individualized dosing using the total amount of urinary 6-β-OHF after cortisol administration produced good results. However, this method is somewhat complicated, and a simpler method would be of great use. We analyzed the expression of CYP3A4 mRNA in the peripheral-blood mononuclear cells of the 29 patients in the individualized arm. No correlation was observed between the expression level of CYP3A4 mRNA and docetaxel CL or the total amount of urinary 6-β-OHF after cortisol administration (data not shown).

In conclusion, the individualized dosing of docetaxel using the total amount of urinary 6-β-OHF after cortisol administration is useful for decreasing the interpatient PK variability compared with the conventional BSA-based method of dosing. This method may be useful for individualized chemotherapy.

Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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Plasma MIP-1β levels and skin toxicity in Japanese non-small cell lung cancer patients treated with the EGFR-targeted tyrosine kinase inhibitor, gefitinib

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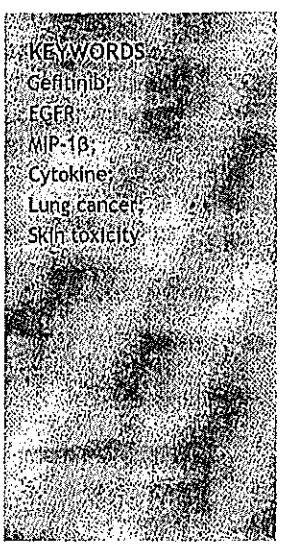

Summary Gefitinib (Iressa®) is an orally active, selective EGFR tyrosine kinase inhibitor that blocks signal transduction pathways. Skin toxicity has been reported to be the major toxicity observed in patients treated with the EGFR-targeted tyrosine kinase inhibitors, such as gefitinib and erlotinib. Although the mechanisms underlying the development of the skin toxicity remain to be precisely clarified, immunological mechanisms are considered to be involved. We examined the correlations between the plasma levels of several cytokines and the risk of development of adverse events, especially skin toxicity, induced by the administration of gefitinib as first-line monotherapy in non-small cell lung cancer (NSCLC) patients.

Paired plasma samples were obtained from a total 28 patients of non-small cell lung cancer; the first before the initiation of gefitinib administration (250 mg/day) (24 patients) and the second 2 or 4 weeks after the initiation of treatment (23 patients). The plasma concentrations of 17 major cytokines were measured using a bead-based multiplex assay. The median concentrations of eight of these cytokines before the start of treatment ranged from 0.06 (IL-5) to 58.26 (MIP-1β) (μg/ml). The concentrations of the remaining nine cytokines were under the detectable limit (<0.01 μg/ml) in more than 50% of the samples. Comparisons of the levels before and after treatment showed no significant differences for any of the cytokines measured.

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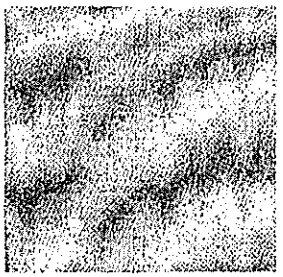

The MIP-1β levels were significantly lower in the patients with skin toxicity (16/24) as compared with those in the patients not showing any skin toxicity (59.1 ± 10.5 versus 119.0 ± 36.8; $p = 0.042$ by the two-sample t-test). The K-Nearest Neighbor Prediction ($K = 3$) showed the classification rate to be 75% for the prediction sets containing MIP-1β, IL-4 and IL-8. There were no significant associations between the levels of any of the cytokines measured and any other parameters, including the tumor response to the drug. In conclusion, the plasma MIP-1β level may be a useful predictor of the development of skin toxicity in patients receiving gefitinib treatment.

1. Introduction

The epidermal growth factor receptor (EGFR) has been found to be expressed, sometimes strongly, in a variety of solid tumors, including non-small cell lung cancer [1,2]. Recognition of the importance of the EGFR in tumor biology provides the rationale for the development of EGFR-targeted cancer therapies. Gefitinib (''Iressa'', ZD1839) is an orally active, selective EGFR tyrosine kinase inhibitor that blocks signal transduction pathways implicated in the proliferation and survival of cancer cells, and also other host-dependent processes that may promote cancer growth [3–5].

Gefitinib has been approved for use as a second-line drug for the treatment of non-small cell lung cancer in Japan, based on evidence collected from large-scale phase II trials (IDEAL 1 and IDEAL 2) [6,7]. In these studies, the adverse effects of gefitinib were mild as compared with those of other cytotoxic agents, and skin toxicity was the most frequently encountered of the adverse events. In some clinical studies, up to 90% of patients treated with gefitinib were reported to suffer from skin toxicity [8]. In others, development of skin toxicity necessitated the discontinuation of gefitinib treatment in some patients [9,10]. Recent publications have reported the development of skin toxicity in patients treated with the anti-EGFR antibody, cetuximab (''Erbitux'', IMC-C225), as well as in those treated with erlotinib (''Tarceva®'', OSI-774), which is an EGFR-targeted small molecule [11–14]. No clear preventive or curative treatment has been established for such drug-induced skin toxicity.

Cytokines mediate numerous physiological and immune reactions, which influence various biological activities, including tumor activity. Activated macrophages secrete many mediators which regulate host defenses by stimulating cellular immunity. Activated macrophages, which produce cytokines such as interleukin (IL)-12, tumor necrosis factor (TNF)-α and interferon (IFN)-α and IFN-β, are powerful activators of natural killer (NK) cells, which have been reported to exert cytotoxic activity against some tumors [15,16]. In non-small cell lung cancer patients, increased production of cytokines such as IL-2, 6, 8 and 10 has been shown to be associated with the response to treatment and survival [17–21]. Other solid tumors have also been shown to possess the ability to produce multiple cytokines [22–25]. These cytokines may act as autocrine growth factors regulating the proliferation and migration of endothelial, tumor, and immune cells. Correlations have been shown between endogenous cytokine levels and the phenotypic manifestations of cancers and prognosis of patients with solid tumors [24–26]. Skin toxicity is the most frequently encountered toxicity in patients treated with EGFR-targeted agents. Some studies have shown evidence of immune reactions in patients developing such skin toxicity, following the administration of other drugs besides the EGFR-targeted agents. In these studies, the levels of various cytokines were elevated after treatment in patients who showed skin toxicity [27–29].

We hypothesized that the serum levels of cytokines may be correlated with the clinical features of patients treated with gefitinib, including the tumor response and adverse effects, especially skin toxicity. To date, no direct comparisons have been made to determine the correlations between cytokine levels and the phenotypic manifestations in cancer patients treated with gefitinib. To investigate the relationship between the cytokine levels and the phenotypic manifestations of cancer in these patients, we measured the plasma concentrations of various cytokines and investigated the roles of these cytokines in NSCLC patients receiving gefitinib as first-line monotherapy.

2. Materials and methods

2.1. Patients and clinical trials

The present study was carried out as a correlative study in a multicenter clinical phase II trial of gefitinib monotherapy, between October 23, 2002, and August 3, 2003. The study was conducted with the

approval of the appropriate ethical review boards, and in accordance with the recommendations of the Declaration of Helsinki for biomedical research involving human subjects. Twenty-eight Japanese patients with histologically or cytologically proven stage IIIb or IV, chemotherapy-naïve NSCLC were enrolled in this trial. Histological subclassification was carried out according to the World Health Organization (WHO) classification (WHO, 1982). Staging was carried out according to the Fourth Edition of the UICC Tumour Node Metastases (TNM) classification. Gefitinib was administrated orally to all patients at a fixed dose of 250 mg daily. Tumor response was evaluated according to the "Response Evaluation Criteria in Solid Tumours" guidelines [30]. Patients were monitored for adverse events during each cycle of therapy, and these events were graded according to NCI-CTC, version 2.0.

2.2. Plasma collection

Blood samples from the 28 NSCLC patients were collected in heparinized tubes before and 14 or 28 days after the initiation of gefitinib administration. After centrifugation of the blood samples at 500 × *g* for 10 min, plasma samples were carefully collected from the top portion of the separated plasma. The separated plasma samples were stocked at −80 °C until use.

2.3. Cytokine assay

A panel of cytokines was measured in duplicate using the Bioplex protein assay kit (Bio-Rad Laboratories, Hercules, CA), in accordance with the instructions of the manufacturer. All samples were diluted by the addition of an equal amount of saline, and 15 μl of the diluted samples were used for this assay. The assay is a novel multiplexed, particle-based, flow-cytometric assay which utilizes anti-cytokine monoclonal antibodies linked to microspheres incorporating distinct proportions of two fluorescent dyes. The assay was customized to detect and quantify IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17, G-CSF, GM-CSF, TNF-α, IFN-γ, monocyte chemotactic protein-1 (MCP-1), and macrophage inflammatory protein-1 beta (MIP-1β). The minimum detectable limit for each of the cytokines was 0.01 μg/ml.

2.4. Statistical analysis

Comparisons of the plasma cytokine levels before and after treatment were carried out with Wilcoxon's signed-rank test, using the Stat View software package (version 5.0, SAS Institute Inc., Cary, NC). The correlations between the cytokine levels and the clinical manifestations were analyzed statistically using the two-sample *t*-test with a random variance model, which was performed using the R software package, version 1.9.0 (The R Foundation, http://www.r-project.org/). The patients were categorized into two groups, depending on the grades of the adverse events (Grade 0 versus >Grade 1). Cytokine values lower than the minimum detectable limit were represented as 0.001 pg/ml. When the significant differences were obtained in the two-sample *t*-test, predictive rates were calculated using the *K*-nearest neighbor prediction analysis ($K = 3$).

3. Results

3.1. Patients

A total 28 patients ware enrolled in this trial. The patients ranged in age from 44 to 87 years, with a median of 64 years, and the male:female ratio was 18:10. Plasma samples were collected before treatment from 24 (85.7%) patients and after treatment from 23 (82.1%) patients. All the patients were evaluated for the presence of drug-related adverse events (Table 1). Skin toxicity was the most frequently encountered drug-related adverse event; 71.4% of the patients receiving gefitinib showed skin toxicity.

3.2. Plasma cytokine levels in the lung cancer patients

The plasma levels of various cytokines in the patients are shown in Table 2. Scatter plots of the levels of individual cytokines are shown in Fig. 1. The levels of IL-2, IL-4, IL-7, IL-12, IL-17, IFN-γ, G-CSF, and GM-CSF in the plasma were lower than the minimal detectable limit (<0.01 μg/ml) in more than 50% of the patients. When the cytokine levels before and after treatment were compared, the MCP-1 levels were significantly higher in the

Table 1. Non-hematological toxicity

	0	1	2	3	4	Percentage of ≥ Grade 1
Skin	8	13	5	2	0	71.4
Hepatitis	22	4	1	1	0	21.4
Pneumonitis	25	0	0	3	0	10.7
Diarrhea	18	7	2	1	0	35.7
Nausea	19	7	2	0	0	32.1

NCI-CTC version 2.0

Table 2 Circulating cytokine levels (pg/mL)

	Pre	Post
Number of patients	24	23
IL-1β	0.09 (0–0.26)	0.02 (0–1.01)
IL-2	0 (0–0)	0 (0–11.58)
IL-4	0 (0–1.45)	0 (0–8.46)
IL-5	0.06 (0–0.85)	0.75 (0.03–1.77)
IL-6	16.45 (9.33–40.61)	28.01 (12.17–44.76)
IL-7	0 (0–0)	0 (0–1.88)
IL-8	7.41 (0–17.29)	8.1 (0–27.45)
IL-10	0.63 (0.11–1.65)	1.13 (0.39–1.96)
IL-12	0 (0–0)	0 (0–0)
IL-13	0.24 (0–2.01)	1.21 (0–4.69)
IL-17	0 (0–0)	0 (0–0)
IFN-γ	0 (0–1.05)	0 (0–14.01)
TNF-α	0.74 (0–1.91)	1.02 (0.07–3.04)
G-CSF	0 (0–17.61)	0 (0–0)
GM-CSF	0 (0–0)	0 (0–0)
MCP-1	0 (0–20.82)	37.45 (0–51.62)
MIP-1β	58.26 (25.49–95.0)	55.71 (32.65–121.42)

Values are expressed as median (interquartile range).
*: Significant difference between pre and post.

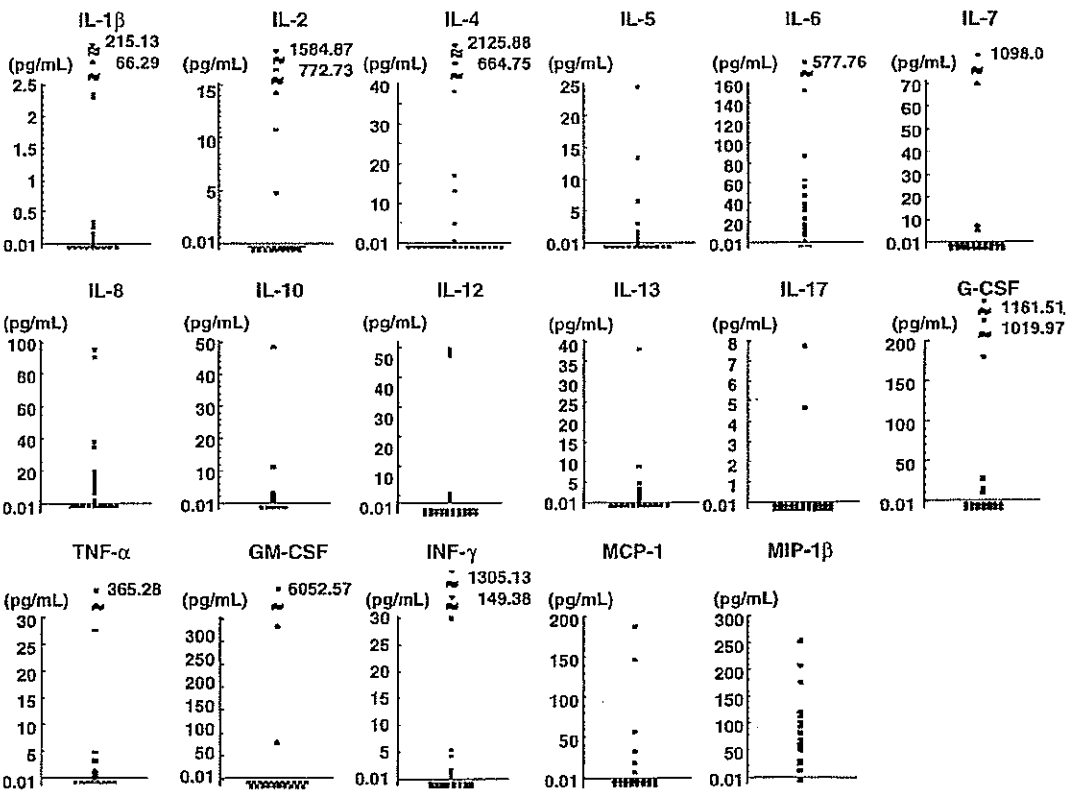

Fig. 1 Plasma concentrations of seventeen cytokines before the commencement of gefitinib in 24 patients. The plots under the line of 0.01 indicate levels lower than the measurement sensitivities.

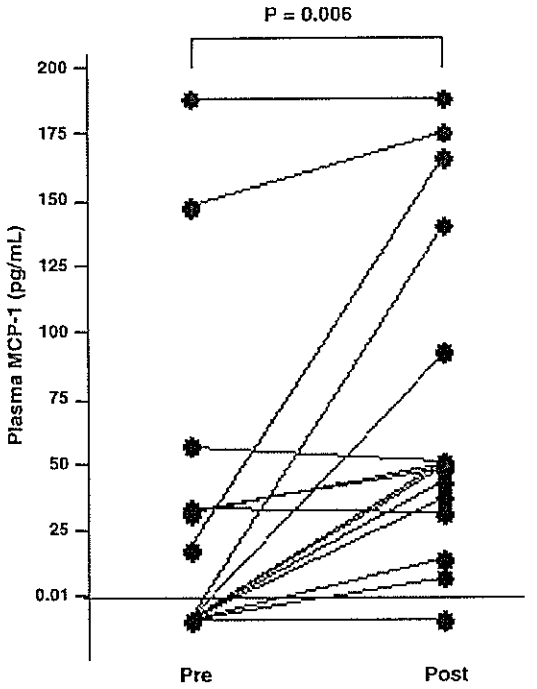

Fig. 2 Plasma concentrations of MCP-1 in 23 patients before and after gefitinib treatment. The differences between the values before and after treatment were significant ($p < 0.05$, paired t-test).

samples obtained after treatment than in those obtained before treatment ($p = 0.006$, by t-test, Fig. 2). There were no significant differences in the levels of any of the other cytokines measured.

3.3. Correlations between cytokine levels and the pharmacodynamic effects of gefitinib

The correlations between the cytokine levels and the clinical features of the patients, including the tumor response, symptomatic improvement, and the development of adverse events, were investigated using the two-sample t-test with a random variance model. There was no significant association between the levels of the various cytokines and the tumor response and symptomatic improvement in any of the patients. When the cytokine levels were comparatively analyzed depending on the grade of adverse events, the patients with skin toxicity (≧ Grade 1) showed significantly lower levels of MIP-1β as compared with those without skin toxicity (Grade 0) ($p = 0.042$, by two-sample t-test, Fig. 3). There was also a trend towards lower levels of IL-8 and IL-4, although the differences were not significant. In addition, the K-nearest neighbor prediction analysis ($K = 3$) showed the classification

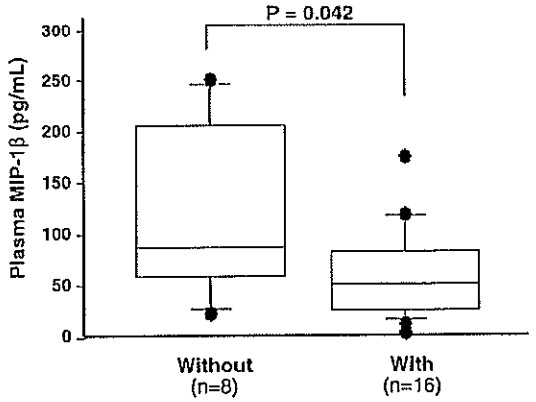

Fig. 3 Box-plots comparing the plasma concentrations of MIP-1β between patients with and without dermatitis; these samples were collected before the initiation of gefitinib. The patients were separated into two groups according to whether or not they developed dermatitis. Patients with skin toxicity showed significantly lower levels of MIP-1β as compared with those without skin toxicity ($p = 0.042$). The top and bottom quartiles and the mean values are depicted as box-plots. Bars indicate the 5th and 95th percentiles of the MIP-1β values.

rate to be 75% for the prediction sets containing MIP-1β, IL-4, and IL-8. There were no significant associations between the cytokine levels and any other parameters.

4. Discussion

The results of this study suggest that a correlation might exist between the serum levels of MIP-1β and the risk of development of skin toxicity during gefitinib administration, with lower levels of the cytokine being associated with a higher risk of appearance of the skin toxicity.

Skin toxicity has been reported to occur commonly in patients treated with EGFR-targeted agents, such as gefitinib, elrotinib, and cetuximab [6–14]. Numerous clinical studies have shown that skin toxicity is more frequently observed as compared to other toxicities during the administration of these drugs. Some studies have described the two major histological findings of the skin toxicity, as follows: presence of keratin plugs with microorganisms in dilated infundibula, and, purulent folliculitis surrounded by an infiltrate composed of lymphocytes and histiocytes, with the superficial portions of some follicles showing dense infiltration with neutrophilic granulocytes [9,31]. The skin toxicity induced by gefitinib has been reported to be similar to that induced by other EGFR-targeted agents, and is believed to result from direct

interference by the drug of the functions of EGFR signaling in the skin [32]. Since the blood levels of cytokines generally reflect the status of immune responses, these histological findings may suggest that the skin toxicity would be correlated with the plasma levels of some cytokines. On the other hand, in normal human skin, EGFR is expressed in the basal epidermal keratinocytes, sweat gland apparatus, and the hair follicle epithelium [33,34]. Therefore, the skin toxicity appears to be related to the mechanism of action of the EGFR-targeted agents and not to allergic reactions [35,36]. These characteristic changes, such as acneform eruptions and skin rashes, are probably secondary to an aberrant differentiation of suprabasal keratinocytes caused by EGFR inhibition.

The results in this study that lower level of plasma MIP-1β were correlated with skin toxicities. MIP-1β is a cysteine–cysteine chemokine that plays a role in inflammation and host defense mechanisms by interacting with its specific receptor CCR1, CCR5 and CCR8 [37,38]. MIP-1β is produced by monocytes, macrophages, lymphocytes and other cell types [39]. MIP-1β is closely related with inflammatory and immune responses. Then, we can arise two possible explanations to our evidence. Immune responses mediated by MIP-1β may play a rule in the healing process of keratinocytes damaged by EGFR-targeted agents. Another is that MIP-1β or its related factors may weaken the inhibiting power of the EGFR-targeted agents, although there is no supporting data for the speculations. Further studies are necessary to clarify the role of MIP-1β for cutaneous reactions.

In this study, 17 kinds of cytokine levels were measured using the bead-based multiplex assay. All of the cytokines were measurable with high sensitivity at once using 15 μl plasma sample volume. It is often difficult to obtain the tumor samples from the advanced non-small cell lung cancer patients. Then, the bead-based multiplex assay might be a useful assay system for biomarkers. This assay system is also able to be customized to detect phosphoproteines such as EGFR and ERK1/2 for the predictive marker for clinical response as the next step.

In conclusion, our results indicate that the plasma MIP-1β level may be a useful predictor of the risk of skin toxicity induced by EGFR-specific tyrosine kinase inhibitors.

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Small In-Frame Deletion in the Epidermal Growth Factor Receptor as a Target for ZD6474

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ABSTRACT

ZD6474 is an inhibitor of vascular endothelial growth factor receptor-2 (VEGFR-2/KDR) tyrosine kinase, with additional activity against epidermal growth factor receptor (EGFR) tyrosine kinase. ZD6474 inhibits angiogenesis and growth of a wide range of tumor models *in vivo*. Gefitinib ("Iressa") is a selective EGFR tyrosine kinase inhibitor that blocks signal transduction pathways implicated in cancer cell proliferation. Here, the ability of gefitinib and ZD6474 to inhibit tumor cell proliferation was examined directly in eight cancer cell lines *in vitro*, and a strong correlation was noted between the IC_{50} values of gefitinib and ZD6474 ($r = 0.79$). No correlation was observed between the sensitivity to ZD6474 and the level of EGFR or VEGFR expression. The NSCLC cell line PC-9 was seen to be hypersensitive to gefitinib and ZD6474, and a small (15-bp) in-frame deletion of an ATP-binding site (exon 19) in the EGFR was detected (delE746-A750–type deletion). To clarify the involvement of the deletional mutation of EGFR in the cellular sensitivity to ZD6474, we examined the effect of this agent on HEK293 stable transfectants expressing deletional EGFR that designed as the same deletion site observed in PC-9 cells (293-pΔ15). These cells exhibited a 60-fold higher sensitivity to ZD6474 compared with transfectants expressing wild-type EGFR. ZD6474 inhibited the phosphorylation of the mutant EGFR by 10-fold compared with cells with wild-type EGFR. In conclusion, the findings suggested that a small in-frame deletion in the EGFR increased the cellular sensitivity to ZD6474.

INTRODUCTION

Gefitinib ("Iressa") is an orally active, selective EGFR-tyrosine kinase inhibitor that blocks the signal transduction pathways implicated in the proliferation and survival of cancer cells and other host-dependent processes promoting cancer cell growth (1–3). Mutation of the EGFR tyrosine kinase in human non–small-cell lung carcinoma (NSCLC) and hyperresponsiveness to gefitinib in patients with NSCLC with this mutation recently were reported (4, 5). The mutations were small, in-frame deletions or substitutions clustered around the ATP-binding site in exons 18, 19, and 21 of the EGFR. The mutant receptors were significantly more sensitive to gefitinib than the wild-type receptor (IC_{50} 0.015 versus 0.1 μmol/L). However, of the 95 other primary tumors and 108 cell lines derived from other tumor types studied, none showed any mutations of this receptor (4). Conversely, Ohm *et al.* (6) reported that all four patients with gefitinib-responsive NSCLC were shown to have mutations of the EGFR near the ATP-binding site compared with none of seven cases showing no response to this drug. These results clearly suggest that the EGFR mutation may be a strong determinant of the tumor response to gefitinib.

ZD6474 is an inhibitor of VEGFR-2 and EGFR signaling that inhibits angiogenesis and tumor growth in a diverse range of tumor models (7). We previously have shown that the NSCLC cell line PC-9 is hypersensitive to gefitinib, with an IC_{50} value of ~0.02 μmol/L (8, 9). It subsequently was established that the PC-9 cells also showed hypersensitivity to ZD6474.

In this report, we discuss the presence of an EGFR deletional mutation and its ability to determine sensitivity to ZD6474.

MATERIALS AND METHODS

Reagents. ZD6474 and gefitinib (Iressa) were provided by AstraZeneca (Cheshire, United Kingdom).

Cell Culture. The human NSCLC cell lines PC-9 and PC-14 were established at the Tokyo Medical University (10, 11). The human epidermal carcinoma cell line A431, breast carcinoma cell line SK-BR-3, ovarian carcinoma cell line SK-OV-3, and colon carcinoma cell lines WiDr and LoVo were obtained from the American Type Culture Collection (Manassas, VA). The SBC-3 cells were supplied by Okayama University School of Medicine. All of the cell lines were maintained in Roswell Park Memorial Institute 1640 medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Rockville, MD), except for the LoVo (F12; Nissui Pharmaceutical, Tokyo, Japan), WiDr (modified Eagle's medium; Nissui Pharmaceutical), and A431 cells (Dulbecco's modified Eagle's medium; Nissui Pharmaceutical). The HEK293 cell line was obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS.

***In vitro* Growth-Inhibition Assay.** The cell growth-inhibitory effect of gefitinib and ZD6474 was determined using the thiazolyl blue tetrazolium bromide (MTT) assay (Sigma). Briefly, 180 μL/well of the cell suspension were seeded onto Sumilon 96-well microculture plates (Sumitomo Bakelite, Akita, Japan) and incubated in 10% FBS-containing medium for 24 hours. The cells were treated with gefitinib or ZD6474 at various concentrations (4 nmol/L to 80 μmol/L) and cultured at 37°C in a humidified atmosphere for 72 hours. After the culture period, 20 μL of MTT reagent were added, and the plates were further incubated for 4 hours. After centrifugation of the plates, the culture medium was discarded, and wells were filled with dimethyl-sulfoxide. The absorbance of the cultures was measured at 562 nm using Delta-soft on a Macintosh computer (Apple, Cupertino, CA) interfaced to a Bio-Tek Microplate Reader EL-340 (BioMetallics, Princeton, NJ). This experiment was conducted in triplicate. The statistical analysis was performed using KaleidaGraph (Synergy Software, Reading, PA).

Plasmid Construction and Transfection. Construction of expression plasmid vector of mock (empty vector), wild-type EGFR, and the 15-bp deletional EGFR (delE746-A750–type deletion; ref. 4) that possess the same deletion site observed in PC-9 cells (Fig. 2*A*) in detail was described in another paper.[4] The plasmids were transfected into the HEK293 cells, and the transfectants were selected by Zeosin (Sigma). The stable transfectants (pooled cultures) of the empty vector, wild-type EGFR, and its deletion mutant were designated as Mock, 293-pEGFR, and 293-pΔ15, respectively.

Immunoblot Analysis. Immunoblot analysis was performed as described previously (3). EGFR antibody was purchased from Santa Cruz Biotechnology (no. 1005; Santa Cruz, CA) and Cell Signaling (Beverly, MA). Phospho-EGFR antibody (specific for Tyr-1068), human epidermal growth factor receptor 2, p44/p42 mitogen-activated protein kinase (MAPK), phospho-p44/p42 MAPK, AKT, phospho-AKT, and antirabbit horseradish peroxidase–conjugated antibody all were purchased from Cell Signaling. The transfected cells cultured in

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[4] Unpublished observation.

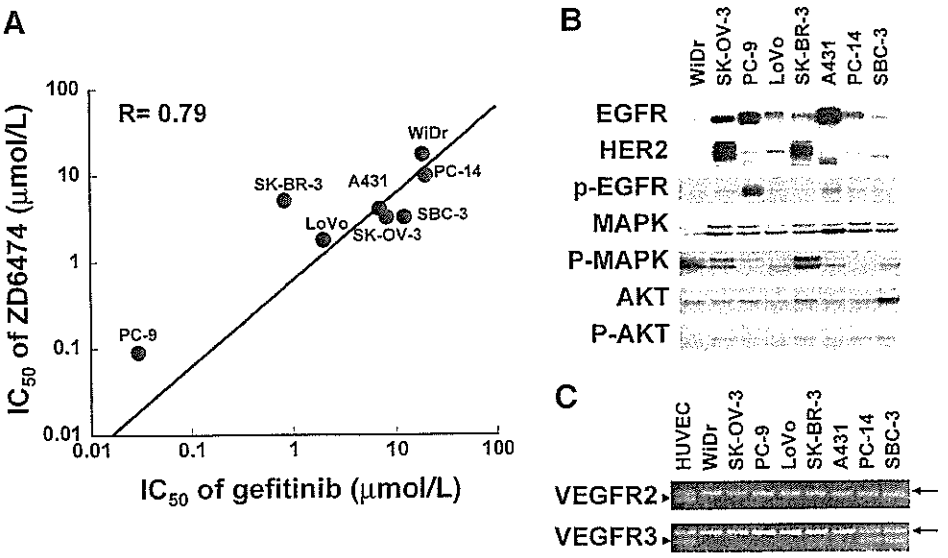

Fig. 1. The cellular characteristics and growth-inhibitory effect of gefitinib and ZD6474. *A*, correlation plot of the IC_{50} values of gefitinib and ZD6474 in human cancer cell lines. The growth-inhibitory effect against PC-9, WiDr, LoVo, PC-14, A431, SK-OV-3, SK-BR-3, and SBC-3 cells was determined by MTT assay (72-hour exposure). The data were obtained from three independent experiments. *B*, expression and phosphorylation status of EGFR and downstream molecules in human cancer cell lines. Data were obtained by immunoblot analysis with anti-EGFR, anti–phospho-EGFR, anti-HER2, anti–phospho-p44/p42 MAPK, anti-p44/p42 MAPK, anti-AKT, anti–phospho-AKT, and anti-AKT. *C*, The mRNA expression level of VEGFR-2 and VEGFR-3 was determined by reverse transcription-PCR. Human umbilical vascular endothelial cell (*HUVEC*) was used as the positive control. Whereas VEGFR-2 expression was not detected in any of the cancer cell lines, VEGFR-3 expression was detected in the PC-14 and SBC-3 cells; *arrows*, β-actin; *arrowheads*, VEGFR-2 or VEGFR-3.

the serum-free medium for 24 hours were stimulated by the addition of EGF (Sigma) at a final concentration of 10 ng/mL. After a 30-minute incubation, the cells were incubated for an additional 3 hours in the presence of ZD6474 and then collected for immunoblot analysis. The subconfluent cancer cell lines were cultured in medium containing 10% FBS and collected for immunoblot analysis.

Reverse-Transcription PCR. Five micrograms of total RNA from each cultured cell line were converted to cDNA using a GeneAmp RNA-PCR kit (Applied Biosystems, Foster City, CA). The primers used for the PCR were as follows: VEGFR-2, 5′-CAGACGGACAGTGGTATGGTTC-3′ (forward) and 5′-ACCTGCTGGTGGAAAGAACAAC-3′ (reverse); and VEGFR-3, 5′-AGCCATTCATCAACAAGCCT-3′ (forward) and 5′-GGCAACAGCTG-GATGTCATA-3′ (reverse). As a control, the following human β-actin primers were used: 5′-GGAAATCGTGCGTGACATT-3′ and 5′-CATCTGCTG-GAAGGTGGACAG-3′. PCR amplification consisted of 35 cycles (95°C for 45 seconds, 62°C for 45 seconds, and 72°C for 60 seconds) followed by incubation at 72°C for 7 minutes. The bands were visualized by ethidium bromide staining.

Sequencing. Sequencing of exons 18 through 21 of EGFR cDNA in the tumor cell lines was performed. The cDNAs were amplified using the following primers: 5′-TCCAAACTGCACCTACGGATGC-3′ (forward) and 5′-CATCAACTCCCAAACGGTCACC-3′ (reverse). PCR amplification consisted of 25 cycles (95°C for 45 seconds, 55°C for 30 seconds, and 72°C for 60 seconds). The sequences of the PCR products were determined using ABI prism 310 (Applied Biosystems). Amplification and sequencing were performed in duplicate for each tumor cell line. The sequences were compared with the GenBank-archived human sequence of EGFR (accession no. NM 005228.3).

RESULTS

Growth-Inhibitory Activity of Gefitinib and ZD6474. We examined the *in vitro* growth-inhibitory activities of gefitinib and ZD6474 on eight cancer cell lines by MTT assay. The IC_{50} values of gefitinib and ZD6474 for each cell line were compared and plotted as shown in Fig. 1*A*. Good correlation ($r = 0.79$) was observed between the IC_{50} values of gefitinib and ZD6474, suggesting that the mechanisms underlying the growth-inhibitory activities of the two drugs *in vitro* might be similar. To clarify the correlation between the cellular sensitivity for gefitinib and ZD6474 and the EGFR status, we examined the expression and phosphorylation levels of EGFR in the cell lines by immunoblot analysis (Fig. 1*B*). No correlation was found between the expression status or the phosphorylation level of EGFR and the IC_{50} value of either drug. There also was no correlation between the cellular sensitivity and the phosphorylation status of any downstream molecules, such as phosphorylated MAPK and phospho-AKT (Fig. 1*B*). To determine the correlation between the VEGFR expression levels and cellular sensitivity, we examined the mRNA levels of the VEGFR-2 and VEGFR-3 in the cell lines by reverse transcription-PCR and detected VEGFR-3 transcripts in PC-14 and SBC-3 cells (Fig. 1*C*). VEGFR-2 was not detectable in all of the cancer cell lines. The results suggested that there was no correlation between the cellular sensitivity to ZD6474 and the VEGFR-2 and VEGFR-3 expression level. Among all of the cell lines examined, the PC-9 cell line was found to be hypersensitive to gefitinib (IC_{50} = 0.03 ± 0.002 μmol/L) and ZD6474 (IC_{50} values = 0.09 ± 0.01 μmol/L). The respective IC_{50} values of gefitinib and ZD6474 for the other cell lines were as follows: WiDr, 18.7 ± 2.5 μmol/L and 17.7 ± 2.3 μmol/L; SK-OV-3, 8.3 ± 1.5 μmol/L and 3.3 ± 0.2 μmol/L; LoVo, 2.0 ± 0.3 μmol/L and 1.8 ± 0.2 μmol/L; A431, 7.1 ± 0.9 μmol/L and 4.1 ± 0.2 μmol/L; PC-14, 20 ± 2.1 μmol/L and 10 ± 1.2 μmol/L; SK-BR-3, 0.8 ± 0.15 μmol/L and 5.2 ± 0.1 μmol/L; and SBC-3, 12.3 ± 2.1 μmol/L and 3.3 ± 0.3 μmol/L.

Fifteen-Base Pair In-Frame Deletion of EGFR in PC-9 Cells. To determine the cellular determinants of the hypersensitivity of the PC-9 cells to gefitinib, we determined the sequence of the EGFR mRNA in the PC-9 cells. The analysis revealed a 15-bp in-frame deletion around the ATP-binding site in exon 19 (Fig. 2*A*). No deletion or mutation was found in the other cell lines. The 15-bp in-frame deletion in the EGFR was consistent with the observations of Ohm *et al.* (6) in four patients with lung cancer.

Deletional Mutation of EGFR Increases the Cellular Sensitivity to ZD6474. We hypothesized that the cellular hypersensitivity of the PC-9 cells to ZD6474 was attributable to the deletional mutation of EGFR in these cells. To confirm the validity of this hypothesis, we examined ZD6474 sensitivity to HEK293 transfectant expressing the 15-bp deletion mutant EGFR or wild-type EGFR. The sequencing of EGFR cDNA obtained from 293-pEGFR and 293-pΔ15 cells was shown (Fig. 2*B*). The sensitivity of the transfectants was examined by 72-hour exposure of ZD6474 using MTT assay. The 293-pΔ15 cells were found to be 60-fold more sensitive to ZD6474 than the mock and wild-type EGFR transfectants (Fig. 3*A*). The IC_{50} of ZD6474 for the 293-pΔ15 cells, 293-pEGFR cells, and the mock transfectants were 0.08, 5.2, and 6.3 μmol/L, respectively.

The EGFR expression levels in the transfectants were quantified by immunoblot analysis using anti-EGFR antibody recognizing the

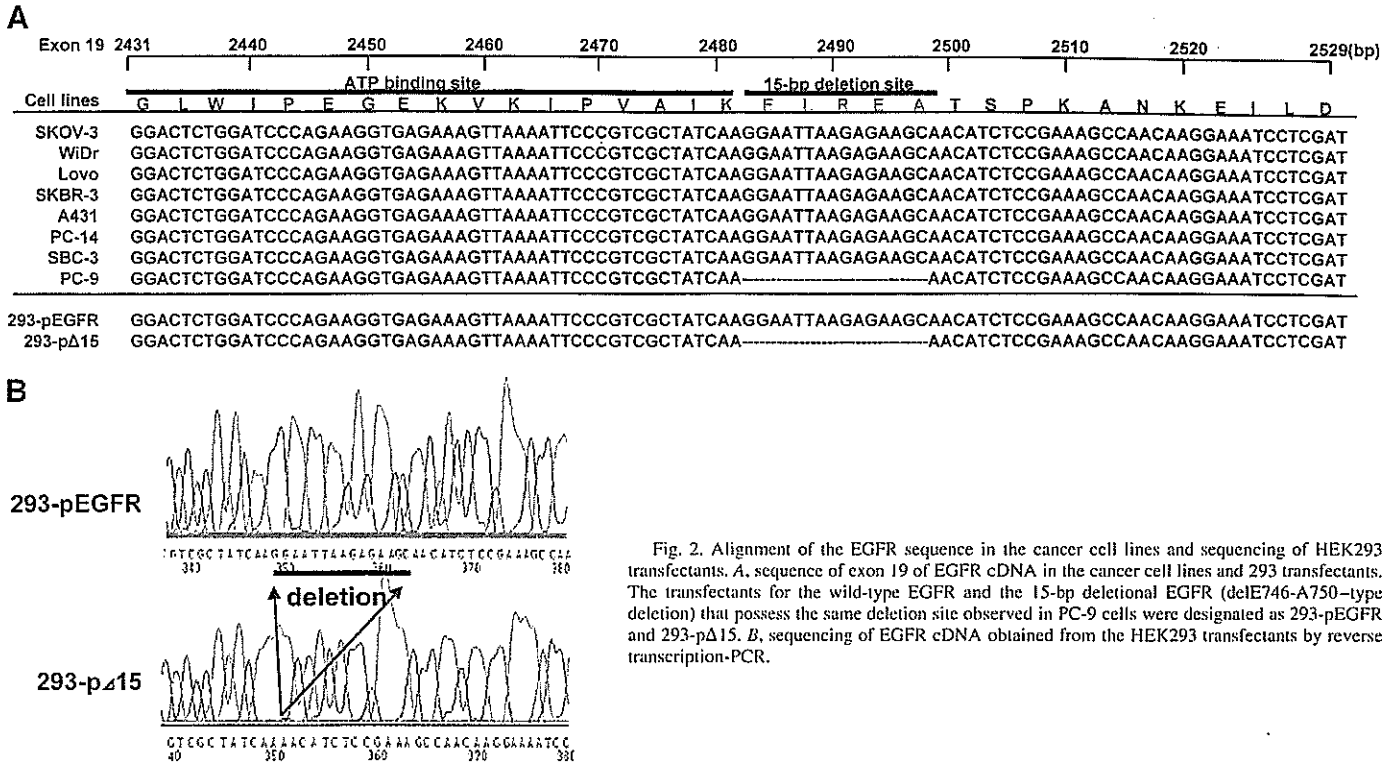

Fig. 2. Alignment of the EGFR sequence in the cancer cell lines and sequencing of HEK293 transfectants. *A*, sequence of exon 19 of EGFR cDNA in the cancer cell lines and 293 transfectants. The transfectants for the wild-type EGFR and the 15-bp deletional EGFR (delE746-A750–type deletion) that possess the same deletion site observed in PC-9 cells were designated as 293-pEGFR and 293-pΔ15. *B*, sequencing of EGFR cDNA obtained from the HEK293 transfectants by reverse transcription-PCR.

COOH-terminus of EGFR. High expression of EGFR proteins was detected in the 293-pΔ15 cells and 293-pEGFR cells but not in the mock cells (Fig. 3*B*). Exposure to ZD6474 did not affect the expression of levels of either the wild-type or the mutant EGFR. EGFR status was quantified by measuring the phosphorylation level of the Tyr-1068 residue, commonly used as a marker of the autophosphorylation of EGFR (12).

Under the condition of serum starvation, wild-type EGFR did not show any autophosphorylation, whereas the addition of EGF activated the receptor. However, marked autophosphorylation of the mutant EGFR was observed, even without the addition of EGF (Fig. 3*B*). ZD6474 exposure inhibited the phosphorylation of wild-type EGFR and mutant EGFR in a dose-dependent manner, with 2 μmol/L and 0.2 μmol/L of ZD6474 completely inhibiting phosphorylation of the wild-type EGFR and mutant EGFR, respectively. These results suggest that cells expressing the deletion mutant of EGFR are markedly more sensitive to the inhibitory effect of ZD6474 than those expressing wild-type EGFR.

DISCUSSION

Recent reports by Paez and Lynch have indicated that deletional mutations of EGFR impact on the therapeutic effects of the molecular-

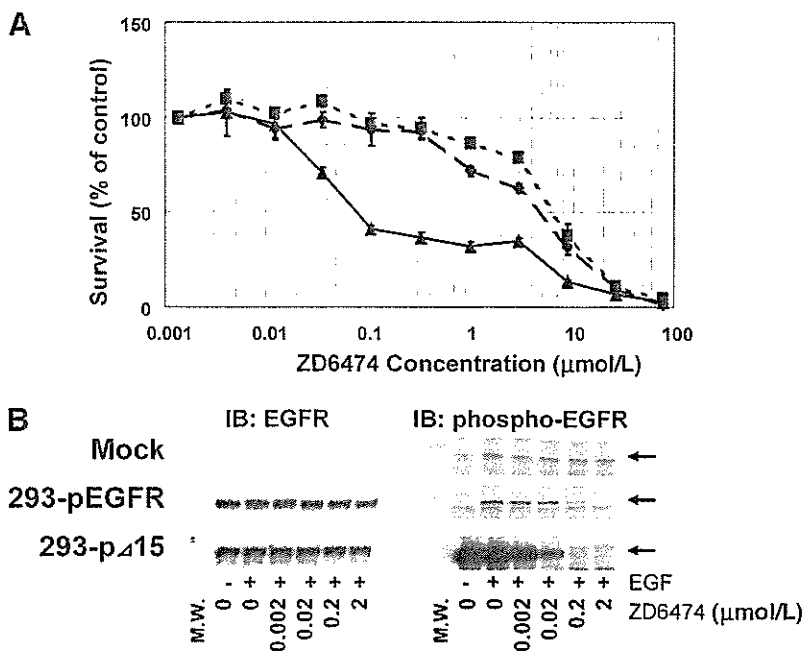

Fig. 3. Effect of ZD6474 on cellular growth inhibition and phosphorylated status of EGFR in the HEK293 transfectants. *A*, The cellular sensitivity of the transfectants against ZD6474 was determined by MTT assay (72-hour exposure). The mean values and SD represent the values obtained from the growth-inhibition curves in three independent experiments; ◆, mock (empty vector); ■, 293-pEGFR (wild-type EGFR); ▲, 293-pΔ15 (deletional-mutant EGFR). *B*, effect of EGF stimulation and ZD6474 exposure on mock, wild-type EGFR, and deletional mutant EGFR-transfected HEK293 cells determined by immunoblot analysis. Cells cultured under serum starvation for 24 hours were exposed to 10 ng/mL EGF for 30 minutes and then treated with 0.002 to 2 μmol/L ZD6474 for 3 hours in the presence or absence of EGF. *Left*, EGFR expression levels; *right*, EGFR phosphorylation levels.

targeted EGFR inhibitor gefitinib (4, 5). Here, we show that a 15-bp deletional mutation residing near the ATP binding site of EGFR in cancer cells also increases the sensitivity of the cells to ZD6474.

ZD6474 is a small molecule inhibitor of VEGFR-2 tyrosine kinase that is in Phase II clinical evaluation. *In vivo*, this compound inhibits VEGF signaling, tumor-induced angiogenesis, and the growth of a histologically diverse panel of tumor xenografts. This includes highly significant activity against tumor xenografts with intrinsic or acquired resistance to EGFR inhibitors (13). However, ZD6474 also has activity against EGFR tyrosine kinase that may give additional therapeutic benefit when tumors have a high dependency on EGFR signaling for growth and/or survival. This has been shown in PC-9 cells that are hypersensitive to treatment with gefitinib (9). PC-9 tumor cells also are hypersensitive to ZD6474 *in vitro* and regress in response to ZD6474 treatment when grown as tumor xenografts *in vivo* (14).

We have shown that PC-9 cells contain a 15-bp in-frame deletional mutation in EGFR, and this mutation may confer increased sensitivity to ZD6474 and gefitinib. The difference in ZD6474 concentration required for complete inhibition of wild-type and mutant EGFR phosphorylation was relatively small (2 versus 0.2 μmol/L), whereas difference in sensitivity to ZD6474 was large (60-fold).

The deletional EGFR was constitutively phosphorylated, and the addition of EGF to the cultures did not result in any additional increase in phosphorylation (Fig. 3*B*). These observations contradict data reported by Lynch *et al.* (4), who showed that a receptor with a similar deletion was still regulated by EGF.

The most possible explanation for this contradiction is that the expression level of deletional EGFR in the 293-pΔ15 cells is much higher than that of the transient transfectant of Del L747-P753insS reported by Lynch *et al.* Ligand-independent oligomerization of the receptor and phosphorylation may have occurred in the 293-pΔ15 cells as a result. This hypothesis is consistent with the result that PC-9 cells harboring the same 15-bp deletion showed a stronger phosphorylation of the EGFR in a 10% FBS medium than other nonhypersensitive cell lines (Fig. 1*B*).

The other possible explanation is that apparent distinct amino acid sequences of EGFR exist between our mutant and that of Lynch *et al.* (293-pΔ15, VAIKELREATSPK>VAIKTSPK; delL747-P753insS, VAIKELREATSPK>VAIKESK). Five amino acids are simply deleted in the 293-pΔ15 cells, whereas six amino acids are deleted and serine is inserted in the delL747-P753insS cells. This small difference may be critical to the ATP-binding properties of 293-pΔ15 and delL747-P753insS, determining whether EGFR is constitutively active. Therefore, it is not surprising that our constitutive active form of EGFR is out of ligand regulation.

The mock-transfected 293 cells and 293-pEGFR cells were not sensitive to the growth-inhibitory effect of ZD6474 (Fig. 3*A*), indicating that these cells were independent of EGFR signaling. The 293 cells are oncogenic transformant. Therefore, the 293 cells were considered to have acquired the dependency on the oncogenic signal. Conversely, the overexpression of the deletional EGFR transduces the excess signal to downstream of EGFR in the 293-pΔ15 cells. If the downstream mutant EGFR signaling pathway were shared with that of the oncogenic signaling pathway in the cells, the excess and constitutive signal from the mutant EGFR would dominate the downstream pathway, possibly influencing the dependency of the cells on the EGFR signal.

A recent report by Sordella *et al.* (15) showed the mutant EGFRs (delL747-P753insS and L858R) expressing a stable transfectant selectively activate AKT and STAT signaling pathways. They also showed that NSCLC cell lines that harboring mutant EGFR transduce survival signals and depend on the acquisition of these signals. Their evidence is consistent with our present speculations. We now are investigating the downstream pathways of the mutant EGFR signaling in the 293-pΔ15 cells.

In summary, inhibition of VEGFR-2 tyrosine kinase by ZD6474 may potentially confer activity against tumors that are not dependent on EGFR signaling. Nevertheless, the additional activity of ZD6474 against EGFR tyrosine kinase could provide further benefit, particularly when EGFR is mutated. Patients with lung adenocarcinoma showing EGFR mutations are likely to be highly sensitive to gefitinib and ZD6474 treatment.

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Anticancer effects of ZD6474, a VEGF receptor tyrosine kinase inhibitor, in gefitinib ("Iressa")-sensitive and resistant xenograft models

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ZD6474 is a novel, orally available inhibitor of vascular endothelial growth factor (VEGF) receptor-2 (KDR) tyrosine kinase, with additional activity against epidermal growth factor receptor (EGFR) tyrosine kinase. ZD6474 has been shown to inhibit angiogenesis and tumor growth in a range of tumor models. Gefitinib ("Iressa") is an selective EGFR tyrosine kinase inhibitor (TKI) that blocks signal transduction pathways. We examined the antitumor activity of ZD6474 in the gefitinib-sensitive lung adenocarcinoma cell line, PC-9, and a gefitinib-resistant variant (PC-9/ZD). PC-9/ZD cells showed cross-resistance to ZD6474 in an *in vitro* dye formation assay. In addition, ZD6474 showed dose-dependent inhibition of EGFR phosphorylation in PC-9 cells, but inhibition was only partial in PC-9/ZD cells. ZD6474-mediated inhibition of tyrosine residue phosphorylation (Tyr992 and Tyr1045) on EGFR was greater in PC-9 cells than in PC-9/ZD cells. These findings suggest that the inhibition of EGFR phosphorylation by ZD6474 can contribute a significant, direct growth-inhibitory effect in tumor cell lines dependent on EGFR signaling for growth and/or survival. The effect of ZD6474 (12.5–50 mg/kg/day p.o. for 21 days) on the growth of PC-9 and PC-9/ZD tumor xenografts in athymic mice was also investigated. The greatest effect was seen in gefitinib-sensitive PC-9 tumors, where ZD6474 treatment (>12.5 mg/kg/day) resulted in tumor regression. Dose-dependent growth inhibition, but not tumor regression, was seen in ZD6474-treated PC-9/ZD tumors. These studies demonstrate that the additional EGFR TKI activity may contribute significantly to the antitumor efficacy of ZD6474, in particular in those tumors that are dependent on continued EGFR-signaling for proliferation or survival. In addition, these results provide a preclinical rationale for further investigation of ZD6474 as a potential treatment option for both EGFR-TKI-sensitive and EGFR-TKI-resistant tumors. (Cancer Sci 2004; 95: 984–989)

ZD6474 is a novel, orally available inhibitor of VEGF receptor-2 (KDR) tyrosine kinase, with additional activity against EGFR tyrosine kinase, and it inhibits angiogenesis and tumor growth in a diverse range of tumor models.[1,2] Phase I clinical evaluation has shown ZD6474 to be generally well tolerated, and tumor responses in patients with non-small cell lung cancer (NSCLC) have been documented.[3,4] Thus, ZD6474 is considered to be a multi-target tyrosine kinase inhibitor active against solid tumors. The purpose of this study is to clarify the mode of antitumor action of ZD6474 as compared with that of gefitinib ("Iressa," ZD1839). Gefitinib is an orally active, selective EGFR tyrosine kinase inhibitor (EGFR-TKI) that blocks signal transduction pathways implicated in the proliferation and survival of cancer cells and other host-dependent processes promoting tumor growth.[5–7] Gefitinib is now available clinically for non-small cell lung cancer patients. In order to elucidate the mode of action of ZD6474, the antitumor activity and pharmacodynamics were investigated in an established human lung cancer cell line resistant to gefitinib (PC-9/ZD cells).[8] This approach allowed us to clarify the common and differential modes of actions of gefitinib and ZD6474 in lung cancer, and this will be important for deciding how to use ZD6474 in non-small cell lung cancer patients in combination with gefitinib.

Materials and Methods

Reagents and cell culture. ZD6474 and gefitinib ("Iressa," ZD1839) were provided by AstraZeneca (Macclesfield, UK). Human NSCLC cell lines PC-9 and PC-14 were used.[9,10] In addition, a gefitinib-resistant subline, PC-9/ZD, was derived from PC-9 cells by short-term exposure to the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, continuous exposure to 0.2–0.5 μM gefitinib for 28 days, and subcloning. The resistant phenotype has been stable for at least 6 months under drug-free conditions.[8] The PC-9/ZD cell line shows no cross-resistance to conventional anticancer drugs.[8] Cells were maintained in RPMI-1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Grand Island, NY).

Antibodies. Anti-vonWillebrand Factor (vWF) antibody was purchased from Chemicon, Temecula, CA. Affinity-purified antibody to EGFR was purchased from Santa Cruz, CA and affinity-purified antibodies to phospho-EGFR specific for Tyr845, Tyr992, Tyr1045, and Tyr1068 were purchased from Cell Signaling Technology, Beverly, MA.

Growth inhibition assay. Cell sensitivity to ZD6474 and gefitinib was estimated by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously.[11] Briefly, PC-9, PC-9/ZD, or PC-14 cells were exposed to 0–10 μM ZD6474 or gefitinib for 72 h before measuring absorbance. Optical density was assessed at 562–630 nm using an EL340 96-well microtiter plate reader (Bio-Tek, Winooski, VT).

Xenograft studies in athymic mice. Suspensions of PC-9 cells (5×10^6) or PC-9/ZD cells (3×10^6) were injected subcutaneously into the backs of 5-week-old female athymic mice (Japan Charles River Co., Atsugi, Japan). After 1 week (tumors >95 mm^3), mice were randomly allocated into groups of six animals to receive ZD6474 (12.5, 25, or 50 mg/kg/day), gefitinib (12.5, 25, or 50 mg/kg/day) or vehicle only by oral gavage. Tumor diameter and body weight were measured twice weekly. The tumor volume was calculated (width$^2\times$length/2) and is presented as a percentage of the pretreatment value. A tumor volume below 100% of the pretreatment volume was defined as "tumor reduction." Experiments were performed in accordance with the UK Coordinating Committee on Cancer Research Guidelines for the welfare of animals in experimental neoplasia (second edition). After 3 weeks of treatment, tumors were removed.

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Abbreviations: VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; NSCLC, non-small cell lung cancer; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Two tumor specimens per group were processed for immunohistochemical analysis.

Immunohistochemical analysis. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections as reported previously.[1,5] An anti-Ki67 monoclonal antibody (clone MIB1; DBA, Milan, Italy) was used and the proportion of positive (proliferating) cells was assessed. At least 1000 cancer cells were counted and scored per slide. Both the percentage of specifically stained cells and the intensity of immunostaining were recorded. Blood vessels were detected with an anti-von Willebrand Factor (vWF) antibody (Chemicon). Microvessel density was determined by calculating the proportion of vWF-positive cells.

Evaluation of apoptosis (TUNEL). Sections were stained with an *in situ* Death Detection POD Kit (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions. At least 1000 tumor cell nuclei from the most evenly and distinctly labeled areas were examined. The TUNEL-positive tumor cell nuclei were counted, and the apoptotic index was calculated as the proportion of cells with apoptotic nuclei.

Immunoprecipitation and immunoblotting. Cells were maintained in medium without serum for 12 h. Then serum-starved cells were exposed to ZD6474 or gefitinib, incubated for 1 h and stimulated in medium including 10% fetal bovine serum for 30 min. The cells were subsequently washed twice with ice-cold PBS, scraped in lysis buffer (50 m*M* Tris-HCl [pH 8.0], 120 m*M* NaCl, 0.5% Nonidet P-40, 100 m*M* NaF, 200 μ*M* Na_3VO_4, and 10 μg/ml each of aprotinin, leupeptin, and PMSF), and incubated on ice for 60 min. The lysates were centrifuged at 8000*g* for 20 min, and total protein was obtained from the supernatants. Protein concentration was measured with the bicinchoninic acid protein assay (Pierce, Rockford, IL). Cell lysates for immunoprecipitates contained 2 mg of total protein. Anti-EGFR antibody (3 μg) was incubated overnight with the lysates at 4°C, and the precipitates were collected with 40 μliters of Protein G Sepharose beads over a 1 h period. Antibody-complexed proteins were washed with lysis buffer, analyzed by SDS-PAGE and visualized using an enhanced chemiluminescence solution (ECL; Amersham Pharmacia Biotech UK, Buckinghamshire, UK). Quantative analysis was performed using Kodak software. Quantified values of phospho-EGFR bands were standardized according to those of EGFR bands.

Results

***In vitro* evaluation of ZD6474 and gefitinib inhibition of tumor cell growth.** The IC_{50} values of gefitinib for growth inhibition of PC-9 and PC-9/ZD cells were 0.038 μ*M* and 6.8 μ*M*, respectively. The IC_{50} values of ZD6474 were 0.14 μ*M* and 5.92 μ*M*, respectively (Fig. 1A). PC-9 cells were 180-fold more sensitive to gefitinib than PC-9/ZD cells, and PC-9/ZD cells were cross-resistant to ZD6474. Experiments with another VEGFR-TKI, SU5416, and PDGFR-TKI, Tyrphostin 9, revealed no cross-resistance (data not shown).

In a separate experiment, the IC_{50} values of gefitinib were 0.006 μ*M* and 20.5 μ*M*, in PC-9 and PC-14 (another human NSCLC cell line), respectively (Fig. 1B). PC-9 cells were therefore approximately 3400-fold more sensitive to gefitinib than PC-14 cells. Corresponding IC_{50} values for ZD6474 were 0.11 μ*M* and 9.81 μ*M*, demonstrating cross-resistance to ZD6474.

Other workers have examined the ability of gefitinib or ZD6474 to inhibit serum-dependent tumor cell growth *in vitro*, and have demonstrated IC_{50} values of gefitinib[12] and ZD6474[13] of >1 μ*M* for tumor cell lines. Therefore, PC-9 is particularly sensitive to *in vitro* growth inhibition by both gefitinib and ZD6474, whereas the sensitivities of both gefitinib-resistant PC-9/ZD and PC-14 fall within the normal range reported for other tumor cell lines.

***In vivo* antitumor effects.** ZD6474 treatment (12.5–50 mg/kg/day) resulted in inhibition of PC-9 tumor growth, with robust tumor regression seen even at the lowest dose tested. ZD6474 treatment also resulted in dose-dependent inhibition of PC-9/ZD tumor xenograft growth, although in this case, regression was not seen (Fig. 2, A and B). This antitumor effect of ZD6474 was very similarly to that of gefitinib we previously reported (Fig. 2, C and D).[8]

Effect of treatment on cell proliferation, apoptosis, and vascularization. ZD6474 treatment resulted in a dose-dependent decrease in the proportion of proliferating cells in the PC-9 tumors, but not in PC-9/ZD xenografts (Fig. 3). No significant

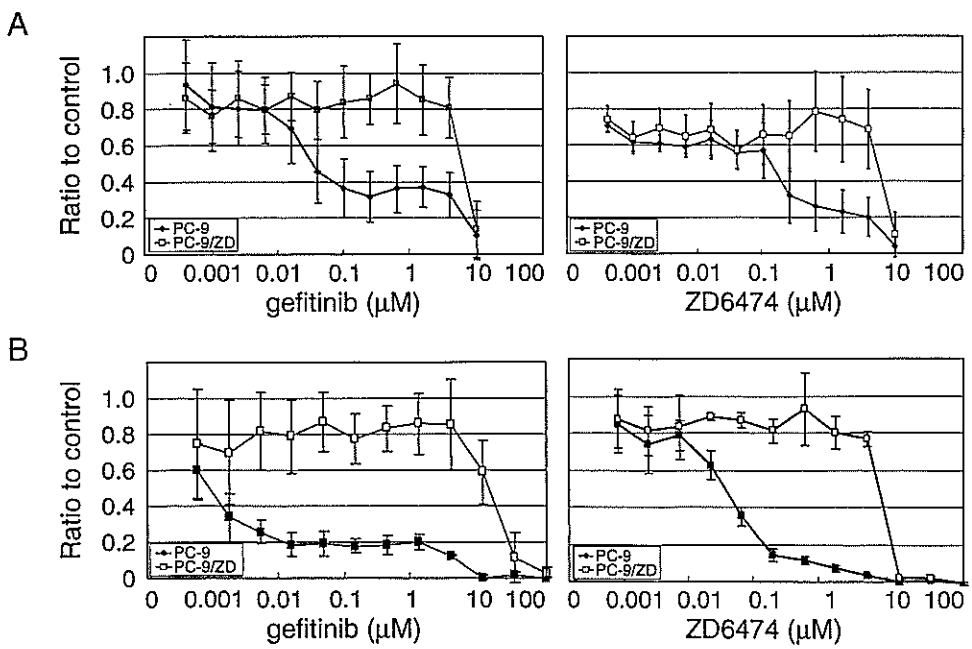

Fig. 1. Growth inhibitory effect of gefitinib (ZD1839) and ZD6474. A: PC-9 and PC-9/ZD, B: PC-9 and PC-14 cells. Data shown are mean values from three experiments (±SD).

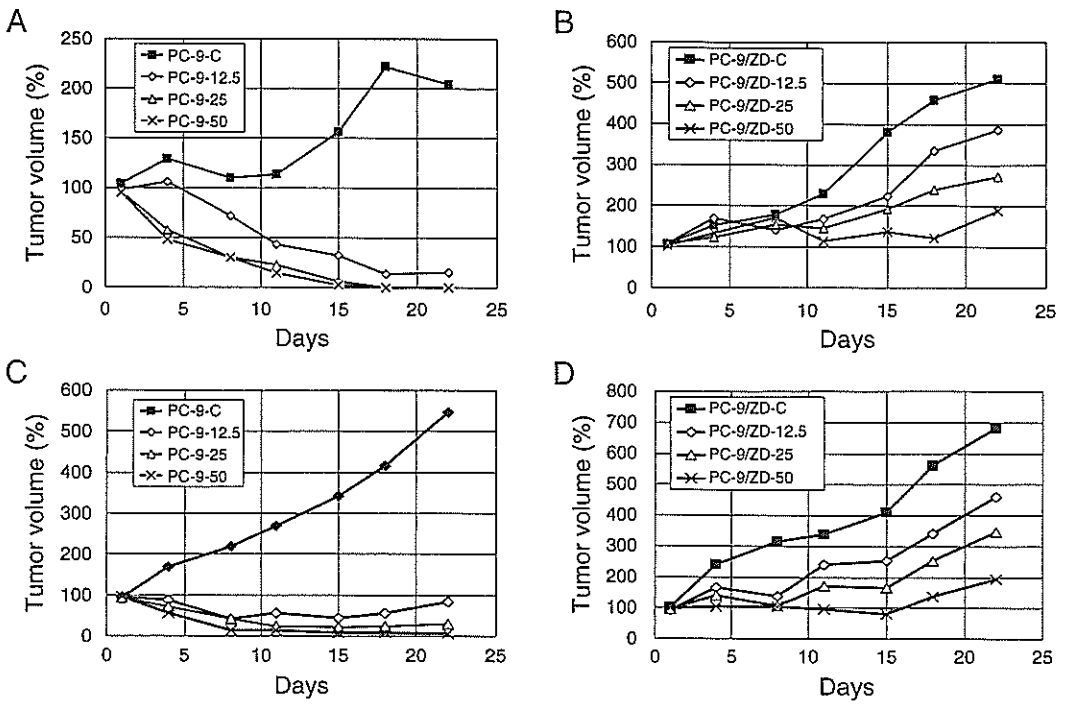

Fig. 2. Antitumor activity of ZD6474 (A, B) and gefitinib (C, D) on established PC-9 (A, C) and PC-9/ZD (B, D) human lung cancer xenografts.

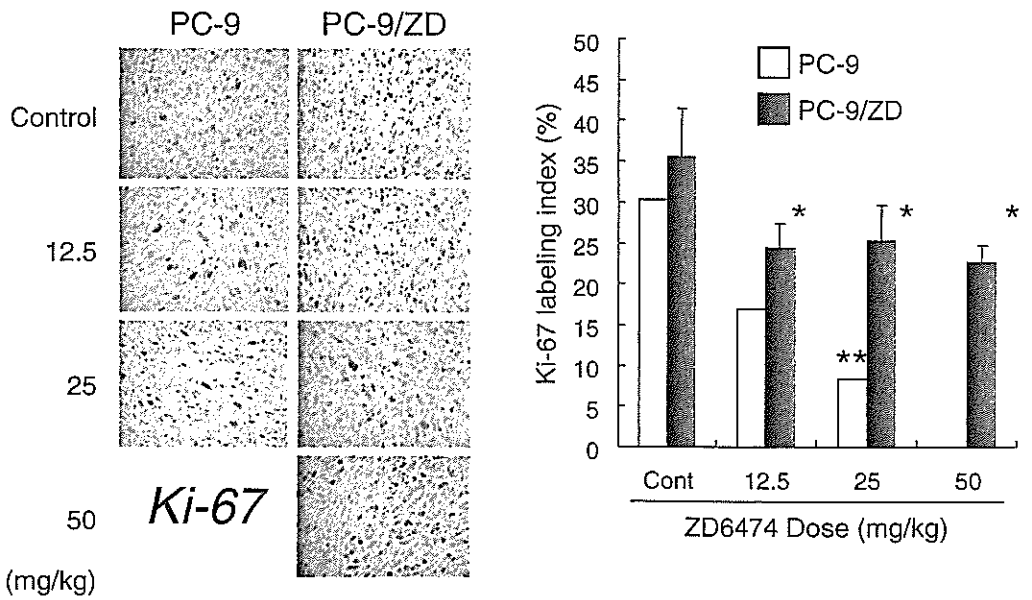

Fig. 3. Effect of ZD6474 on the Ki67 labeling index of PC-9 and PC-9/ZD tumors *in vivo*. Data represent mean values (±SD). Significant difference from control shown by the Dunnett test (* $P<0.05$, ** $P<0.01$).

increase in apoptosis was observed in either tumor type (Fig. 4).

Assessment of tumor vascularization showed a significant reduction in vascular density following ZD6474 treatment of PC-9 tumor xenografts, although no effect was seen in PC-9/ZD tumors (Fig. 5). Differences in the action of ZD6474 on PC-9 and PC-9/ZD tumors are summarized in Table 1.

Inhibition of EGFR activity. It is possible that the antitumor activity of ZD6474 is partly attributable to EGFR inhibition based on the evidence of cross-resistance to gefitinib (Figs. 1–3). Therefore, site-specific anti-phosphorylated-EGFR antibodies were used to investigate inhibition of EGFR phosphorylation by ZD6474 in PC-9 and PC-9/ZD cells at four different tyrosine phosphorylation sites (Tyr845, Tyr992, Tyr1045, and Tyr1068; Fig. 6). ZD6474 dose-dependently inhibited phosphorylation of the four EGFR tyrosine residues in PC-9 cells (Fig. 6). In PC-9/ZD cells, drug-related inhibition of phosphorylation at the Tyr992 site was highly resistant to ZD6474 treatment (Fig. 6), and the Tyr845 and Tyr1045 sites were moderately resistant, while the effect of phosphorylation at the Tyr1068 site did not differ significantly between the sensitive and resistant cell lines (Table 1). The spectrum of activity of ZD6474 on the

four EGFR tyrosine residues examined in PC-9/ZD cells differed from that of gefitinib. ZD6474 displayed a variety of actions on each tyrosine residue, which may be responsible for the wide range of biological activities.

Discussion

In the NSCLC xenograft model reported here, ZD6474 treatment significantly inhibited PC-9 tumor growth, inducing tumor regression. In addition, ZD6474 caused dose-dependent PC-9/ZD tumor growth inhibition. These data indicate that ZD6474 exerts potent antitumor activity against gefitinib-sensitive and resistant lung cancers *in vivo*. Although PC-9/ZD cells are less sensitive to gefitinib than PC-9 cells, the *in vitro* sensitivity of these cells falls within the normal range for other tumor cell lines. Accordingly, gefitinib has significant *in vivo* activity against PC-9/ZD, producing a dose-dependent inhibition of xenograft growth, rather than the tumor regression seen with PC-9 xenografts. Therefore, the antitumor activity of ZD6474 appeared to parallel that of gefitinib in PC-9 and PC-9/ZD tumor cells, both *in vitro* and *in vivo*. Since gefitinib is a TKI with a high degree of selectivity for EGFR,[1,2,4] inhibition of EGFR autophosphorylation is likely to contribute to the antitumor activity of ZD6474, particularly in tumor cells which are dependent on EGFR signaling for continued growth and survival. This was shown *in vitro*, as ZD6474 inhibited EGFR

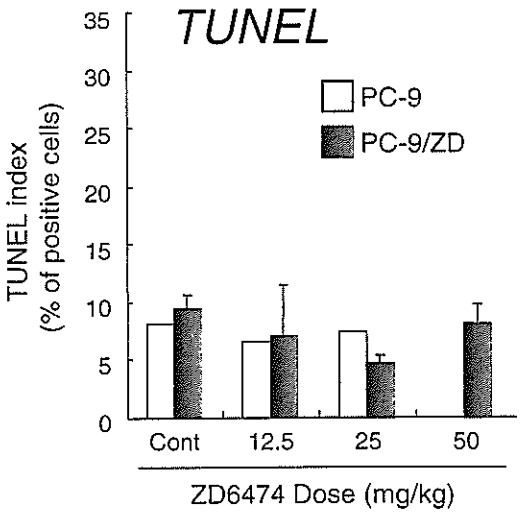

Fig. 4. Effect of ZD6474 on the TUNEL index of PC-9 or PC-9/ZD tumors *in vivo*. Data represent mean values (±SD).

Table 1. Site-specific effect of ZD6474 on EGFR tyrosine residues in PC-9 and PC-9/ZD cells

Tyr residue of EGFR	Inhibition of phosphorylation			
	ZD6474		Gefitinib	
	PC-9	PC-9/ZD	PC-9	PC-9/ZD
845	++	+	++	+
992	++	–	++	++
1045	++	+	–	–
1068	++	++	++	+

++ strong; + moderate; – not significant.

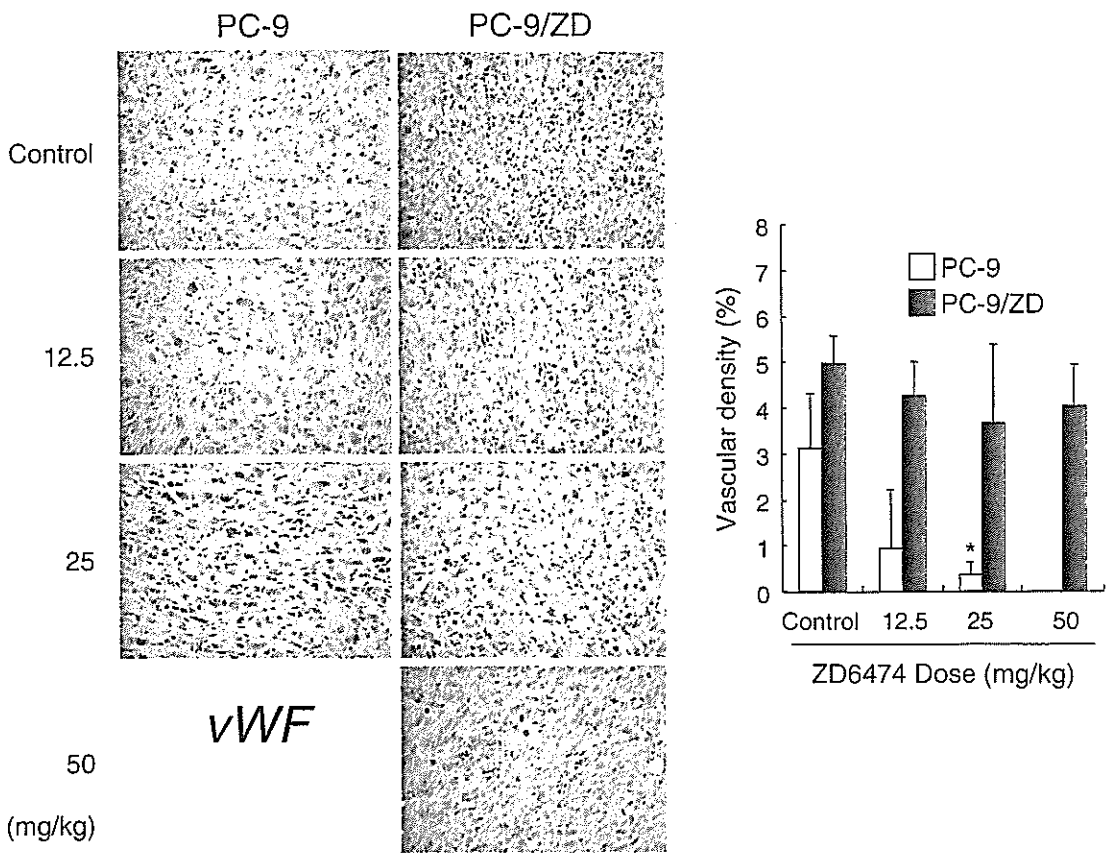

Fig. 5. Effect of ZD6474 on the vascular density of PC-9 and PC-9/ZD tumors stained *in vivo* with anti-vWF. Values are means±SD. Significant difference from the control by the Dunnett test (* $P<0.05$).

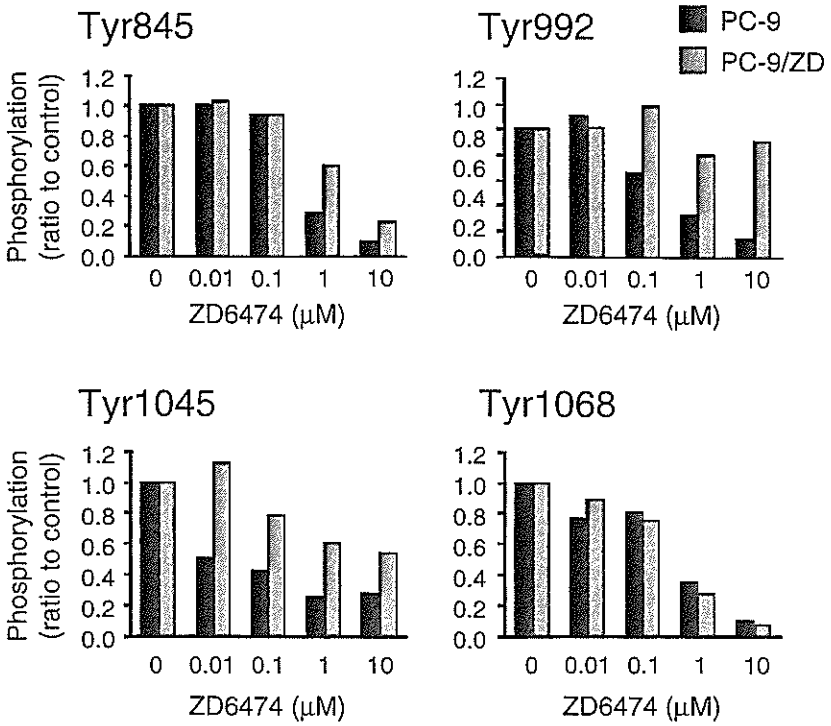

Fig. 6. Phosphorylation of EGFR tyrosine residues in PC-9 and PC-9/ZD cells after exposure to ZD6474.

phosphorylation in a dose-dependent manner. These results are consistent with previous reports[1] and indicate that ZD6474 is a potent EGFR TKI. *In vivo*, ZD6474 decreased vascular density in PC-9 tumors but not in PC-9/ZD cells, suggesting that ZD6474 may affect the angiogenic process via EGFR blockade. This could be mediated by inhibition of EGFR-induced paracrine production of angiogenic growth factors, such as VEGF, bFGF, and TGF from cancer cells, but the exact mechanism of action is unclear. This activity is, however, likely to be of less significance than the VEGFR-2-mediated antiangiogenic effect, since ZD6474 has been shown to have consistent *in vivo* antitumor activity in a range of histologically diverse human tumor xenografts, including activity in tumor models which do not respond to treatment with an EGFR TKI.[13] In addition, any change, or lack of change in microvessel density needs to be interpreted with caution as a either positive or negative indication of antiangiogenic activity, since the efficacy of antiangiogenic agents may not be related to microvessel density measurements.[14] ZD6474 was expected to induce increased apoptosis in tumor cells; although no induction of apoptosis was in fact observed, this may have been due to experimental factors.

Phosphorylations of Tyr845 and Tyr1045 of PC-9 and PC-9/ZD cells are similarly inhibited by ZD6474. On the other hand, while the inhibition pattern of Tyr845 phosphorylation by ZD6474 is coincident with that by gefitinib, the patterns at Tyr1045 are different. Therefore, we considered that the Tyr1045 is more important than Tyr845 for assessing the distinctive mode of action of ZD6474. In searching for a common mode of action of ZD6474 and gefitinib, Tyr845 seems to be the most promising site.

Phosphorylation of Tyr992 has been reported to transduce the signal to phospholipase C and protein kinase C.[15–17] In contrast, no inhibition of pan-phospho-PKC (the downstream signal of Tyr992) by gefitinib or ZD6474 was observed (data not shown). Tyr1045 has been reported to be linked to the Cbl-ubiquitin signaling pathway.[18] We have previously reported that Tyr1068 is a possible target site of EGFR for gefitinib[7], and gefitinib inhibited phosphorylation of Tyr1068 to varying degrees in PC-9 and PC-9/ZD cells, whereas ZD6474 inhibited Tyr1068 in both cell lines. These results suggest that the mode of inhibition of phosphorylation of EGFR by ZD6474 is subtly different to that of gefitinib. Therefore, although ZD6474 shows cross-resistance to gefitinib in these PC-9/ZD tumor cells, it has the potential for activity against gefitinib-resistant tumors through at least two mechanisms: (i) inhibition of EGFR-dependent downstream signaling pathways through differential effects on the phosphorylation status of tyrosine residues in the intracellular domain of EGFR, and (ii) inhibition of tumor angiogenesis through inhibition of VEGFR2 tyrosine kinase activity, which has not been examined in the present study. Site-directed mutagenesis studies are now under way to elucidate the biological significance of these sites.

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Multi-institutional phase II trial of irinotecan, cisplatin, and etoposide for sensitive relapsed small-cell lung cancer

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Irinotecan (CPT-11) has been shown to exhibit excellent antitumour activity against small-cell lung cancer (SCLC). A multi-institutional phase II study was therefore conducted to evaluate the efficacy and toxicity of CPT-11 combined with cisplatin (CDDP) and etoposide (ETOP) (PEI regimen) for the treatment of sensitive relapsed SCLC. Patients who responded to first-line chemotherapy but relapsed more than 8 weeks after the completion of first-line therapy ($n = 40$) were treated using the PEI regimen, which consisted of CDDP (25 mg m^{-2}) weekly for 9 weeks, ETOP (60 mg m^{-2}) for 3 days on weeks 1, 3, 5, 7, and 9, and CPT-11 (90 mg m^{-2}) on weeks 2, 4, 6, and 8 with granulocyte colony-stimulating factor support. Five complete responses and 26 partial responses were observed, and the overall response rate was 78% (95% confidence interval 61.5–89.2%). The median survival time was 11.8 months, and the estimated 1-year survival rate was 49%. Grade 3/4 leucocytopenia, neutropenia, and thrombocytopenia were observed in 55, 73, and 33% of the patients, respectively. Nonhaematological toxicities were mild and transient in all patients. In conclusion, the PEI regimen is considered to be highly active and well tolerated for the treatment of sensitive relapsed SCLC.
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Small-cell lung cancer (SCLC) is one of the most chemosensitive solid tumours, and first-line combination chemotherapy improves survival. However, despite a high response rate to chemotherapy, the majority of SCLC patients relapse. At the time of recurrence, the tumour is broadly resistant to second-line chemotherapy and is lethal within a few to several months (Glisson, 2003). The further development of not only first-line chemotherapy but also of effective salvage chemotherapies is needed.

In predicting the efficacy of salvage chemotherapy, two major factors are important: the response to the initial chemotherapy and the duration of time between the last exposure to chemotherapy and the confirmation of recurrence (Postmus *et al*, 1987; Giaccone *et al*, 1988; Ardizzoni *et al*, 1997; Ebi *et al*, 1997). Based on these factors, relapsed SCLC is now commonly classified into two main groups. Patients who both respond to the initial chemotherapy and relapse more than 2 or 3 months after the completion of chemotherapy are considered to be 'sensitive relapse' patients, while patients whose tumour is stable or progresses during the initial chemotherapy or who have a recurrence within 2 or 3 months after the completion of chemotherapy are considered to be 'refractory relapse' patients (Giaccone *et al*, 1988). Since the outcomes of salvage chemotherapy for relapsed SCLC patients are different between these two groups, the ratios of sensitive and refractory cases must be carefully considered when evaluating the results of clinical trials for second-line chemotherapy.

The combination of cisplatin (CDDP) and etoposide (ETOP) (PE regimen) has been the standard chemotherapeutic regimen for SCLC (Fukuoka *et al*, 1991; Ihde, 1992; Roth *et al*, 1992; Aisner, 1996). Moreover, PE is a reasonable second-line chemotherapy for relapsed SCLC after combination chemotherapy consisting of cyclophosphamide, doxorubicin (ADM), and vincristine (VCR) (CAV regimen); the likelihood of a response to this regimen is 40–50% (Evans *et al*, 1984; Porter *et al*, 1985). Since PE has a relatively mild toxicity profile, other cytotoxic agent can be combined with PE.

Irinotecan (CPT-11), a camptothecin derivative topoisomerase I inhibitor, has been shown to exhibit excellent antitumour activity against SCLC in monotherapy and in combination with CDDP (Masuda *et al*, 1992; Kudoh *et al*, 1998). Based on these results, the Japan Clinical Oncology Group (JCOG) conducted a randomised phase III trial comparing CPT-11 and CDDP (IP regimen) with standard PE for previously untreated extensive stage (ED) SCLC (JCOG 9511) (Noda *et al*, 2002). The response rates were significantly higher for IP than for PE, and overall survival was also significantly better for IP than for PE. This was the first study to show the superiority of any one regimen over PE for the

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