reach this conclusion. Therefore, we propose the use of EGFR mutation analysis from serum DNA, which is easily collected and repeatable, to show that EGFR mutation status using the EGFR Scorpion kit correlates with the responsiveness to gefitinib.

EGFR mutation in NSCLC is reported to be somatic (3, 4). It is well known that the concentration of free circulating DNA in serum is higher in patients with tumors than in healthy volunteers (18) and it seems that the detected mutational EGFR in serum was tumor derived. This is the first report analyzing EGFR mutations from serum DNA and evaluating EGFR mutation status and clinical outcome (response and survival) with gefitinib. No other studies have analyzed EGFR mutations from samples other than actual tumor samples. The mutation in two patients was positive in the tumor and negative in the serum, and the mutation in one patient was negative in the tumor and positive in the serum. We have tried to explain the discrepancy why tumor and serum were not better correlated as follows. In cases of positive in the tumor and negative in the serum, the volumes of mutant DNA extracted from the serum were under the detectable limit using the Scorpion ARMS method, or a very small amount of DNA derived from an actual tumor was circulating in the bloodstream. A previous study showed that 73% of patients with at least one molecular event, such as a hypermethylation of the tumor suppressor gene *p16*, in their tumor DNA had the same alteration in plasma DNA (10). In a case of negative in the tumor and positive in the serum, wild-type DNA interfered with

Table 4. EGFR mutation status in tumor samples and serum samples. Pairs of both tumor samples and serum samples were obtained from 11 patients

Gender	Histology	Response	EGFR mutation status				
			Tumor sample	EGFR Scorpion kit (serum sample)			
				Exon 19		Exon 21	
				Wild	Mutation	Wild	Mutation
M	Large	SD	Wild	+	–	+	–
F	Scc	PD	Wild	+	–	+	–
M	Ad	PD	Wild	+	–	+	–
M	Ad	PR	L858R	+	–	+	+
F	Ad	SD	Wild*	+	+	+	–
M	Large	PD	E746-A750del	+	+	+	–
M	Ad	PD	Wild	+	–	+	–
M	Ad	PD	Wild	+	–	+	–
M	Ad	SD	E746-A750del*	+	–	+	–
F	Ad	PR	E746-A750del*	+	–	+	–
M	Ad	PD	Wild	+	–	+	–

*Patients who have different states of EGFR mutation from tumor-derived DNA and serum-derived DNA.

the detection of mutant DNA in the tumor samples using the direct sequencing method. The rate of the mutations in serum DNA detected by the Scorpion ARMS was compared with that in tumor tissues detected by the direct sequencing method as a current standard method. DNA from tumor samples consisted of a mixture of the mutant DNA and wild-type DNA because the EGFR mutation status was always heterogeneous, and the complete removal of normal cells, such as normal epithelial cells and inflammatory cells, from tumor specimens is very difficult. Parallel tumor tissue investigations were done on only a small subset of these patients, which is a recognized limitation in the present study. A larger study is necessary to evaluate the consistency of the mutation status from tumor and serum. On the other hand, it is sometimes difficult to obtain tumor samples from patients with inoperable NSCLC in prospective studies. We showed that patients who were EGFR mutation positive in the serum DNA using the Scorpion ARMS method seem to have better outcomes with gefitinib treatment in terms of progression-free survival, overall survival, and response, despite the nonconformity between the mutation states of tumor and serum DNA in some of the patients. We anticipate that the detection of EGFR mutations in serum DNA using the Scorpion ARMS will be equivalently useful as a feasible approach for predicting tumor response to gefitinib.

Two groups have reported alternative methods for detection of EGFR mutations. One group used the LightCycler PCR assay (19) and the other postulated that the SSCP assay was more sensitive than direct sequencing and was a rapid method (20). Further studies are needed to validate these assays for detection of EGFR mutations and to clarify the most sensitive assay. Although the direct sequence method is common in reported studies, the EGFR mutation status in serum DNA by direct sequencing did not correlate with the responsiveness to and survival benefit of gefitinib in our study. These results indicate that the EGFR Scorpion kit is superior to the direct sequencing method for detection of an EGFR mutation in serum as a predictive marker.

One limitation of the EGFR Scorpion kit is that it is only able to detect mutations targeted by the designed Scorpion primers. EGFR mutations are not solely at these two sites but clustered around the ATP-binding site in exons 18, 19, and 21 (3–8). Moreover, the 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 treatment with gefitinib (21–23). Mutations in *K-ras*, a known downstream signaling molecule in the EGFR signaling pathway, are more frequent in patients who develop disease progression with treatment with either gefitinib or erlotinib (24). These mutation states may also be critical factors for the treatment of gefitinib. To clarify the usefulness of serum DNA as a source of genotypic information, the Scorpion primers need to be designed for detection of these mutations, and further studies using these primers are required.

In conclusion, the two major mutations of EGFR, E746_A750del and L858R, were detected in serum DNA with the EGFR Scorpion kit from patients with NSCLC. These results suggest that patients who were EGFR mutation positive seem to have better outcomes with gefitinib treatment, in terms of progression-free survival, overall survival, and response, than those patients who were EGFR mutation negative. In the near future, a controlled clinical trial is necessary to confirm these conclusions.

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ORIGINAL ARTICLE

Yasuhide Yamada · Tomohide Tamura
Noboru Yamamoto · Tatsu Shimoyama
Yutaka Ueda · Haruyasu Murakami · Hitoshi Kusaba
Yoshikazu Kamiya · Hideo Saka · Yusuke Tanigawara
J. Patrick McGovren · Yutaka Natsumeda

Phase I and pharmacokinetic study of edotecarin, a novel topoisomerase I inhibitor, administered once every 3 weeks in patients with solid tumors

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Abstract *Purpose*: Edotecarin (J-107088) is a potent indolocarbazole topoisomerase I inhibitor which is structurally distinct from the camptothecins. This study aimed to determine the maximum tolerated dose (MTD), the recommended dose for future Phase II studies and the safety, pharmacokinetic profile, and preliminary antitumor activity of edotecarin in a population of patients with advanced solid tumors. *Experimental design*: Edotecarin was administered as a single dose by IV infusion over 2 h every 21 days (with 1 week permitted for recovery from toxicities, if needed) in patients with advanced solid tumors. Doses ranged from 8 to 15 mg/m^2. Pharmacokinetic assessments were performed during and after the first administration. *Results*: Twenty-four patients received 61 cycles of therapy. Dose-limiting toxicities (infection, febrile neutropenia, constipation, ileus, and prolonged grade 4 granulocytopenia) were observed in 3 of 5 evaluable patients at the 15 mg/m^2 dose, defining the MTD. The most commonly reported non-hematologic toxicities were anorexia, nausea, malaise, and constipation. Diarrhea was neither frequent nor severe. Neutropenia was the most common hematologic toxicity (grade 3–4 in 21/23 patients during cycle 1). Plasma concentrations of edotecarin rose rapidly following the start of the 2-hour infusion, reaching C_{max} values of 103 ± 17 ng/ml at the 13 mg/m^2 dose, and decreased steeply after the end of the infusion. Plasma concentrations declined to approximately 1–2 ng/ml at 26 h post start of infusion, the last PK sampling time point. The mean apparent plasma half-life of the drug was 20 h, which should be considered a preliminary estimate until results from studies with a longer duration of plasma sampling are available. A mean of 1.4–3.6% of the dose was recovered as unchanged drug in the urine over 48 h. Unconfirmed tumor regression ≥50% was observed in 2 patients, 1 with metastatic gastric carcinoma and 1 with esophageal cancer. *Conclusions*: The MTD of edotecarin administered IV over 2 h every 21 days was 15 mg/m^2. The recommended dose for Phase II studies with a 3-week schedule (with 1 week permitted for recovery from toxicities, if needed) is 13 mg/m^2. The observed safety profile and preliminary evidence of antitumor activity warrant further investigation of this drug in solid tumors.

Keywords Edotecarin · Pharmacokinetics · Maximum tolerated dose · Dose-limiting toxicity · Topoisomerase I inhibitor

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Y. Yamada (✉) · T. Tamura · N. Yamamoto · T. Shimoyama
Y. Ueda · H. Murakami · H. Kusaba
Department of Medical Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, 104-0045 Tokyo, Chuo-ku, Japan
E-mail: yayamada@ncc.go.jp
Tel.: +81-3-35422511
Fax: +81-3-35423815

Y. Kamiya · H. Saka
Department of Respiratory Medicine and Clinical Research Center, National Hospital Organization, Nagoya Medical Center, Tokyo, Japan

Y. Tanigawara
Department of Hospital Pharmacy, School of Medicine, Keio University, Tokyo, Japan

J. P. McGovren
Pfizer Corporation, New York, NY, USA

Y. Natsumeda
Banyu Pharmaceuticals Co. Ltd, Tokyo, Japan

Introduction

DNA damage mediated by topoisomerase I (topo I) inhibitors is an important mechanism of antineoplastic activity [1]. During DNA replication, topo I relieves torsional strain by causing a reversible single-strand

break in the DNA [2]. Topo I binds to DNA at the break site to form a cleavable complex. Topo I inhibitory drugs bind to the topo I-DNA complex, stabilizing it and preventing the religation of the single-strand breaks. Cell death appears to be due to double-strand DNA damage that occurs during DNA synthesis when replication enzymes interact with the stabilized cleavable complex. Currently in the USA, two topo I inhibitors, both camptothecin derivatives, are available for the treatment of cancer patients: topotecan, approved for ovarian carcinoma and small cell lung cancer indications, and irinotecan, approved for metastatic colorectal carcinoma [3–6]. The remarkable anticancer activity of the campothecins and the possibility that other topo I inhibitors could exhibit different activity, better tolerability, or a more favorable pharmacokinetic profile have led to the search for new topo I inhibitors.

Edotecarin (J-107088) is a new derivative of NB-506, an indolocarbazole antitumor agent with a chemical structure completely different from the camptothecins (Fig. 1) [7]. Like camptothecin and its derivatives, edotecarin inhibits topo I [7, 8], but because of the distinct structure of this agent, its interaction with the target enzyme differs significantly from the camptothecin derivatives [9]. Edotecarin is a more potent inhibitor of topo I than camptothecin. The cleavable complex formed with edotecarin is more stable than that with camptothecin and persists significantly longer after removal of drug from cell culture medium [7]. The activity of edotecarin does not appear to be cell-cycle dependent. In vitro edotecarin has demonstrated a wide spectrum of activity against human cancer cell lines [7] and is active in vivo against a variety of human and murine tumor-derived xenografts as well as experimental liver metastases [10, 11]. Preclinical studies in animal species have shown that edotecarin is largely eliminated as unchanged parent drug via biliary excretion (unpublished Banyu data), in marked contrast to the camptothecin analog, irinotecan, which is characterized by a very complex disposition and metabolic pathways. In vitro studies showed that edotecarin was not metabolized by liver microsomes or by hepatocytes from humans or several animal species (unpublished Banyu data). The unique in vitro and in vivo pharmacological profile of edotecarin relative to other topo I inhibitors makes this compound a potentially useful antineoplastic agent.

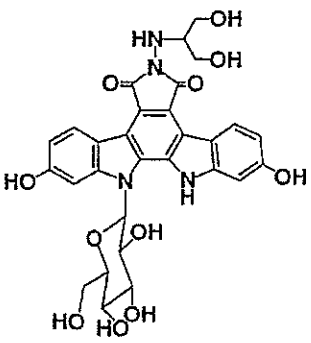

Fig. 1 Structure of edotecarin, an indolocarbazole. Molecular weight: 608.56 Da

When this study was initiated in Japan, edotecarin had been previously investigated in 2 Phase I studies in the USA. In the first [12], edotecarin was administered as a 2 h IV infusion once every 21 days (with an additional week permitted for recovery to ≥grade 1 toxicity, if necessary) at doses of 6, 8, 11, 13 and 15 mg/m^2. Nausea, vomiting, headache, fatigue, febrile neutropenia and neutropenia were dose-limiting at 15 mg/m^2. Treatment could be administered repeatedly for 2 or more courses in 24 of 29 patients (83%). One patient with metastatic bladder cancer had a confirmed partial response of long duration and 12 patients showed stabilization of disease. In the second study [13], edotecarin was administered as a 1 h IV infusion twice-weekly (on days 1, 4, 8, and 11) in cycles of at least 28 days at doses of 2, 4, 5.5, and 7.5 mg/m^2/day. Mucositis, neutropenia and thrombocytopenia were dose-limiting at 7.5 mg/m^2/day. Only two patients received treatment beyond cycle 2.

Based on these results, this Phase I study was designed to evaluate ascending doses of edotecarin administered by 2 h IV infusion every 21 days. A more sensitive assay was utilized in this study compared to the earlier study, of similar design, conducted in the USA. Objectives of the study were to determine the MTD (where the MTD is the maximum dose administered) and the dose to be recommended in future Phase II studies, and to assess its safety, pharmacokinetic profile, and the preliminary antitumor activity in a population of Japanese patients with advanced solid tumors.

Patients and methods

Patients

Japanese patients with histologically or cytologically confirmed evaluable malignant solid tumors refractory to conventional chemotherapy or tumors for which no effective therapy existed were candidates for this study. Inclusion criteria also included the following: age ≥20 years; Eastern Cooperative Oncology Group (ECOG) performance status 0, 1, or 2; life expectancy ≥12 weeks; absolute granulocyte count ≥1,500/mm^3, platelet count ≥100,000/mm^3, hemoglobin ≥9 g/dl, and serum creatinine < 1.5 mg/dl. Additional entry criteria were serum total bilirubin within the normal limit and serum AST, ALT, and alkaline phosphatase less than twice the upper limit of normal. A 4-week interval was required for chemotherapy, radiation therapy, or immunotherapy treatments (6-week interval for patients previously treated with mitomycin C or nitrosoureas). A 2-week interval after major surgery was required.