recommended dose was determined after an overall review of the results obtained for the following: status of manifestation of DLT in cycle 1; status of manifestation and disappearance of toxicity in cycle 2 and subsequent cycles; frequency and nature of treatment delay/discontinuation; pharmacokinetics; and antitumor effect. Tumor responses were evaluated according to RECIST (response evaluation criteria in solid tumors) criteria.

PHARMACOKINETICS

Pharmacokinetic (PK) evaluation was performed in all patients during the initial cycle of treatment, and in patients who could be administered repeatedly during the second cycle of treatment. Venous blood samples (5 ml, anticoagulant: EDTA) were taken before dosing, at the end of infusion and 1, 4, 8, 24, 34, 48, 96, 168 and 240 h after completion of infusion, and then before dosing, at the end of infusion in the second cycle. Blood samples were immediately placed in ice water and centrifuged at 4°C, 1000×g for 10 min, and plasma was aliquoted and stored at −20°C or below in polyethylene tubes until analysis.

The concentrations of total (encapsulated plus non-encapsulated) doxorubicin and its major metabolite doxorubicinol in plasma were measured by validated reverse-phase high-performance liquid chromatography (HPLC) with fluorescence detection (excitation wavelength: 480 nm and emission wavelength: 560 nm) which is a modification of the measurement method previously reported (18).

The PK parameters (C_{max}, maximum plasma concentration; $t_{1/2}$, elimination half-life; AUC, area under the concentration–time curve; V_c, volume of distribution; CL, total clearance) were calculated by non-compartmental analysis using WinNonlin™ (Pharsight) software.

In addition, an assessment was made of the correlation between C_{max} and AUC with the dose (mg/m^2) of liposomal doxorubicin administered. Moreover, the presence or absence of accumulation was verified by comparing the individual plasma concentrations of doxorubicin and doxorubicinol determined before dosing and at the end of infusion in the second cycle with the corresponding measured values in the initial cycle.

RESULTS

Patients' Characteristics

From April 2003 to January 2004, 15 patients were entered in this study. Their characteristics are listed in Table 1. There were five men and 10 women with good performance status and the median age was 56 (range, 49–69) years. The predominant types of tumor were ovarian cancer and non-small cell lung cancer. Seven patients had received surgical resection for primary tumors, all 15 patients had received prior chemotherapy and 11 had more than three prior regimens. Two patients had received anthracycline; one at a cumulative dose of 273 mg/m^2 and the other at 100 mg/m^2. A total of 67 cycles of PLD was administered, and the median number of cycles administered per patient was three (range, 1–15). All patients were included in the toxicity evaluation.

Table 1. Patients' characteristics

	Number of patients
Total number of patients	15
Male/female	5/10
Age (years)	
Median	56
Range	49–69
ECOG* performance status	
0	5
1	10
Primary cancer	
Ovary	6
Non-small cell lung	6
Breast	1
Esophagus	1
Thymic cancer	1
Prior treatment	
Surgery	7
Chemotherapy	15
Radiation	3
Number of prior chemotherapy regimens	
1	3
2	4
≥3	8

*Eastern Cooperative Oncology Group.

Toxicity

The major toxicities in the first cycle and all cycles are listed in Table 2. The principal non-hematological toxicities were skin toxicities consisting of HFS and skin rash, and stomatitis.

HFS and rash as major skin toxicities occurred in 12 (80.0%) and 10 (66.7%) patients, respectively. These toxicities were generally mild (≤grade 2, Table 2) with clinical symptoms including erythema, swelling, itching, pain and desquamation. The median time to onset of HFS and grade 2 HFS were 39 days (cycle 2) and 96 days (cycle 3.5) after treatment initiation, and the median duration of grade 2 HFS was 7 days. The median time to onset of rash and grade 2 rash were 29 days (cycle 1.5) and 64.5 days (cycle 2.5) after treatment initiation and the median duration of grade 2 rash was 5 days. These skin toxicities increased in

Table 2. Major toxicities

Dose (mg/m^2)	No. of patients	CTC grade																					
		HFS			Rash			Stomatis			Nausea			Anorexia			Leu			Neu			Anemia		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1st cycle																									
30	6	–	–	–	1	1	–	–	2	–	3	–	–	2	1	–	3	2	–	2	1	–	3	2	–
40	3	1	1	–	2	–	–	1	–	–	1	–	–	1	–	–	–	2	–	–	1	1	1	–	–
50	6	–	–	–	–	1	–	2	2	–	2	–	–	2	–	–	2	3	–	–	2	3	3	–	–
All cycles																									
30	6	1	2	–	2	1	–	1	2	–	4	–	–	4	1	–	2	3	–	1	2	–	3	2	–
40	3	1	2	–	1	1	–	–	1	–	2	–	–	1	–	–	–	2	–	–	1	1	1	–	–
50	6	4	2	–	1	4	–	2	2	–	2	–	–	2	–	–	2	3	1	–	2	3	1	3	–

HFS, hand–foot syndrome; Leu, leukemia; Neu, neutropenia; anemia, hemoglobin decrease.

frequency and severity at high dose or with multiple doses of PLD. In level 1 and 3 cohorts, treatment delays owing to skin toxicities were observed in six of 29 cycles and 10 of 32 cycles, respectively. However, these skin toxicities were manageable by delay of the next infusion and commonly used dermatologic medications including vitamin B_2, B_6 tablets, antihistamine and steroid tablets/ointment.

Stomatitis was observed in eight patients (53.3%) and was generally mild (≤grade 2, Table 2). The median times to onset of stomatitis and grade 2 stomatitis were 15 days (cycle 1) and 17 days (cycle 1) after treatment initiation, and the duration of grade 2 stomatitis was 7 days. This toxicity tended to occur after cycle 1, but resolved relatively promptly.

The principal hematological toxicities were leukopenia and neutropenia, and there was only one patient with grade 3 leukopenia in level 3 and there were 4 patients with grade 3 neutropenia in level 2 and 3 (1 and 3 patients, respectively, Table 2). The nadir time to leukopenia and neutropenia was approximately 3 weeks after treatment initiation. Although leukopenia and neutropenia increased in severity at high dose (50 mg/m^2) compared with at low dose (30 mg/m^2), they were manageable with just delay of subsequent treatment. No patient developed neutropenic fever, thrombocytopenia or grade 4 hematological toxicities in any dose levels. No patient required administration of granulocyte colony-stimulating factor or blood transfusion.

The left ventricular ejection fraction (LVEF) was determined at baseline and serially by heart ultrasonography. There was one patient each with grade 1 LVEF decrease after the administration of PLD cumulative dose of 40 and 100 mg/m^2, respectively. Seven patients developed cardiotoxicity that was reported as an adverse event (Table 3). All of them were grade 1. One patient, who had received 100 mg/m^2 anthracycline as previous treatment, experienced supraventricular arrhythmia, AV block and sinus arrhythmia after the administration of a PLD cumulative dose of 150 mg/m^2 (total doxorubicin dose of 250 mg/m^2). No patient required treatment for cardiotoxicity.

Table 3. Cardiotoxicities

	30 mg/m^2 (n = 6)	40 mg/m^2 (n = 3)	50 mg/m^2 (n = 6)
Cardiac disorder	2	2	3
Supraventricular arrhythmia	0	0	3
AV block	0	0	1
Myocardial	0	1	0
Palpitation	1	1	0
Pericardial effusion	1	1	0
Sinus arrhythmia	0	0	1
Ventricular	0	1	1

Grade 1 or 2 infusion reactions developed in 4 patients and they appeared within 10 min after initiation of infusion. All symptoms caused by infusion reaction disappeared within 60 min without any medication, interruption of infusion or infusion rate adjustment.

Three DLTs were recognized in one patient administered 30 mg/m^2 of PLD with grade 3 diarrhea, grade 3 infection not accompanied by neutropenia, and grade 3 hypoxia. Diarrhea and infection were recovered and improved at the end of the observation period, respectively, while hypoxia lasted. There was no DLT at the level of 40 or 50 mg/m^2. There were no treatment-related deaths in this study.

Antitumor Activity

All of 15 patients were evaluable for antitumor response. One and eight out of 15 evaluable patients had achieved

partial response and stable disease, respectively. The patient who achieved partial response (PR) was a 53-year-old female diagnosed as ovarian cancer with three lesions in peritoneum and one instance of pelvic lymph node metastasis. The duration of response was 441 days. In the case of the other patient with ovarian cancer who was evaluated as not evaluable (NE), the elevated tumor marker CA125 (241 U/ml) prior to the study entry was normalized (11 U/ml) after the second cycle of PLD.

PHARMACOKINETICS

Pharmacokinetic evaluation was performed using plasma samples obtained from all 15 patients during the initial cycle of treatment, and for 11 patients during the second cycle of treatment. Pharmacokinetic parameters are summarized in Table 4 and the mean plasma doxorubicin concentration–time profiles are illustrated in Fig. 1. Plasma doxorubicin concentrations after administration of PLD showed a monophasic decline, consistent with a one-compartment model. Total doxorubicin exhibited a long $t_{1/2}$ (range of mean values: 86.3–95.3 h), slow clearance (range of mean values: 11.0–13.1 ml/h/m^2), and small volume of distribution (range of mean values: 1.47–1.57 l/m^2) that was similar to the plasma volume.

The plasma C_{max} and AUC values increased proportionally with the dose of PLD ($P < 0.0001$ respectively, Fig. 2), suggesting linear pharmacokinetics in this dose range. Moreover, PLD did not significantly accumulate in plasma when administered at intervals of 4 weeks or longer. Plasma concentrations of doxorubicinol, the major metabolite of doxorubicin, were lower than the lower limit of quantitation in most samples (data not shown).

DISCUSSION

We report a phase 1 study of pegylated liposomal doxorubicin (PLD) given every 4 weeks in Japanese patients with solid tumors. The major non-hematological toxicities were HFS, rash and stomatitis. Myelosuppression especially, leukopenia and neutropenia were the most common hematological toxicities. HFS is rarely seen with standard doses of conventional doxorubicin and other liposomal anthracycline agents (19–21). In our study, grade 3 or higher skin toxicities were not observed but it was indicated that they increased in frequency and severity by multiple administration of PLD. These skin toxicities were manageable by delay of next infusion and commonly used dermatologic medications. Lyass et al. reported that severity of HFS was correlated with $t_{1/2}$ of PLD ($P = 0.0083$), and prevention of recurrence of HFS was best achieved by delay of the next infusion (22). The effect of dose interval on skin toxicity may be related to the turnover time of keratinocytes and epidermal transit time that are in the order of 3–4 weeks (23). Thus, prevention of recurrence of skin toxicity seems to be best achieved by delay of the next infusion because of allowing adequate time for recovering of keratinocytes.

The severity of stomatitis and the nadir leukocyte count were reported as correlated with dose level and C_{max} of PLD. In our study, these toxicities observed in Japanese patients tended to increase in severity along with dose escalation. As the results of our PK analysis revealed that C_{max} increased linearly with dose, it can be suggested that the toxicity profile observed in Japanese patients is similar to that reported by Lyass et al. (22). Prevention of increase in severity of these toxicities seems to be best achieved by dose reduction.

Cardiotoxicities observed in Japanese patients were all grade 1 in our study. The most serious toxicity of conventional doxorubicin therapy is cumulative-dose-related cardiotoxicity (24). Although no retrospective and prospective studies have identified a maximum 'cardiac safe' cumulative dose of PLD which may induce chronic heart failure, the result of a recent direct comparison study conducted in patients with metastatic breast cancer between PLD and conventional doxorubicin therapy showed that the risk of cardiotoxicity with PLD was significantly lower than that with conventional doxorubicin (21). Our study result and previous clinical studies suggest that PLD can be used in place of conventional doxorubicin to reduce the risk of cardiotoxicity without reducing the efficacy of therapy.

All infusion reactions appeared within 10 min after initiation of PLD infusion at rate of 1 mg/min and all of them were generally mild. However some cases that required discontinuation of treatment were reported (21). So it is very important to monitor the patients' condition carefully during the initial 10–15 min after start of PLD infusion. Infusion reaction was correlated with the initial PLD infusion rate—a lower infusion rate reduces the risk of infusion reaction (25).

Only one patient treated at level 1 developed DLTs and no patients developed DLT in level 2 and 3. However, the

Table 4. PK parameters

Dose (mg/m^2)	No. of patients	C_{max} (μg/ml)		AUC (μg h/ml)		$t_{1/2}$ (h)		CL (ml/h/m^2)		Vd (l/m^2)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
30	6	19.312	(2.502)	2512.7	(783.5)	89.50	(24.05)	13.14	(4.84)	1.569	(0.187)
40	3	25.605	(2.866)	3228.0	(789.6)	86.30	(14.72)	12.99	(3.70)	1.568	(0.174)
50	6	34.057	(3.293)	4663.3	(1061.8)	95.33	(25.32)	11.10	(2.05)	1.471	(0.130)

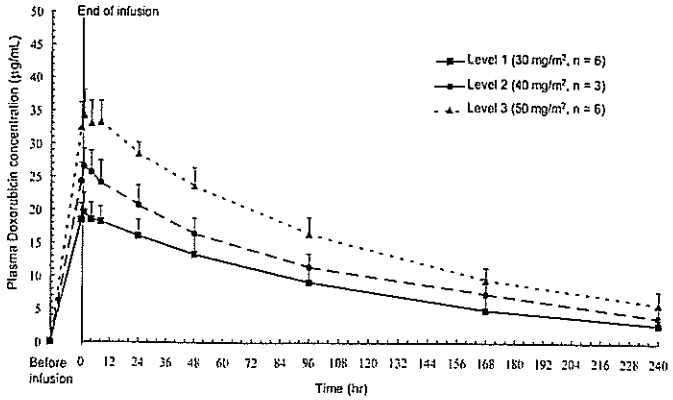

Figure 1. Mean plasma concentration–time curve for doxorubicin infused as PLD.

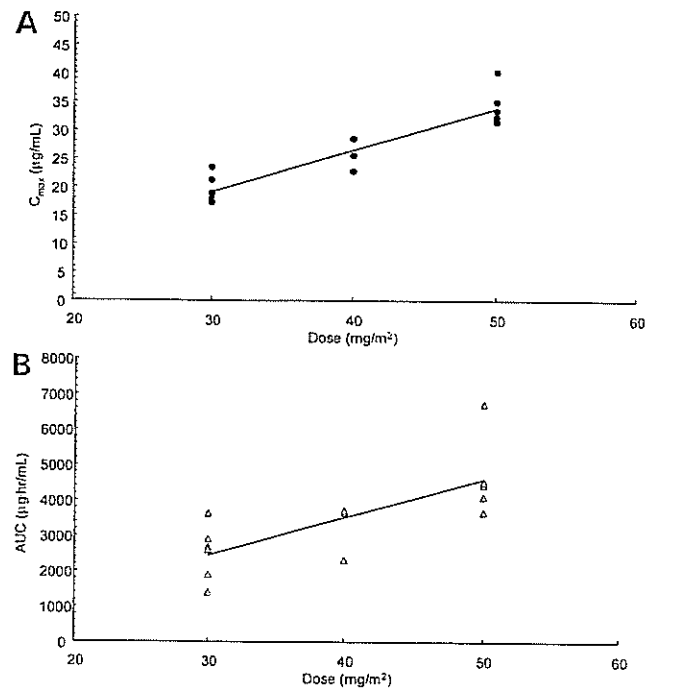

Figure 2. Relationships between (A) dose and C_{max} ($r^2 = 0.861$) and (B) dose and AUC ($r^2 = 0.575$) for doxorubicin infused as PLD.

independent data monitoring committee did not recommended further dose escalation beyond level 3. We accepted this recommendation for the following reasons. First, the repeated dosing toxicity of PLD in level 3, which was the approved dosage established in Europe and the USA, was acceptable. Second, among six patients treated at level 3, delay of therapy was required in three patients because of leukopenia in the present phase 1 study. Of these three, two patients also developed HFS leading to delay of therapy. In the level 3 cohort, HFS causing delay of therapy was observed in 10 of 32 cycles in total. Based on these findings, further dose escalation over level 3 seemed to be difficult as PLD requires multiple dosing to show antitumor activity. Third, from the results of PK analysis, PLD did not significantly accumulate in plasma when administered at intervals of 4 weeks or longer by level 3. Fourth, antitumor effect was already obtained in patients with ovarian cancer in the present study. From the above-mentioned facts, we concluded that level 3 (50 mg/m^2) was the recommended dose for subsequent phase 2 study. HFS showed an aggravating trend with repeated JNS002 treatment in our study, but did not lead to a severe toxicity. However, repeated JNS002 treatment in the previous phase 1 study in USA and Israel resulted in a severe dose-limiting toxicity. Therefore, further studies should be carefully conducted in a greater number of patients paying attention to the severity of HFS.

Regarding pharmacokinetics of PLD, the profile clarified in our study is largely consistent with previous findings in overseas studies indicating that PLD has an extremely long circulation time with a slow clearance and a small volume of distribution (22,26,27). Lyass et al. provided the results of correlation analysis that dose and C_{max} are strongly correlated with stomatitis and nadir leukocyte count, whereas plasma $t_{1/2}$ is significantly correlated with HFS which is one of the important cause for prolongation of dosing interval leading to delay of treatment for consequent cycle (22). The half-life values in the present study (86–95 h) are comparable to those reported previously (80–84 h, Hamilton et al. (26); 62–86 h, Lyass et al. (22); 75–91 h, Hubert et al. (27)).

PLD is already approved for the treatment of AIDS-KS and ovarian cancer in Europe and the USA, and breast cancer in Europe. Also in our study of six patients with ovarian cancer, one had achieved partial response and one had achieved normalization of the tumor marker CA125. This result is very encouraging in planning for further clinical studies in Japanese patients with ovarian cancer.

In conclusion, we confirmed the tolerance of the recommended dose (50 mg/m^2) in Europe and the USA, which was intravenous infusion of PLD every 4 weeks in Japanese patients, and one partial response and one normalization of CA125 were observed in patients with ovarian cancer. We concluded that the recommended dose in phase 2 clinical study was 50 mg/m^2 every 4 weeks. At present, a phase 2 clinical study in Japanese patients with ovarian cancer is ongoing.

Acknowledgments

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A Phase I Dose-Escalation Study of ZD6474 in Japanese Patients with Solid, Malignant Tumors

Tomohide Tamura, MD, Hironobu Minami, MD,† Yasuhide Yamada, MD, PhD,* Noboru Yamamoto, MD, PhD,* Tatsu Shimoyama, MD,* Haruyasu Murakami, MD,* Atsushi Horiike, MD,* Yasuhito Fujisaka, MD,* Tetsu Shinkai, MD, PhD,† Makoto Tahara, MD, PhD,† Kenji Kawada, MD,† Hiromichi Ebi, MD,† Yasutsuna Sasaki, MD, PhD,† Haiyi Jiang, MD,‡ and Nagahiro Saijo, MD, PhD**

Introduction: ZD6474 (vandetanib) is an orally available inhibitor of vascular endothelial growth factor receptor, epidermal growth factor receptor, and RET receptor tyrosine kinase activity. This study assessed the safety and tolerability of escalating doses of ZD6474 in Japanese patients with solid, malignant tumors.

Methods: Adult patients with solid tumors refractory to standard therapy received a once-daily oral dose of ZD6474 (100–400 mg) in 28-day cycles, until disease progression or unacceptable toxicity was observed.

Results: Eighteen patients were treated at doses of 100 mg ($n = 3$), 200 mg ($n = 6$), 300 mg ($n = 6$), and 400 mg ($n = 3$). Dose-limiting toxicities at the completion of cycle 2 were hypertension ($n = 3$), diarrhea ($n = 1$), headache ($n = 1$), toxic skin eruption ($n = 1$), and alanine aminotransferase increase ($n = 1$). A dose of 400 mg/day was considered to exceed the maximum tolerated dose (MTD). Toxicities were manageable with dose interruption and/or reduction. Objective tumor response was observed in four of nine patients with non-small cell lung cancer (NSCLC) at doses of either 200 or 300 mg. Terminal half-life was about 90–115 hours. Plasma trough concentrations achieved steady-state conditions after approximately 1 month of daily dosing.

Conclusions: It was concluded that a dose of 400 mg/day was considered to exceed the MTD, and doses for phase II study were thought to be not more than 300 mg/day. The objective response observed in some NSCLC patients is encouraging for further studies in this tumor type.

Key Words: Phase I study, ZD6474, Vandetanib, Non-small cell lung cancer

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*National Cancer Center Hospital, Tokyo, Japan; †National Cancer Center Hospital East, Chiba, Japan; and ‡AstraZeneca KK, Osaka, Japan.

Address for correspondence: Tomohide Tamura, MD, National Cancer Center Hospital, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. E-mail: ttamura@ncc.go.jp

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Vascular endothelial growth factor (VEGF) is a potent stimulator of angiogenesis and plays an essential role in the formation and maintenance of the vasculature by activating protease expression, endothelial cell proliferation and migration, and capillary vessel formation.[1–4] Enhanced secretion of VEGF from tumor tissue induces vascular permeability and results in the development of a network of highly permeable, immature vessels that are characteristic of pathological angiogenesis.[5] Although VEGF binds to VEGFR-1 (Flt-1) and VEGFR-2 (KDR or Flk-1) on vascular endothelial cells, activation of VEGFR-2 alone is sufficient to stimulate VEGF-mediated angiogenesis.[6] Pathological angiogenesis is necessary for the progression of solid, malignant tumors,[7] and inhibition of VEGF-dependent signaling has been identified as a key antiangiogenic strategy.[8,9] The clinical value of inhibiting VEGF signaling in colon cancer,[10] non-small cell lung cancer (NSCLC),[11] and breast cancer[12] has been confirmed with bevacizumab, an anti-VEGF antibody.

Epidermal growth factor receptor (EGFR)-dependent signaling is an important pathway contributing to the growth and metastasis of tumor cells, and aberrant EGFR tyrosine kinase activity has been reported in a number of human tumors.[13,14] One consequence of upregulated EGFR tyrosine kinase activity is increased expression of proangiogenic factors, including VEGF,[15,16] which may lead to possible paracrine and autocrine stimulation of angiogenesis.

ZD6474 (vandetanib; ZACTIMA) is a novel inhibitor of VEGFR, EGFR, and RET tyrosine kinase activity.[17–20] As such, ZD6474 has the potential to inhibit two key pathways in tumor growth: VEGF-dependent tumor angiogenesis, and EGFR- and RET-dependent tumor cell proliferation and survival. Indeed, preclinical evaluation of ZD6474 has demonstrated potent inhibition of VEGF-dependent signaling and angiogenesis in vivo, as well as dose-dependent inhibition of tumor growth, including profound regression in established PC-3 prostate tumors. More recently, the results of a phase I study of ZD6474 conducted in the United States and Australia showed that once-daily continuous oral dosing was generally well tolerated in patients with advanced tumors.[21]

We report the results of a phase I, open-label, nonrandomized, multicenter clinical study of ZD6474 in Japanese patients with advanced solid tumors. The primary objective

of the study was to assess the safety and tolerability of escalating oral doses of ZD6474, with the aim of establishing the maximum tolerated dose (MTD) and the recommended doses for further phase II study assessment. Additional objectives included evaluation of antitumor activity and assessment of single- and multiple-dose pharmacokinetics.

PATIENTS AND METHODS

Patients

Adult patients between 20 and 74 years of age with solid, malignant tumors refractory to standard therapies, or for which no appropriate therapy exists, were eligible for inclusion. Patients were required to have a life expectancy ≥3 months and a World Health Organization performance status of 0 or 1. The main exclusion criteria were significant cardiac, hematopoietic, hepatic or renal dysfunction; severe complications (including active double cancers); any gastrointestinal disease that would affect drug bioavailability; poorly controlled hypertension; CNS tumors and metastases; systemic anticancer therapy or radiotherapy within the previous 4 weeks; unresolved adverse effects from prior anticancer therapy or radiotherapy; and incomplete recovery from prior surgery. All patients provided written informed consent. The trial was approved by the ethics committee of institutional review board and was conducted in accordance with the Declaration of Helsinki and guidelines for good clinical practice.

Study Design

This was an open-label, nonrandomized, multicenter dose-escalation study. Patients received a single oral dose of ZD6474 (100, 200, 300, or 400 mg), which was followed by a 7-day observation period (cycle 0; Figure 1). On day 8, patients started a once-daily ZD6474 dosing regimen at the same dose as they had received in cycle 0 for a total of 28 days (cycle 1). Further 28-day treatment cycles were repeated at the same dose. A dose-limiting toxicity (DLT) was defined as any toxicity of at least grade 3 according to common toxicity criteria (CTC version 2.0) that was related to ZD6474 treatment, or grade 2 diarrhea daily for >7 days or grade 3 diarrhea despite maximum antidiarrheal support; ≥grade 2 skin toxicity for >7 days that affected the patient's subjective well-being and required cessation of treatment, despite supportive care; and QT or corrected QT (QTc) prolongation ≥490 msec, or a rise of ≥60 msec from baseline QT or QTc to ≥460 msec. QTc values were obtained using Bazett's[22] method of correction.

The initial dose of ZD6474 was set at 100 mg/day, based on the minimum toxic effect dose in rats as well as safety data from U.S./Australian phase I study. Dose escalation was performed when a minimum of three patients per dose level had completed cycle 1 (28 days) without experiencing a DLT. The MTD was defined as the dose of drug at which 33.3% of patients experienced a DLT during cycle 1 that was not controlled with symptomatic therapy. Once the MTD was established, three or more additional patients were enrolled at the two highest dose levels below the MTD. This was to further characterize the safety, tolerability, and biological activity of ZD6474.

Assessment of Safety and Tolerability

The primary objective was to assess the safety and tolerability of escalating oral doses of ZD6474. After full physical examination at enrollment, adverse events (AEs) were recorded at each scheduled study visit.

Electrocardiograms (ECGs) were recorded at the screening visit, on days 1 (baseline) and 2 of cycle 0, and three times per week up to day 21 of cycle 1. If no prolongation of QT or QTc occurred, ECGs were performed weekly up to day 14 of cycle 2, every 2 weeks until the end of cycle 3 and monthly during subsequent cycles; and 29 days after the last dose. Vital signs (blood pressure, pulse rate, and body temperature) were measured before and 2, 4, 6, 8, and 10 hours after the drug administration on day 1, and then every 24 hours until day 7 of cycle 0; every 24 hours until day 15 of cycle 1; weekly thereafter until the end of cycle 2; once every 2 weeks during subsequent cycles; and at withdrawal.

Blood chemistry and hematological assessments were performed at the screening visit; predose of cycle 0; predose and on days 8, 15, 22, and 29 of cycles 1 and 2; every 2 weeks (days 15 and 29) during subsequent cycles; at withdrawal; and on days 15 and 29 after the last dose. Electrolytes were measured weekly for patients who experienced diarrhea or vomiting. Urinalysis was performed at the screening visit; on day 2 of cycle 0; on days 15 and 29 of cycle 1; on day 29 during subsequent cycles; at withdrawal; and on days 15 and 29 after the last dose.

Pharmacokinetic Assessment

The pharmacokinetic profile of ZD6474 was assessed after both single and multiple dosing. During cycle 0, blood

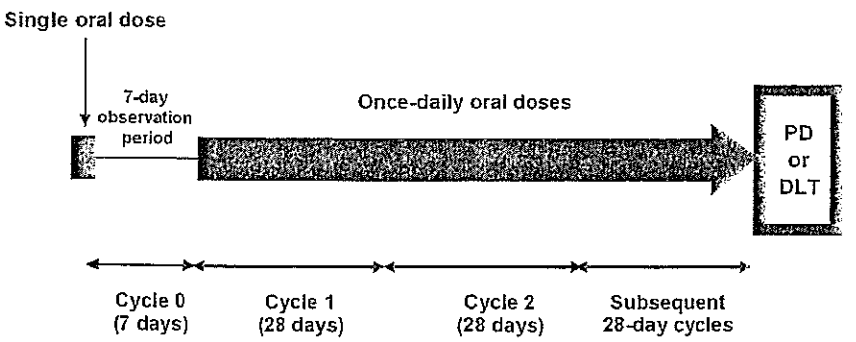

FIGURE 1. Study design. PD, progressive disease; DLT, dose-limiting toxicity.

samples were collected before and 1, 2, 4, 6, 8, 10, 24, 48, 96, 120, and 144 hours after administration. During cycle 1, blood samples were collected before administration on days 1, 8, 14, 22, and 28 and 2, 4, 6, 8, 10, and 24 hours after administration on day 28. Samples were also collected before administration on days 15 and 29 of cycles 2 and 3, before administration on day 29 of subsequent cycles, and at withdrawal. Plasma concentrations of ZD6474 were determined using high-performance liquid chromatography with mass spectrometry (LC-MS/MS). C_{max} and t_{max} were determined by visual inspection of the plasma concentration time data for ZD6474 for each patient on each sampling occasion. Where there were adequate data, ZD6474 plasma elimination half-life ($t_{½}$) was determined by log-linear regression of those points considered to constitute the terminal phase. The area under the plasma concentration time curve (AUC_{0-t}) was calculated using the linear trapezoidal rule. The accumulation ratio based on AUC_{0-24} was calculated by ratio of AUC_{0-24} after 28-day multiple doses to AUC_{0-24} after a single dose.

Assessment of Tumor Response

Tumor response was evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines[23] at the end of each treatment cycle. Baseline tumor assessments were performed before the start of single dosing.

Statistical Analyses

All analyses were descriptive, with no formal statistical analysis performed on the data from this study. AEs were coded according to both the Medical Dictionary for Regulatory Activities (MedDRA) coding system and the CTC grading system.

RESULTS

Patient Characteristics

All 18 patients (11 male, 7 female) enrolled in the study received ZD6474 treatment and were evaluable for safety, efficacy, and pharmacokinetics. Initially, three patients each were enrolled in the 100-, 200-, 300-, and 400 mg groups. Subsequently, three additional patients were enrolled in the 200- and 300-mg groups. Overall, 3, 6, 6, and 3 patients received ZD6474 100, 200, 300, and 400 mg, respectively.

The overall patient population profile is summarized in Table 1. Median duration of ZD6474 treatment was 56.5 (22–556) days. The median duration (range) of each dose group was 43.0 (30–45), 191.5 (29–556), 76.5 (25–124), and 37.0 (22–42) days in the 100-, 200-, 300-, and 400-mg groups, respectively. The reasons for discontinuation were radiological or clinical disease progression (n = 12), AEs (n = 5), or disease-related postrenal failure (n = 1).

Safety and Tolerability

All patients experienced at least one AE. Drug-related AEs by CTC grade with an incidence of at least 20% of the overall population are summarized in Table 2. The most common drug-related AEs were rash (n = 13), prolongation of QTc interval (n = 12), diarrhea (n = 11), and proteinuria (n = 11). There were various types of rash such as acne, dermatitis acneform, macular rash, maculopapular rash, pustular rash, erythema, folliculitis, photosensitivity rash, follicular rash, and skin eruption. Although there were no skin disorders of grade 3 or 4 severity, one patient in the 300-mg group developed grade 2 toxic skin eruption, which persisted for 7 days despite medical treatments and local supportive care. Because of this, the event was defined as DLT, and the study treatment was discontinued.

TABLE 1. Patient Characteristics

	ZD6474 Dose				
	100 mg (n = 3)	200 mg (n = 6)	300 mg (n = 6)	400 mg (n = 3)	Total (n = 18)
Male/female	1/2	5/1	3/3	2/1	11/7
Median age, yr (range)	50 (44–67)	52.5 (41–72)	55.5 (31–68)	53 (40–62)	52 (31–72)
Performance status (0/1)	1/2	2/4	2/4	1/2	6/12
Primary tumor diagnosis (n)					
NSCLC	1	3	3	2	9
Colorectal	1	1	1	1	4
Breast	0	1	0	0	1
Stomach	0	0	1	0	1
Other*	1	1	1	0	3
Number of prior cancer treatments†	3	6	6	3	18
Chemotherapy	3	6	5	3	17
Radiotherapy	1	1	3	1	6
Median duration of ZD6474 treatment, days (range)	43 (30–45)	191.5 (29–556)	76.5 (25–124)	37.0 (22–42)	56.5 (22–556)

*Various other tumor types.
†Includes surgery, chemotherapy, immunotherapy, hormonal therapy, and radiotherapy.
NSCLC, non-small cell lung cancer.

TABLE 2. Common Drug-Related Adverse Events by CTC Grade

	ZD6474 Dose								
	100 mg (n = 3)		200 mg (n = 6)		300 mg (n = 6)		400 mg (n = 3)		Total
Adverse Event*	G1/2	G3	G1/2	G3	G1/2	G3	G1/2	G3	(n = 18)
Rash (NOS)	1	0	6	0	4	0	2	0	13
Electrocardiogram QT corrected interval prolonged	2	0	4	0	4	0	2	0	12
Diarrhea (NOS)	1	0	4	0	3	1	2	0	11
Proteinuria	1	0	4	0	4	0	2	0	11
Fatigue	1	0	1	1	2	0	3	0	8
Hypertension† (NOS)	0	0	1	2	1	1	1	1	7
Blood lactate dehydrogenase increased	0	0	4	0	1	0	2	0	7
ALT increased	0	0	3	0	1	0	1	1	6
Anorexia	1	0	2	0	2	0	1	0	6
AST increased	0	0	3	0	1	0	2	0	6
β-N-acetyl-D-glucosaminidase increased	0	0	4	0	1	0	1	0	6
Hematuria	1	0	2	0	0	0	2	0	5
Headache	0	0	1	0	1	1	2	0	5
Lymphopenia	0	1	2	0	1	0	1	0	5
Blood alkalinephosphatase	0	0	3	0	1	0	1	0	5
Nausea	0	0	1	0	2	0	1	0	4

*Medical dictionary for regulatory activities (MedDRA) preferred term.
†Includes one patient with an adverse event reported as blood pressure increased.
CTC, common toxicity criteria; NOS, not otherwise specified; ALT, alanine aminotransferase; AST, asparate aminotransferase.
No grade 4 drug-related adverse events were reported.

All episodes of QT or QTc prolongation in this study were asymptomatic and considered by the investigator to be drug related. QTc prolongation necessitated dose interruption in 7 of 12 patients, 6 of whom were able to resume ZD6474 treatment at a reduced dose. The remaining patient was discontinued from the study after experiencing QTc prolongation, despite resuming treatment at a reduced dose.

No grade 4 drug related AE was observed. Seven patients experienced grade 3 drug-related AEs. The most common grade 3 drug-related AE was hypertension. One patient who had grade 3 hypertension in the 300-mg group was urgently hospitalized for hypertension and headache (both of grade 3) at 6 weeks after the start of multiple dosing. The symptoms were relieved 3 weeks after dose interruption, and the treatment with ZD6474 was resumed at a reduced dose of 150 mg/day. Eight patients had dose interruption, and five patients discontinued study treatment because of AEs. Drug-related AEs that led to treatment discontinuation were increased alanine aminotransferase, fatigue, hypoacusis, prolonged QTc interval, and toxic skin eruption (all n = 1).

Mean arterial blood pressure increased in most patients after multiple dosing with ZD6474. Hypertension or increased blood pressure was reported as an AE in eight patients (n = 4, grade 1 or 2; n = 4, grade 3). In five of these eight patients, the AE required treatment with standard antihypertensive medication (primarily Ca^{2+}-channel blockers or ACE inhibitors). There were no clinically relevant hematological toxicities. Elevations of ALT, asparate aminotransferase, and alkalinephosphatase reported as AE were in 6, 6, and 5 patients, respectively. Urinalysis revealed raised β-N-acetyl-D-glucosaminidase (n = 6) and proteinuria (n = 11), but all of these events were classified as CTC grade 1. Elevations of serum creatinine level were observed in three patients.

In total, five patients experienced drug-related DLTs up to the completion of cycle 2 (Table 3). Because 33.3% of patients in the 400-mg cohort developed a DLT during cycle 1, 400 mg was considered to exceed the MTD.

Pharmacokinetic Evaluation

Pharmacokinetic parameters following a single oral dose and multiple oral doses of ZD6474 (100–400 mg) are shown in Tables 4. Plasma concentration of ZD6474 decreased biphasically (Figure 2A). The terminal half-life seemed to be independent of the dose and was estimated to be approximately 100 hours; this may be underestimated because up to 40% of the AUC was extrapolated. Mean plasma trough concentrations of ZD6474 during continuous oral dosing indicate that steady state is achieved after about 1 month of treatment (Figure 2B). Based on the $AUC_{0-24\ h}$ on days 1 and 28, exposure to ZD6474 increased approximately sixfold after multiple dosing compared with a single dose. The relationship between AUC and dose after a single dose and 28-day multiple dosing was shown in Figure 3A and B, respectively. Exposure to ZD6474 as assessed by AUC after a single oral dose seemed to show an increase with dose. There was an approximately threefold interindividual variability in AUC at the same dose level.

TABLE 3. Drug-Related Dose-Limiting Toxicity (DLT) at the Completion of Cycle 2

ZD6474 (mg)	Patients Enrolled	Patients Developing DLT	DLT*	
			Cycle 1	Cycle 2
100	3	0/3	None	None
200	3	0/3	None	None
	3	1/3	Hypertension	None
	(additional cohort)			
300	3	1/3	None	Hypertension, diarrhea, headache†
	3	1/3	None	Toxic skin eruption
	(additional cohort)			
400	3	2/3	Hypertension	Alanine aminotransferase increased

*All DLTs were CTC grade 3 except for grade 2 toxic skin eruption.
†Observed in the same patient.
CTC, common toxicity criteria; ALT, alanine aminotransferase.

Tumor Response

Tumor responses were evaluated in 18 patients. No complete response was observed, but four patients achieved a confirmed partial response (three patients in the 200-mg group and one patient in the 300-mg group), all of whom had NSCLC with adenocarcinoma. Prior cancer treatments in these four patients included chemotherapy ($n = 4$), surgery ($n = 2$), and radiotherapy ($n = 2$). Each of the responders experienced dose interruptions/reduction because of AEs, but their responses were maintained at a reduced dose of 100 or 200 mg/day; the individual time to onset of response was 36, 64, 70, and 103 days, with a respective duration of response of 90, 230, 246, and 438 days (Table 5). Three of the four responders subsequently discontinued treatment because of AEs. Representative CT scans from two responders are shown in Figure 4.

DISCUSSION

In this phase I dose-escalation study, once-daily oral dosing with ZD6474 was generally well tolerated at doses up to and including 300 mg in Japanese patients with solid, malignant tumors. Pharmacokinetic analyses confirmed that once-daily oral dosing was appropriate for ZD6474, which had an estimated half-life of approximately 5 days. Notably, partial tumor response was observed in four out of nine patients with refractory NSCLC.

The most common drug-related AEs were rash, QTc prolongation, diarrhea, and proteinuria. QTc prolongation was reported at all doses studied, with no clear evidence of dose dependency. All patients with QTc prolongation were asymptomatic, and most did not require withdrawal of ZD6474 treatment. QTc prolongation was reversible and can be managed through dose interruption or dose reduction.

TABLE 4. Pharmacokinetic Parameters of ZD6474 After a Single Dose (Cycle 0) and After Multiple Dosing for 28 Days (Cycle 1)

	ZD6474 Dose			
Parameters After a Single Dose	100 mg ($n = 3$)	200 mg ($n = 6$)	300 mg ($n = 6$)	400 mg ($n = 3$)
Mean C_{max}, ng/mL (SD)	103 (42)	186 (92)	392 (198)	447 (240)
Median t_{max}, hr (range)	6 (4–6)	4 (4–6)	5 (4–8)	6 (2–6)
Mean $AUC_{0-24\ h}$, μg·hr/ml (SD)	1.5 (0.5)	2.8 (1.5)	5.6 (2.5)	6.7 (3.0)
Mean AUC, μg·hr /ml (SD)	10.1 (3.5)	16.8 (6.9)	29.4 (11.8)	32.1 (4.7)
Mean $t_{½}$, hr (SD)	115 (46)	101 (14)	90 (14)	114 (45)
Parameters After Multiple Dosing	**100 mg ($n = 3$)**	**200 mg ($n = 4$)**	**300 mg ($n = 3$)**	**400 mg ($n = 1$)**
Mean C_{max}, ng/mL (SD)	1200 (583)	922 (259)	1580 (302)	2050
Median t_{max}, hr (range)	4 (4–6)	6 (4–10)	6 (6–6)	4
Mean AUC_{0-24h}, μg·hr/ ml (SD)	20.5 (5.0)	18.3 (5.7)	29.9 (4.6)	44.6
Accumulation index* (SD)	14.2 (1.8)	6.2 (1.9)	5.3 (1.2)	6.5

*Day 28 $AUC_{0-24\ h}$/day 1 $AUC_{0-24\ h}$
AUC, area under the curve to infinity; $AUC_{0-24\ h}$, area under the curve to 24 hr; C_{max}, maximum concentration; SD, standard deviation; t_{max}, time to maximum concentration; $t_{½}$, terminal half-life.

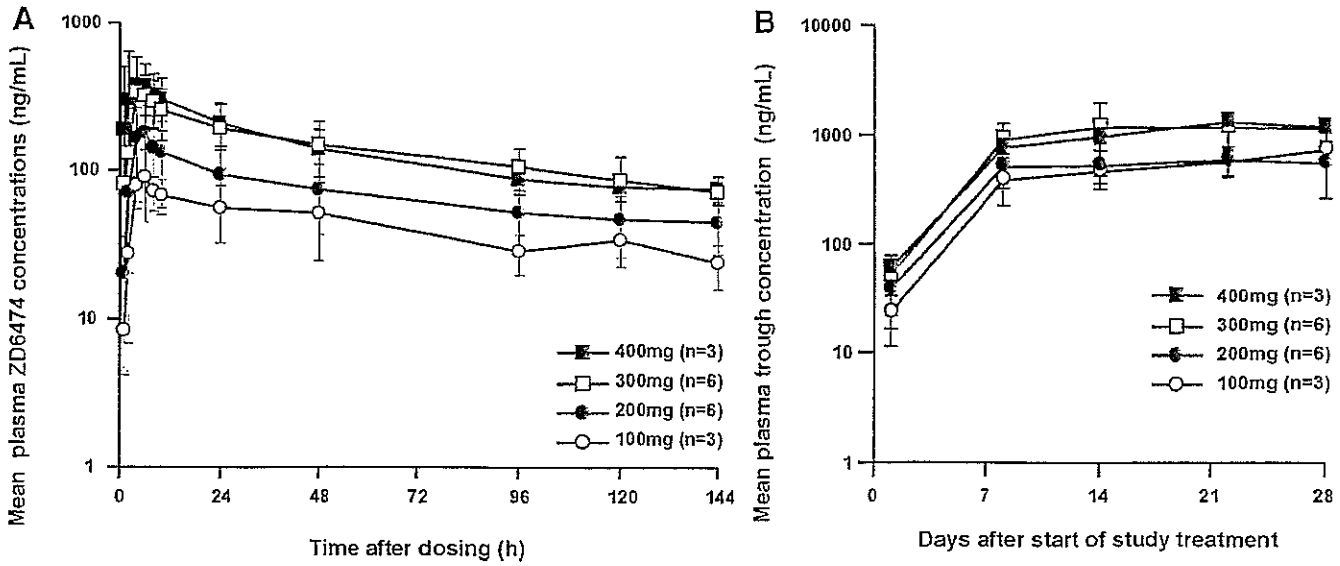

FIGURE 2. (*A*) Mean (±SD) plasma concentration of ZD6474 after a single oral dose. (*B*) Mean (±SD) plasma trough concentration of ZD6474 during continuous oral dosing for 28 days (cycle 1).

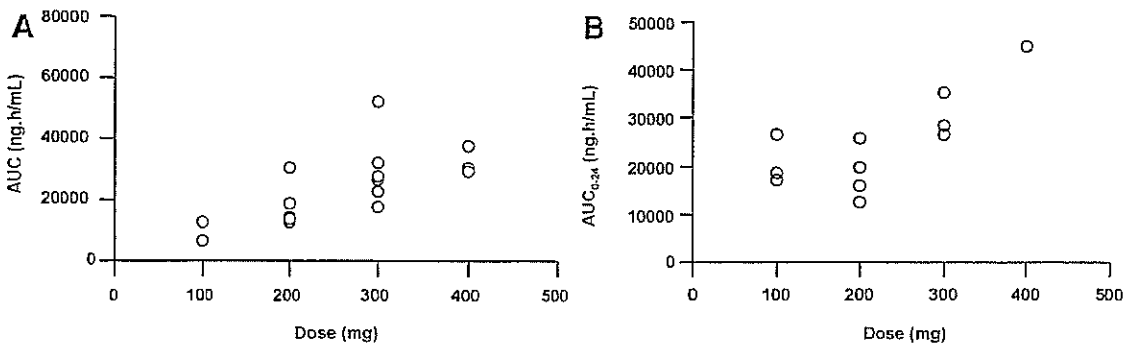

FIGURE 3. (*A*) Relationship between AUC and dose after a single oral dose of ZD6474. (*B*) Relationship between AUC_{0-24} and dose after 28-day multiple doses of ZD6474. AUC, area under the curve from zero to infinity; AUC, area under the curve from 0 to 24 hours.

There were some T-wave and U-wave changes in ECG, but there was no consequent arrhythmia finding in ECG. However, ECG monitoring should continue in future clinical trials.

Hypertension was also reported as a drug-related AE in seven patients, but no patients withdrew from the study as a result of hypertension, and all cases were controllable with dose adjustment or appropriate drug therapy. Rash and hypertension were also reported as relatively common AEs in a larger phase 1 study of ZD6474, which was conducted in the United States and Australia.[21] These events could be indicative of target inhibition by ZD6474. Also, because synthesis

TABLE 5. Summary of Partial Responders

Patient No.	Age (yr)	Sex	Initial ZD6474 Dose (mg)	Dose Reduction[a]	Partial Response: Time to Onset (days)	Partial Response: Duration (days)[b]
301	72	M	200	200→100 mg (day 28)	64	+230
304	54	M	200	200→100 mg (day 42)	103	438
305	41	M	200	200→100 mg (day 276)	70	+246
406	50	F	300	300→200 mg (day 79)	36	+90

[a]Dose reduction was attributable to AEs: QT/QTc prolongation (#301); hypertension (#304); rash (#305); toxic skin eruption (#406).
[b]Dose discontinuation was attributable to: hypoacusis (#301); disease progression (#304); fatigue (#305); toxic skin eruption (#406).

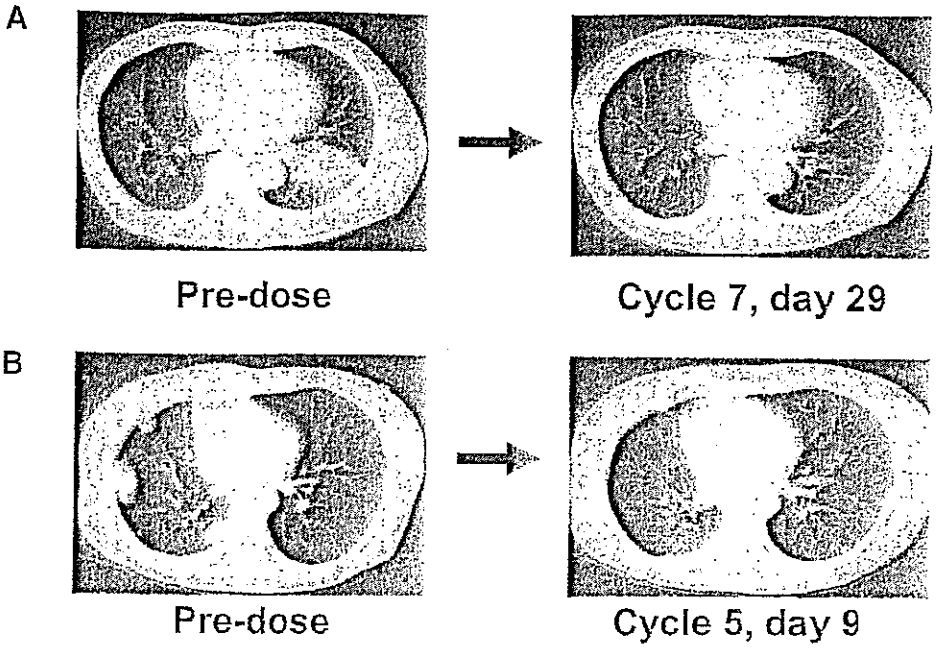

FIGURE 4. Representative CT scans before and after ZD6474 treatment in two NSCLC (adenocarcinoma) patients with partial responses. Baseline scans were performed within 4 weeks before the first dose. Male, 72 years (#301), initial ZD6474 dose = 200 mg. Female, 50 years (#406), initial ZD6474 dose = 300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

H Kimura[1,2,6], Y Fujiwara[3,6], T Sone[2], H Kunitoh[3], T Tamura[3], K Kasahara[2] and K Nishio[*,1,4,5]

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

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).

*Correspondence: Dr K Nishio, Shien-Lab, National Cancer Center Hospital, Tsukiji 5-1, Chuo-ku, Tokyo, Japan;
E-mail: knishio@gan2.res.ncc.go.jp
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[6]These authors contributed equally to this work
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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 (≥100 cigarettes/lifetime, no smoking at present), and current smokers (≥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

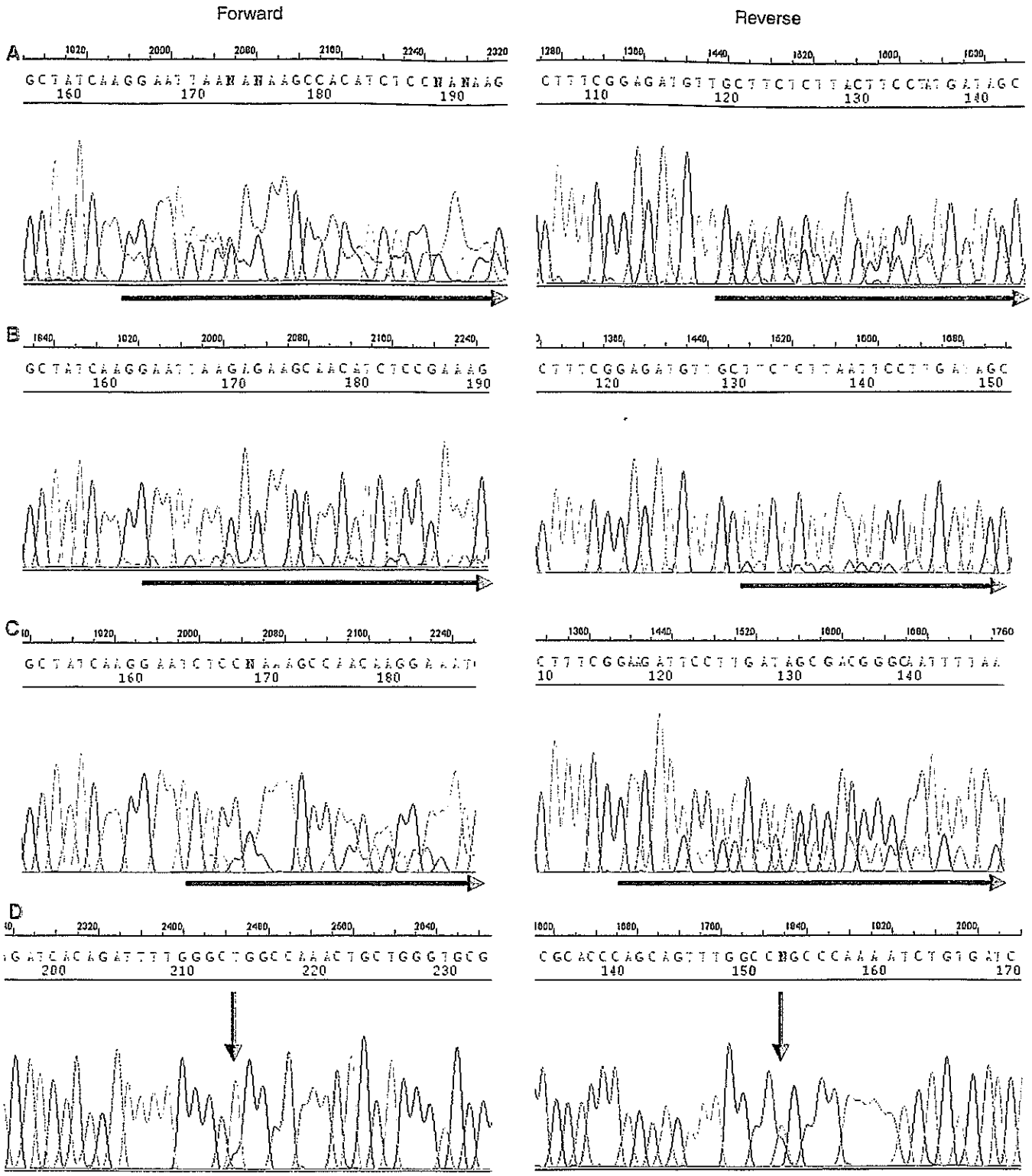

Figure 1 The wave figures of the nucleotide sequence of the *EGFR* gene with heterozygous mutations obtained by direct sequencing (see 'Patients and Methods') are shown. Horizontal arrows in both the sense and the antisense directions are shown to demonstrate the two breakpoints of the deletion. The 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

Ikuo Sekine, MD, PhD, Minoru Takada, MD,† Hiroshi Nokihara, MD, PhD,* Seiichiro Yamamoto PhD,‡ and Tomohide Tamura, MD**

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.

*Division of Internal Medicine and Thoracic Oncology, National Cancer Center Hospital, Tokyo, Japan; †Division of Pulmonary Medical Oncology, Kinki-chuo Chest Medical Center, Osaka, Japan; ‡ Statistics and Cancer Control Division, Research Center for Cancer Prevention and Screening, Tokyo, Japan

Address for correspondence: Ikuo Sekine, M.D., Ph.D., Division of Internal Medicine & Thoracic Oncology, National Cancer Center Hospital, Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045, Japan; E-mail: isekine@ncc.go.jp

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