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

Tokuo Arao,1 Hisao Fukumoto,1 Masayuki Takeda,1 Tomohide Tamura,2 Nagahiro Saijo,2 and Kazuto Nishio1,3
1Shien-Lab, 2Medical Oncology, National Cancer Center Hospital, Tsukiji, Japan; and 3Pharmacology Division, National Cancer Center Research Institute, Tokyo, Japan

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 IC50 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 (IC50 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 IC50 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 deletion 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. 2A) in detail was described in another paper. 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 anti-rabbit horseradish peroxidase–conjugated antibody all were purchased from Cell Signaling. The transfected cells cultured in

4 Unpublished observation.
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 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'-ACCTGCTGTTGGAAAGAACAAC-3' (reverse); and VEGFR-3, 5'-AGCATTCTCAACAGGCTT-3' (forward) and 5'-GGCAACAGCTGAGTGTCAATA-3' (reverse). As a control, the following human β-actin primers were used: 5'-GGAAATCGTGCGTGACATT-3' (forward) and 5'-GACTTCCCTAAAGGCACTC-3' (reverse). 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'-TCCAAACTGCACTTACGGATGC-3' (forward) and 5'-CATCATTSCACAAAAGCCGTACC-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. 1A. 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. 1B). 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. 1B). 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. 1C). 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, 20.0 ± 0.3 μmol/L and 18.0 ± 0.2 μmol/L; A431, 7.1 ± 0.9 μmol/L and 4.1 ± 0.2 μmol/L; PC-14, 20.2 ± 1.5 μmol/L and 10.1 ± 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. 2A). 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 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 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.
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. 3B). 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. 3B). 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-
IN-FRAME DELETION IN THE EGFR AS A TARGET FOR ZD6474

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. 3B). 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 nonhyposensitive cell lines (Fig. 1B).

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; dellL747-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 dellL747-P753insS cells. This small difference may be critical to the ATP-binding properties of 293-pΔ15 and dellL747-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. 3A), 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 (dellL747-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 are now 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.

REFERENCES


Downloaded from cancerres.aacrjournals.org on April 20, 2017. © 2004 American Association for Cancer Research.
Small In-Frame Deletion in the Epidermal Growth Factor Receptor as a Target for ZD6474

Tokuzu Arao, Hisao Fukumoto, Masayuki Takeda, et al.

*Cancer Res* 2004;64:9101-9104.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/24/9101

Cited articles
This article cites 11 articles, 7 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/24/9101.full.html#ref-list-1

Citing articles
This article has been cited by 23 HighWire-hosted articles. Access the articles at:
/content/64/24/9101.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.