Enhancement of Sensitivity to Tumor Necrosis Factor α in Non–Small Cell Lung Cancer Cells with Acquired Resistance to Gefitinib

Koichi Ando,[1] Tohru Ohmori,[1,2] Fumiko Inoue,[2] Tsuyoki Kadofuku,[2] Takamichi Hosaka,[1] Hiroo Ishida,[1] Takao Shirai,[1] Kentaro Okuda,[1] Takashi Hirose,[1] Naoya Horichi,[1] Kazuto Nishio,[3] Nagahiro Saijo,[3] Mitsuru Adachi,[3] and Toshio Kuroki[4]

Abstract Tumor cells that have acquired resistance to gefitinib through continuous drug administration may complicate future treatment. To investigate the mechanisms of acquired resistance, we established PC-9/ZD2001, a non-small-cell lung cancer cell line resistant to gefitinib, by continuous exposure of the parental cell line PC-9 to gefitinib. After 6 months of culture in gefitinib-free conditions, PC-9/ZD2001 cells reacquired sensitivity to gefitinib and were established as a revertant cell line, PC-9/ZD2001R. PC-9/ZD2001 cells showed collateral sensitivity to several anticancer drugs (vinorelbine, paclitaxel, camptothecin, and 5-fluorouracil) and to tumor necrosis factor α (TNF-α). Compared with PC-9 cells, PC-9/ZD2001 cells were 67-fold more sensitive to TNF-α and PC-9/ZD2001R cells were 1.3-fold more sensitive. Therefore, collateral sensitivity to TNF-α was correlated with gefitinib resistance. PC-9/ZD2001 cells expressed a lower level of epidermal growth factor receptor (EGFR) than did PC-9 cells; this down-regulation was partially reversed in PC-9/ZD2001R cells. TNF-α-induced autophosphorylation of EGFR (cross-talk signaling) was detected in all three cell lines. However, TNF-α-induced Akt phosphorylation and IκB degradation were observed much less often in PC-9/ZD2001 cells than in PC-9 cells or PC-9/ZD2001R cells. Expression of the inhibitor of apoptosis proteins c-IAP1 and c-IAP2 was induced by TNF-α in PC-9 and PC-9/ZD2001R cells but not in PC-9/ZD2001 cells. This weak effect of EGFR on Akt pathway might contribute to the TNF-α sensitivity of PC-9/ZD2001 cells. These results suggest that therapy with TNF-α would be effective in some cases of non-small-cell lung cancer that have acquired resistance to gefitinib.

Gefitinib (Iressa, ZD1839), a small-molecule epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, has been approved for the treatment of refractory and relapsed non-small-cell lung cancer (NSCLC) patients in a number of countries around the world. This drug, which is given continuously as a once-daily oral dose, showed antitumor activity in patients with relapsed or recurrent NSCLC; however, tumor responses were observed in 12% to 18% of patients with chemotherapy-refractory advanced NSCLC (1, 2). Even in cases sensitive to gefitinib, resistance might be acquired through continuous drug administration. Additional treatments for cases of NSCLC relapsing during treatment with gefitinib are urgently needed.

To investigate the mechanism of acquired resistance to gefitinib, we previously established gefitinib-acquired resistant cells, PC-9/ZD2001, from a NSCLC, PC-9, which is hypersensitive to gefitinib and has a 15-del mutation in exon 19 of EGFR (data not shown). After >6 months of culture in gefitinib-free conditions, the sensitivity of PC-9/ZD2001 cells to gefitinib was restored, and the cells were subsequently established as a revertant cell line, PC-9/ZD2001R. The active mutation of EGFR was sustained in both the resistant and the revertant cell lines and the existence of revertant cell line suggests the additional mutation of EGFR, such as a secondary mutation of T790M in EGFR that causes resistance to gefitinib (3, 4), is unlikely to be contribute to this gefitinib resistance. In the gefitinib-resistant cells, the expression levels of EGFR and mRNA decreased to 30% to 50% of those in parental cells. A ligand-induced EGFR activation minimally activated mitogen-activated protein kinase signaling pathways and the inhibitory effect of gefitinib on this

Authors' Affiliations: [1]First Department of Internal Medicine and [2]Institute of Molecular Oncology, Showa University, Tokyo, Japan; [3]Internal Medicine, Pharmacology Division, National Cancer Center Hospital, National Cancer Center Research Institute, Tokyo, Japan; and [4]Gifu University, Gifu, Japan
Received 4/12/05; revised 8/10/05; accepted 8/26/05.
Grant support: Grant-in-Aid for a High-Technology Research Center Project from the Ministry of Education, Science, Sports, and Culture of Japan; Showa University Grant-in-Aid for Innovative Collaborative Research Projects; and Special Research Grant-in-Aid for Development of Characteristic Education from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Requests for reprints: Tohru Ohmori, Institute of Molecular Oncology, Showa University, Hatanodai, 1-5-8, Shinagawa-ku, Tokyo 142-8555, Japan. Fax: 81-3-3784-2299; E-mail: ohmorit@med.showa-u.ac.jp.

doi:10.1158/1078-0432.CCR-05-0811

pathway was significantly decreased in the resistant cells.[5] To elucidate the cross-resistance to other anticancer agents, we examined the sensitivity to the conventional anticancer agents and tumor necrosis factor α (TNF-α). PC-9/ZD2001 showed cross-resistance to another EGFR inhibitor, AG1478. Interestingly, gefitinib-resistant cells were ~3-fold more sensitive than PC-9 cells to the cytotoxic effects of vinorelbine, paclitaxel, camptothecin, 5-fluorouracil, and a cytokine, TNF-α.[5] The same tendency was confirmed in the other gefitinib-resistant clones established along with PC-9/ZD2001. The restoration of these collateral sensitivities (except 5-fluorouracil) in revertant PC-9/ZD2001R cells suggests that such sensitivities are correlated with the mechanism of gefitinib resistance.

TNF-α is the prototype of ~20 related cytokines that act through specific members of the TNF receptor (TNFR) super family (5–7). Several cancer therapies exploiting the cytotoxic effect of TNF-α on solid tumors and soft-tissue sarcomas have recently been examined in clinical trials (8, 9). The TNF-α stimulates inflammation by turning on gene transcription through signaling cascades such as the Akt/nuclear factor κB (NF-κB) pathway. This signaling subsequently serves as the primary mechanism to protect cells against apoptotic stimuli through several transcriptional genes, such as inhibitor of apoptosis proteins (IAP), the specific inhibitor of caspases (10, 11). In contrast, TNF-α-mediated signaling also triggers apoptosis through the activation of caspase-8 and the downstream caspase-3 or caspase-7 in a wide variety of cells (12). From these observations, it is possible to say that TNF-α has two different signaling pathways that contradict each other. The cytotoxic effect of TNF-α might be determined by ratios between the apoptosis-inducing and the apoptosis-inhibiting effects.

Akt/NF-κB signaling also occurs downstream of EGFR and this signaling mediates cell proliferation and antiapoptotic signaling through this pathway (13). In the case of the antiapoptotic signaling of TNF-α, TNFR is known to activate Akt/NF-κB in three ways: directly through phosphatidylinositol 3-kinase activation, or indirectly through cross-talk signaling to EGFR, or both together (5–7, 12, 14, 15). Moreover, several recent articles report that the TNFR-mediated cross-talk signaling to EGFR occurs in a ligand-dependent and -independent manner (16–21). Therefore, to investigate the mechanisms of the collateral sensitivity to TNF-α in gefitinib-acquired resistant cells, we focused on TNF-α-induced cross-talk signaling to EGFR and analyzed the Akt/NF-κB signaling pathway in response to TNF-α.

In this article, we show that a weakness of Akt/NF-κB signaling from TNF-α-mediated cross-talk signaling via EGFR causes the collateral sensitivity to TNF-α in the gefitinib-acquired resistant cell line. Moreover, this cross-talk signaling is thought to be a dominant pathway of TNF-α-mediated Akt activation.

Materials and Methods

Chemicals and antibodies. Gefitinib was donated by AstraZeneca Pharmaceuticals (Wilmington, DE). An anti-phospho-EGFR antibody (Tyr1068) was purchased from Cell Signaling Technology (Beverly, MA). Other antibodies and chemicals were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Sigma-Aldrich Co. (St. Louis, MO), respectively, unless otherwise specified.

Cell lines and cultures. The PC-9 human NSCLC cell line, established from a previously untreated patient, was kindly donated by Prof. K. Hayata (Tokyo Medical College, Tokyo, Japan.). The PC-9 cells were cultured with RPMI 1640 supplemented with 10% FCS and maintained in a 5% CO_2 incubator at 37°C under humidified conditions.

Establishment of gefitinib-resistant cell lines. To establish gefitinib-resistant cell lines, PC-9 cells were continuously exposed to increasing dosages of gefitinib for >1 year. The surviving cells were cloned and three gefitinib-resistant cell lines, designated as PC-9/ZD2001, PC-9/ZD2002, and PC-9/ZD2003, were established. These cell lines can survive exposure to 200 nmol/L gefitinib. Sensitivity to gefitinib was restored by culture of PC-9/ZD2001 in gefitinib-free conditions for >6 months. The restored cells were cloned and subsequently established as a revertant cell line, PC-9/ZD2001R.

Established resistant cell lines were maintained by culture in a medium containing 200 nmol/L gefitinib. To eliminate the effects of gefitinib, the resistant cells were cultured in a drug-free medium for at least 2 weeks before all experiments. As the relative resistance values of these cell lines were stable for at least 3 months after culture under drug-free conditions (data not shown), we used the cells for experiments during this period.

Growth inhibition assay. To measure sensitivity to gefitinib, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was done (Cell Titer 96 assay kit, Promega Corp., Madison, WI). In brief, PC-9, PC-9/ZD2001, and PC-9/ZD2001R cells were seeded onto 96-well plates and preincubated overnight. The cells were continuously exposed to the indicated concentrations of gefitinib for 4 or 5 days. Absorbance was measured at 570 nm with a microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA).

Analysis of tumor necrosis factor α–induced apoptotic cell death. The PC-9, PC-9/ZD2001, and PC-9/ZD2001R cells were treated with 100 ng/mL TNF-α for the indicated time periods. They were then fixed with 4% paraformaldehyde at 4°C for 30 minutes. After 100 μL of 70% ethanol were added, the cells were permeabilized by incubation overnight at −20°C. Apoptotic DNA fragments were probed with the terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling method (MEBSTAIN Apoptosis TUNEL Kit Direct, Medical & Biological Laboratories, Nagoya, Japan) and subpopulations of apoptotic cells were measured with a flow cytometer (FACSCalibur, BD Biosciences Immunocytometry Systems, San Jose, CA).

Activity assays for CPP32/caspase-3 and FLICE/caspase-8. Activities of CPP32/caspase-3 and FLICE/caspase-8 were measured with caspase-3 and caspase-8 colorimetric assay kits (MRL Diagnostics, Cypress, CA) according to the instructions of the manufacturer. The PC-9, PC-9/ZD2001, and PC-9/ZD2001R cells were incubated for 12 hours with 10 ng/mL TNF-α and then resuspended in 50 μL of chilled cell lyses buffer. The cells were incubated on ice for 10 minutes and the protein concentration of the supernatant was assayed with a bicinchoninic acid protein assay kit (Sigma-Aldrich). A certain amount of each sample was added to 50 μL of 2× reaction buffer containing the respective substrates DEVD-pNA and IETD-pNA, then incubated at 37°C for 1 hour. After incubation, absorbance was measured at 400 and 405 nm with a microtiter plate reader (Model 550, Bio-Rad Laboratories).

Immunoblot analysis. Cells were treated with 10 ng/mL of TNF-α for 30 minutes, then washed twice with ice-cold PBS and lysed in EBC buffer [50 mmol/L Tris-HCl (pH 8.0), 120 mmol/L NaCl, 0.5% NP40, 100 μmol/L NaF, 200 μmol/L Na orthovanadate, and 10 μg/mL of leupeptin, aprotinin, and phenylmethylsulfonyl fluoride] with an ultrasonic disrupter (Tomy Digital Biology Co., Ltd., Tokyo, Japan). The cell lysate was precleared by centrifugation, resolved by 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with antibodies against EGFR, phospho-EGFR (Tyr1045), phosphatase and tensin homologue, Akt, phospho-Akt, IκB, c-IAP1, and c-IAP2. Bound antibodies were detected with horseradish peroxidase-linked immunoglobulin (Amersham Biosciences, Buckinghamshire, United Kingdom)

[5] T. Yamaoka, T. Ohmori, F. Inoue, et al. Characteristics of gefitinib-acquired resistance in non–small cell lung cancer cell lines, submitted for publication.

and enhanced chemiluminescence reagents (Perkin-Elmer Life and Analytical Sciences, Boston, MA).

Real-time reverse transcription-PCR method. Total RNA was isolated with the guanidium isothiocyanate method using an RNA purification kit (RNeasy Mini Kit, Qiagen, Venlo, the Netherlands) according to the instructions of the manufacturer. After RNA isolation, cDNA was prepared in the presence of random 9-mers with a reverse transcription-PCR (RT-PCR) kit (Takara Shuzo Co., Ltd., Kyoto, Japan). Expression levels of EGFR, c-IAP1, and c-IAP2 mRNA were quantified with a fluorescence-based real-time detection method (GeneAmp 5700 Sequence Detection System, Applied Biosystems, Foster City, CA). Cycling conditions were 40 cycles at 94°C for 20 seconds, 55°C (EGFR) and 64°C (c-IAPs) for 20 seconds, and 72°C for 30 seconds. Expression of the mRNA was measured with the following primer sets: EGFR, 5′-ACGAATGGGCCTAAGATC-3′ and 5′-TGCTTACCCGGATTCTAGG-3′; c-IAP1, 5′-ATGTGGGTAACAGTGATGATGTCA-3′ and 5-AAACCAC-TTGGCATGTTGAAC-3′; and c-IAP2, 5′-CTAGTGTTCATGTTGAAC-3′ and 5′-CCTCAAGCCACCATCACAAC-3′. The expression of β-actin mRNA was used as an internal control.

Statistical analysis. Statistical analysis was done with the StatView II software program (Abacus Concepts, Berkeley, CA). Activities of CPP32/caspase-3 and FLICE/caspase-8 were analyzed with paired Student's t test. $P < 0.05$ was considered significant.

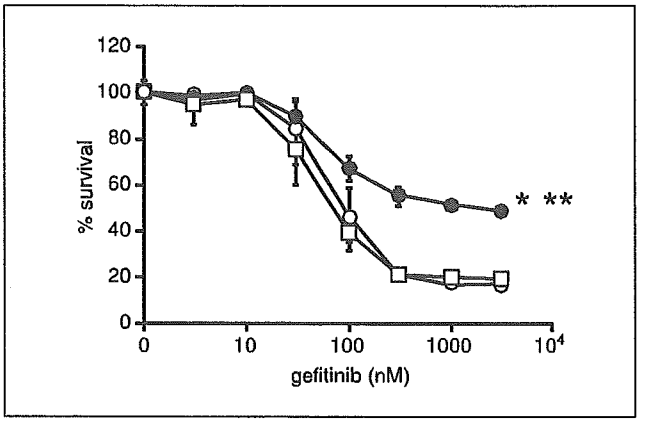

Fig. 1. Cytotoxic effects of gefitinib in a gefitinib-resistant NSCLC cell line. The cells (2×10^3 per well) were seeded onto a 96-well plate and preincubated overnight, then continuously exposed to the indicated concentrations of gefitinib for 4 or 5 days. The growth inhibition rate was analyzed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in Materials and Methods. ○, PC-9; ●, PC-9/ZD2001; □, PC-9/ZD2001R. Points, mean of three different experiments; bars, SD. *, $P < 0.001$, PC-9 versus PC-9/ZD2001; **, $P < 0.001$, PC-9/ZD2001R versus PC-9/ZD2001.

Results

Establishment of acquired gefitinib-resistant cell lines. To elucidate the mechanism of acquired resistance against gefitinib, we established gefitinib-resistant NSCLC cell lines through continuous exposure of this drug. Resistance against gefitinib developed quite slowly; the relative resistant values of 3- to 4-fold were reached after >1-year exposure to gefitinib. We picked the clones of gefitinib-resistant cell lines named PC-9/ZD2001, PC-9/ZD2002, and PC-9/ZD2003. These cell lines can survive in 200 nmol/L gefitinib-contained medium. Sensitivities to gefitinib were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. In the case of PC-9/ZD2001 cells, the cell line was able to survive by >50% at the concentration of >500 nmol/L gefitinib. This concentration caused maximum inhibition in PC-9. The IC_{40} value of gefitinib in PC-9 cells was 53.0 ± 8.1 nmol/L. The gefitinib-resistant cell line PC-9/ZD2001 showed a 4-fold higher resistance to gefitinib than PC-9 cells (IC_{40} = 211.1 ± 32.4 nmol/L; Fig. 1). Culture of the cells in gefitinib-free conditions for 6 months restored sensitivity to gefitinib in PC-9/ZD2001 and subsequently established a revertant cell line, PC-9/ZD2001R, in which sensitivity to gefitinib was completely restored (IC_{40} = 46.3 ± 10.2 nmol/L).

Analysis for tumor necrosis factor α–induced apoptotic cell death. TNF-α-induced cytotoxic effect was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The IC_{40} values of TNF-α in PC-9, PC-9/ZD2001, and PC-9/ZD2001R cell lines were 815.0 ± 44.8, 12.2 ± 1.4, and 626.2 ± 18.5 ng/mL, respectively. PC-9/ZD2001 cells acquired new sensitivity to TNF-α. PC-9/ZD2001 was ~67-fold more sensitive to TNF-α as compared with PC-9, but this sensitization was restored to 1.3-fold in PC-9/ZD2001R (Fig. 2A). This collateral sensitivity to TNF-α was confirmed in the other gefitinib-resistant cell lines, PC-9/ZD2002 and PC-9/ZD2003 (data not shown).

Additionally, we measured TNF-α-induced apoptotic cell death by flow cytometry. The apoptotic cells were stained by the terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling method. No significant apoptosis was observed in these three cell lines until 24 hours of exposure to TNF-α (10 ng/mL). Forty-eight hours of TNF-α exposure induced a 6-fold higher apoptotic cell death in PC-9/ZD2001 cells (70.3%) as compared with the parental PC-9 cells (11.8%). This enhancement was completely recovered in PC-9/ZD2001R cells (16.6%; Fig. 2B; Table 1). These results suggest that the collateral sensitivity to TNF-α might be correlated with the resistance to gefitinib in these cell lines.

Analysis of tumor necrosis factor α–mediated activations of CPP/caspase-3 and FLICE/caspase-8. To clarify the difference of TNF-α-induced apoptotic cell death in these cell lines, we analyzed TNF-α-mediated CPP32/caspase-3 and its upstream FLICE/caspase-8 activations by caspase-8 and caspase-3 colorimetric protease assay kits (Medical and Biological Laboratories), respectively. PC-9, PC-9/ZD2001, and its revertant PC-9/ZD2001R cells were incubated with the indicated concentrations of TNF-α for 12 hours. In the case of caspase-3, TNF-α did not cause any increases in the activity in PC-9 and PC-9/ZD2001R cells even at the highest concentration of 100 ng/mL. In contrast, TNF-α significantly enhanced caspase-3 activity in PC-9/ZD2001 cells even at the concentration of 1 ng/mL within this time course (Fig. 3A). In the case of caspase-8, TNF-α enhanced the activities in all three cell lines from 10 ng/mL (Fig. 3B). TNF-α at 100 ng/mL activated caspase-8 ~1.6-, 2.9-, and 1.9-fold higher in PC-9, PC-9/ZD2001, and PC-9/ZD2001R, as compared with the respective untreated cells. In PC-9/ZD2001 cells, TNF-α caused the highest relative induction of caspase-8 (Fig. 3B).

Immunoblot analysis for the tumor necrosis factor α–induced cross-talk signaling to epidermal growth factor receptor and Akt/nuclear factor κB pathway activation. EGFR expression was significantly lower in PC-9/ZD2001 than in PC-9 cells (Fig. 4A). When measuring the expression of EGFR protein by a densitometer (calculated by the NIH image software), the expression was decreased to 52.4 ± 2.6% of that in parental cell line. Moreover, we measured the expression levels of EGFR mRNA by a real-time RT-PCR method. The expression level in PC-9/ZD2001 was decreased to 37.0 ± 3.2% of that in parental

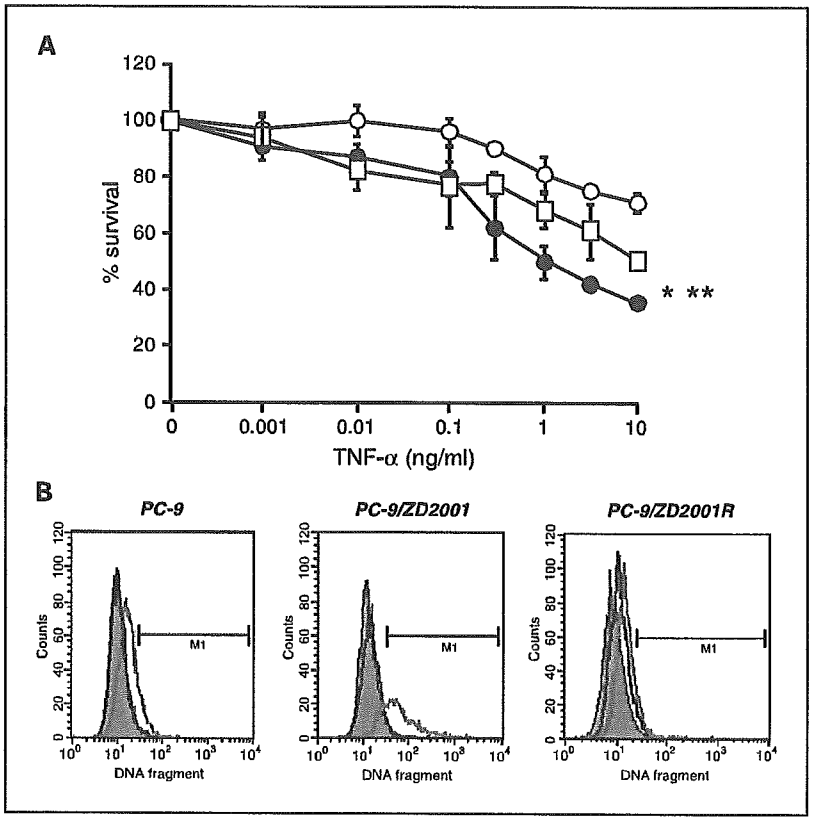

Fig. 2. Gefitinib-resistant cells acquired sensitivity to TNF-α. *A*, the cells were continuously treated with the indicated concentrations of TNF-α for 4 or 5 days. The growth inhibition rate was analyzed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in Materials and Methods. ○, PC-9; ●, PC-9/ZD2001; □, PC-9/ZD2001R. PC-9/ZD2001 cells were ~67-fold more sensitive to TNF-α than were PC-9 cells but the sensitivity of revertant PC-9/ZD2001R cells decreased to 1.3-fold that in PC-9 cells. Points, mean of three different experiments; bars, SD. *, $P < 0.001$, PC-9 versus PC-9/ZD2001, **, $P < 0.001$, PC-9/ZD2001R versus PC-9/ZD2001. *B*, the cells were treated with 10 ng/mL TNF-α for the indicated time periods. After treatment, the cells were fixed with 4% paraformaldehyde at 4°C and permeabilized with 70% ethanol. Fragments of apoptotic DNA were stained with the terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling method and measured with flow cytometry as described in Materials and Methods.

cells. The same down-regulation of EGFR was seen in the other resistant cell lines (data not shown). In the case of PC-9/ZD2001R, expression levels of EGFR protein and mRNA were also decreased to 69.3 ± 1.1% and 56.8 ± 2.2%, respectively, as compared with PC-9. The expression of EGFR was restored, but not completely, in the revertant cell line.

In PC-9 cells, cross-talk signaling from TNFR to EGFR was observed and treatment with 10 ng/mL TNF-α for 30 minutes induced significant autophosphorylation of EGFR (Fig. 4A). According to the autophosphorylation of EGFR, definite phosphorylation of Akt and a decrease in IκB content were observed. The activation of Akt and down-regulation of IκB were inhibited by gefitinib at concentrations <10 nmol/L. Because gefitinib (100 nmol/L) mostly inhibited this signaling, we concluded that the cross-talk signaling from TNFR to EGFR might be the dominant pathway of TNF-α-mediated Akt/NF-κB activation in this cell line rather than the direct signaling from TNFR to Akt. In contrast, although EGFR autophosphorylation was observed, only partial phosphorylation of Akt and down-regulation of IκB, compared with those in PC-9, were observed after TNF-α exposure in PC-9/ZD2001 cells (Fig. 4A and B). Treatment with gefitinib inhibited this cross-talk signaling to EGFR but had no effect on downstream Akt phosphorylation.

These observations suggest that TNF-α-mediated EGFR signaling has less effect on the Akt/NF-κB pathway in the gefitinib-resistant PC-9/ZD2001 cell line. Other stimuli might activate Akt in an EGFR-independent manner. In the revertant PC-9/ZD2001R cell line, this weak effect of EGFR was largely reversed and TNF-α exposure induced autophosphorylation of EGFR and subsequent activation of the Akt/NF-κB pathway. The expression levels of phosphatase and tensin homologue, a suppressor of Akt signaling, did not differ significantly among PC-9, PC-9/ZD2001, and PC-9/ZD2001R cells. This decreased effect of EGFR might be partially caused by the down-regulation of EGFR expression in PC-9/ZD2001. However, although the EGFR-mediated signaling and the resistance to gefitinib were mostly restored, EGFR expression remained only partially restored in PC-9/ZD2001R. For this reason, we speculated that the down-regulation of EGFR expression might not fully explain the weak EGFR signaling to Akt pathway in PC-9/ZD2001 cells.

To clarify the decreased EGFR signaling in PC-9/ZD2001, we examined the inhibitory effect of a phosphatidylinositol 3-kinase inhibitor, wortmannin, on the TNF-α-induced activation of this pathway (Fig. 4B). Interestingly, wortmannin inhibited the TNF-α-mediated phosphorylation of Akt in PC-9/ZD2001 cells at the same level as it did in PC-9 and PC-9/ZD2001R cells.

Expresstion of c-IAP1 and c-IAP2 on treatment with tumor necrosis factor α. After treatment with TNF-α (10 ng/mL) for 30 minutes, expression of c-IAP1 and c-IAP2 proteins was

Table 1. Percentage of apoptotic subpopulations

%Apoptosis	PC-9	PC-9/2001	PC-9/2001R
Control	1.1	1.2	1.1
24 h	1.4	2.3	3.2
48 h	11.8	70.3	6.6

NOTE: After 72 hours of exposure to TNF-α, significant apoptotic cell death was observed in PC-9/ZD2001 cells but not in PC-9 or PC-9/ZD2001R cells.

significantly increased in PC-9 and PC-9/ZD2001R cells but not in PC-9/ZD2001 cells (Fig. 4A and B). According to the results of Akt phosphorylation, induction was inhibited by gefitinib in PC-9 and PC-9/ZD2001R cells but not in PC-9/ZD2001 cells. Wortmannin could inhibit induction in all three cell lines. Consistent with the results of protein expression, treatment with TNF-α increased the expression level of c-IAP1 and c-IAP2 mRNA in PC-9 and PC-9/ZD2001R cells in a dose-dependent manner (Fig. 5A and B). After treatment with 100 ng/mL TNF-α for 12 hours, the expression levels of both c-IAP1 and c-IAP2 mRNA were significantly increased in PC-9 cells (c-IAP1, 7.05 ± 0.62; c-IAP2, 18.22 ± 0.25) and PC-9/ZD2001R cells (c-IAP1, 7.02 ± 0.54; c-IAP2, 11.56 ± 0.75) but not in PC-9/ZD2001 cells (c-IAP1, 2.60 ± 0.58; c-IAP2, 2.83 ± 0.66). These observations suggest that TNF-α-induced apoptotic signaling is not inhibited by its own antiapoptotic effects, such as IAPs induction, owing to the weak effect of TNF-α-mediated signaling and the Akt/NF-κB pathway via EGFR in this gefitinib-resistant cell line.

Discussion

We have shown that the gefitinib-acquired resistant NSCLC cell line PC-9/ZD2001 acquired collateral sensitivity to the apoptotic effect of TNF-α. Because this collateral sensitivity was significantly diminished in the revertant PC-9/ZD2001R, it might be correlated with gefitinib resistance. As described before, PC-9/ZD2001 also acquired collateral sensitivities to some anticancer drugs, such as vinorelbine, paclitaxel, camptothecin, and 5-fluorouracil. However, this cell line did not show the collateral sensitivities to cisplatin, etoposide, mitomycin C, and cyclophosphamide.[5] Moreover, there was no difference of susceptibility to serum-starved condition between PC-9 and PC-9/ZD2001 (data not shown). From these observations, it can be concluded that the collateral sensitivities of the gefitinib-resistant cells are specific to some cell stresses and are not caused by the fragility of the cells. Because the same tendency of sensitivity was seen in the other resistant clones, PC-9/ZD2002 and PC-9/ZD2003, the acquired sensitivity to the anticancer drugs and TNF-α could be a general phenomenon even in the clinical gefitinib-resistant cells.

TNF-α activates not only apoptotic signaling but also antiapoptotic signaling via the Akt/NF-κB activation (22, 23). Activation of the downstream transcription factor NF-κB inhibits various types of apoptotic cell death by inducing apoptotic inhibitory proteins (22, 23), such as bcl-2 (24), bcl-xl (25), forkhead (26), and IAPs (10, 11, 27, 28). As described before, it is thought that the cytotoxic effect of TNF-α is determined by ratios between the apoptosis-inducing and the apoptosis-inhibiting effects (5–7, 12, 14, 15).

In parental PC-9 cells, TNF-α induced EGFR autophosphorylation and subsequent Akt/NF-κB pathway activation (Fig. 4A and B). This autophosphorylation was completely inhibited by a low concentration of gefitinib (10 nmol/L). From these observations, we think that TNF-α-induced Akt/NF-κB pathway activation occurs mainly through cross-talk from TNFR to EGFR in this cell line. Because the expression level of EGFR was significantly decreased in PC-9/ZD2001 as compared with the parental PC-9, the decline of the cross-talk signaling might partially diminish the TNF-α-induced activation of the Akt/NF-κB pathway. Our results are supported by those of an earlier study showing that resistance to the cytotoxic effect of TNF-α is associated with high expression of Her family receptors, such as EGFR (Her1), erbB2/Her2/neu, or Her3, in a panel of human tumor cell lines (29). However, the decreased EGFR signaling from the Akt/NF-κB pathway could not be fully explained by the lower EGFR expression in PC-9/ZD2001 because EGFR expression remained only partially restored in the revertant PC-9/ZD2001R cell line. In light of these observations, to clarify the mechanisms of collateral sensitivity to TNF-α in the gefitinib-resistant cells, we focused on the cross-talk signaling from TNFR to EGFR in PC-9, PC-9/ZD2001, and PC-9/ZD2001R cells.

Several recent articles have reported that TNFR mediates cross-talk signaling to EGFR through a ligand-dependent and -independent manner (16–19, 21, 23). Chan et al. (17) have reported that exposure of human mammary epithelial cells to TNF-α results in transactivation of EGFR through metalloprotease-dependent shedding of EGFR ligand(s). Hirota et al. (18) reported that EGFR transactivation by TNF-α is

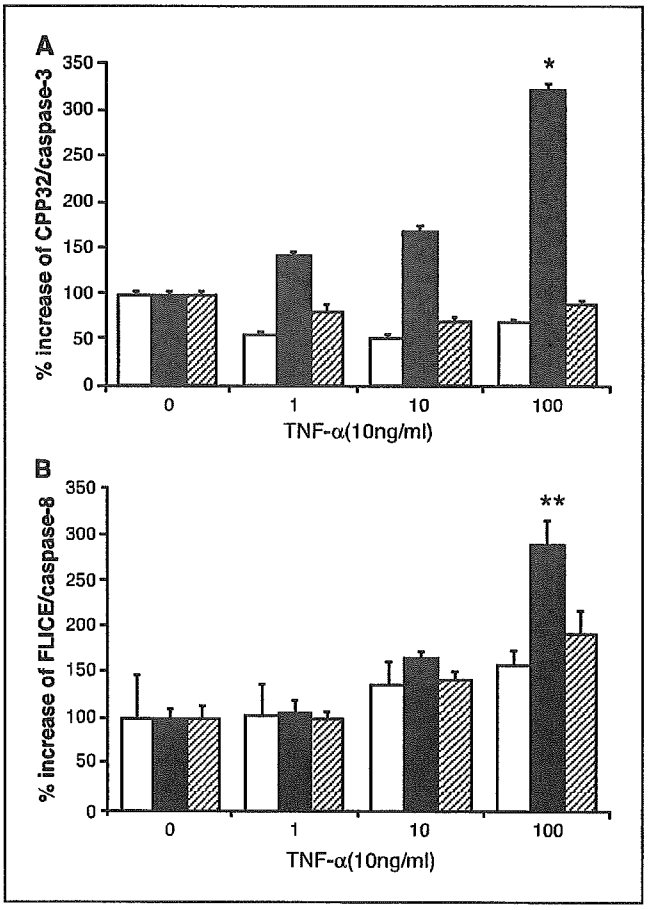

Fig. 3. TNF-α-mediated activation of CPP32/caspase-3 and FLICE/caspase-8 in PC-9, PC-9/ZD2001, and PC-9/ZD2001R cells. Activation of CPP32/caspase-3 and FLICE/caspase-8 was measured as described in Materials and Methods. The cells were exposed to the indicated concentrations of TNF-α for 12 hours; after which equivalent amounts of samples were reacted with the substrates DEVD-pNA and IETD-pNA. Absorbance was measured at 400 and 405 nm with a microtiter plate reader. *A*, CPP32/caspase-3. *B*, FLICE/caspase-8. TNF-α activated FLICE/caspase-8 in all three cell lines but activated CPP32/caspase-3 only in PC-9/ZD2001 cells. Data calculated as the percentage increase compared with respective untreated controls. Points, mean of three different experiments each done in triplicate; bars, SD. Open columns, PC-9; closed columns, PC-9/ZD2001; hatched columns, PC-9/ZD2001R. *, $P < 0.001$, PC-9 versus PC-9/ZD2001. **, $P = 0.02$, PC-9 versus PC-9/ZD2001.

regulated by means of redox-dependent mechanisms. The transactivation of EGFR was observed to occur quickly, after <30 minutes of exposure to TNF-α in PC-9 cells (Fig. 4A and B). No additional induction of ligands, EGF and transforming growth factor-α, were detected by ELISA in the culturing medium of the cells even after 6 hours of 100 ng/mL TNF-α exposure (data not shown). From these observations, we think that this activation could occur independently of ligands but not through TNF-α-mediated ligands synthesis or proteolytic releasing of preexisting ligands from the disrupted cells. Although TNF-α induced the same levels of EGFR autophosphorylation in all three cell lines, this EGFR activation is minimally transmitted to the downstream Akt/NF-κB pathway in the resistant PC-9/ZD2001 cells (Fig. 4A). Moreover, an inhibitory effect of gefitinib on TNF-α-induced Akt/NF-κB activation was not observed although wortmannin, a phosphatidylinositol 3-kinase inhibitor, completely inhibited this signaling in PC-9/ZD2001 cells (Fig. 4B). These results suggest that the weak effect of EGFR on Akt/NF-κB signaling could occur between EGFR and phosphatidylinositol 3-kinase in PC-9/ZD2001 cells.

Several articles reported that the sensitivity to gefitinib is regulated by active mutant EGFR (30, 31), by the expression level of phosphatase and tensin homologue/MMAC/TEP (32), and by levels of Akt phosphorylation (13, 33, 34). Because the gefitinib-hypersensitive PC-9 cells originally had 15-bp deletion mutation in exon 19 of EGFR, they were thought to have a gefitinib-sensitive active mutant EGFR (35); however, because we found no alteration of the EGFR mRNA sequence in PC-9/ZD2001 cells (data not shown), we conclude that this gefitinib-resistant cell line was a good model for acquired gefitinib resistance. In our previous study, EGFR signaling mediated by transforming growth factor-α, an EGFR ligand, could not activate the mitogen-activated protein signaling pathway but could partially activate the Akt signaling cascade in PC-9/ZD2001. In PC-9/ZD2001R cells, the association between EGFR and mitogen-activated protein kinase signaling was completely reconstituted. On the basis of this result, we conclude that the decrease of EGFR signaling to the mitogen-activated protein kinase signaling pathway might contribute to acquired gefitinib resistance.[5] In this study, TNF-α significantly induced EGFR autophosphorylation but subsequent activation of the Akt signaling cascade was little observed in PC-9/ZD2001 (Fig. 4A and B). This decreased EGFR signaling on Akt could be partially caused by the decrease in EGFR expression but we have

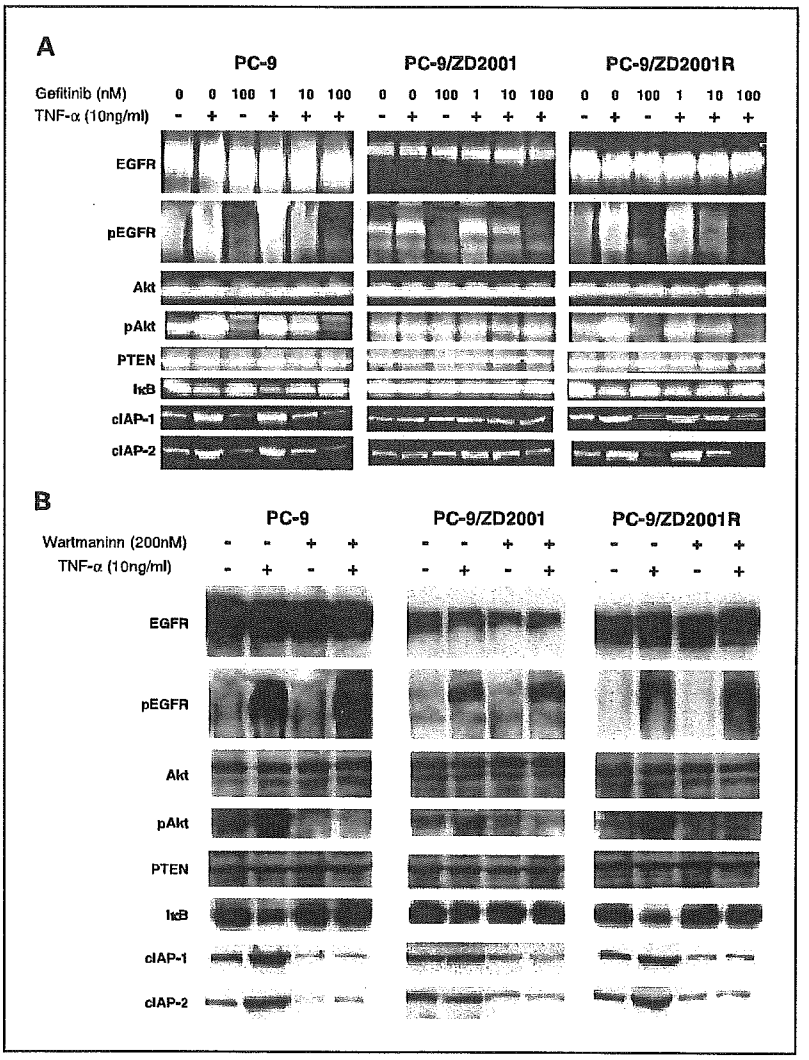

Fig. 4. Inhibitory effect of gefitinib on TNF-α-induced phosphorylation of Akt1 and degradation of IκB. Cells were treated with TNF-α with or without gefitinib (*A*) or wortmannin (*B*) simultaneously for 30 minutes at 37°C. Cell lysates were prepared and equivalent amounts of protein from each cell lysate were resolved with 10% SDS-PAGE, transferred to nitrocellulose membranes, and subjected to Western blotting with specific antibodies (as described in Materials and Methods). The EGFR and Akt1 membranes were stripped and reblotted with antibodies against phospho-EGFR (Tyr1045) and phospho-Akt, respectively. Expression of β-actin was used as internal control. Although treatment with TNF-α significantly phosphorylated EGFR in all three cell lines, downstream Akt/NF-κB activation was observed in PC-9 and PC-9/ZD2001R but weakly in PC-9/ZD2001. Gefitinib inhibited cross-talk signaling in PC-9 and PC-9/ZD2001R cells but not in PC-9/ZD2001 cells (*A*). A phosphatidylinositol 3-kinase inhibitor, wortmannin, completely inhibited this signaling in all three cell lines (*B*).

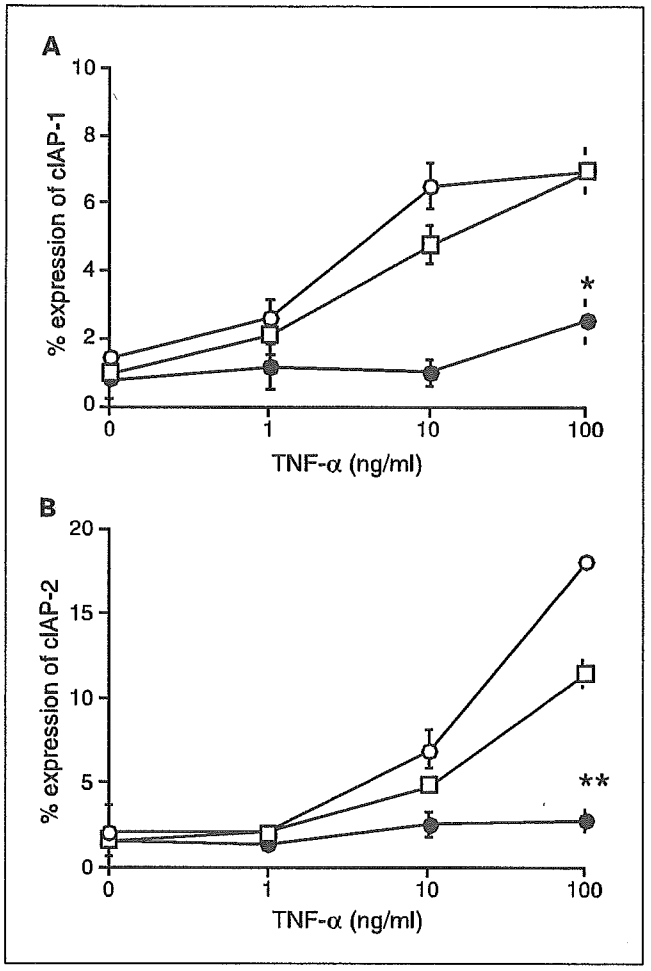

Fig. 5. TNF-α induced c-IAP1 and c-IAP2 mRNA expression in PC-9 and PC-9/ZD2001R cells but not in PC-9/ZD2001 cells. The cells were exposed to the indicated concentrations of TNF-α for 12 hours; after which mRNA was isolated with the guanidium isothiocyanate method. Induction of c-IAP1 (*A*) and c-IAP2 (*B*) mRNA was measured with a fluorescence-based real-time RT-PCR method using specific primer sets (as described in Materials and Methods). The expression levels of c-IAP1 and c-IAP2 mRNA were significantly and dose-dependently increased by exposure to TNF-α in PC-9 and PC-9/ZD2001R cells but this enhancement was rarely observed in PC-9/ZD2001 cells. Results expressed as the percentage of each cell line compared with the internal control, expression of β-actin mRNA. ○, PC-9; ●, PC-9/ZD2001; □, PC-9/ZD2001R. Points, mean of three different experiments; bars, SD. *, $P < 0.001$.

no data to explain the discrepancy between transforming growth factor-α-mediated and TNF-α-mediated EGFR signaling in this cell line. Nevertheless, TNF-α-mediated cross-talk signaling to EGFR, although ligand independent, seems to cause downstream activation in a different way from that caused through ligand-mediated direct EGFR activation. Akt/NF-κB signaling is also known to be downstream of other receptors, such as other Her family receptors (36), platelet-derived growth factor receptor (37), and IFN receptor (38). We previously confirmed the expression of other Her family receptors, Her2 and Her3, in PC-9 cells. Possibly, signaling of these receptors may be able to modulate the TNF-α-mediated cross-talk signaling and Akt/NF-κB signaling. Various aspects of TNF-α-induced cross-talk signaling to EGFR, such as EGFR heterodimer formation with other Her family receptors and downstream signaling specificity, require further investigation.

Human IAPs, c-IAP1 and c-IAP2, have been reported to block the apoptotic events caused by caspase-8 activation by directly combining with caspase-3 and caspase-7 and restraining them (10, 27). IAPs play a key role in the resistance to apoptotic effect of TNF-α superfamily of proteins (39) and various anticancer drugs (40, 41); for this reason, IAPs are considered promising targets in anticancer therapy (42, 43). To evaluate TNF-α-mediated antiapoptotic signaling, we measured IAP induction in these cell lines by means of Western blotting analysis and real-time RT-PCR. As might be expected, IAPs and their mRNAs were markedly induced by TNF-α in PC-9 and PC-9/ZD2001R cells but not in PC-9/ZD2001 cells (Fig. 5A and B). TNF-α-induced activation of caspase-3, but rarely of caspase-8, was significantly lower in PC-9 and PC-9/ZD2001R as compared with PC-9/ZD2001 (Fig. 3A and B). These results suggest that TNF-α precisely activates apoptotic signaling through caspase-8 in all three cell lines and that induction of IAPs blocks downstream signaling by inhibiting caspase-3 in PC-9 and PC-9/ZD2001R. In these cell lines, the induction of IAPs likely plays a key role in determining the sensitivity to TNF-α-mediated apoptosis among the antiapoptotic proteins that are induced by NF-κB-mediated transcription.

Several clinical studies of TNF-α as an anticancer treatment have been done, mainly in the 1970s; however, treatment with TNF-α was greatly limited by its side effects, particularly its toxicity to previously healthy organs (44–49). Recently, several new anticancer therapies using TNF-α have been developed, such as RGD-V29 (F4614) and TNF-erade (Biologic), in an attempt to reduce adverse effects (8, 9, 50, 51). We have shown that a NSCLC cell line with acquired resistance to gefitinib acquired collateral sensitivity to TNF-α. These data strongly suggest that treatment with TNF-α might be effective against tumors that have acquired resistance to gefitinib after long-term administration of this drug. Further analysis is required before clinical application.

In summary, the cross-talk signaling from TNFR to EGFR and subsequent IAP induction play important roles in the resistance to TNF-α-induced apoptosis in PC-9 cells. Because this signaling cascade is decreased in the gefitinib-resistant PC-9/ZD2001 cells, TNF-α did not activate the Akt/NF-κB cascade. This decrease of EGFR signaling to Akt/NF-κB pathway, which is related to gefitinib-acquired resistance, may contribute to the acquisition of hypersensitivity to TNF-α in this cell line.

References

1. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial) [corrected]. J Clin Oncol 2003;21:2237–46.
2. Kris MG, Natale RB, Herbst RS, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. JAMA 2003;290:2149–58.
3. Kobayashi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 2005;352:786–92.
4. Pao W, Miller VA, Politi KA, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med 2005;2:e73.
5. Ashkenazi A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nat Rev Cancer 2002;2:420–30.
6. Basile JR, Zacny V, Munger K. The cytokines tumor

necrosis factor-α (TNF-α) and TNF-related apoptosis-inducing ligand differentially modulate proliferation and apoptotic pathways in human keratinocytes expressing the human papillomavirus-16 E7 oncoprotein. J Biol Chem 2001;276:22522–8.
7. Chen G, Goeddel Dv. TNF-R1 signaling: a beautiful pathway. Science 2002;296:1634–5.
8. Mundt AJ, Vijayakumar S, Nemunaitis J, et al. A Phase I trial of TNFerade biologic in patients with soft tissue sarcoma in the extremities. Clin Cancer Res 2004;10:5747–53.
9. Senzer N, Mani S, Rosemurgy A, et al. TNFerade biologic, an adenovector with a radiation-inducible promoter, carrying the human tumor necrosis factor α gene: a phase I study in patients with solid tumors. J Clin Oncol 2004;22:592–601.
10. Deveraux QL, Roy N, Stennicke HR, et al. IAPs block apoptotic events induced by caspase-8 and cytochrome *c* by direct inhibition of distinct caspases. EMBO J 1998;17:2215–23.
11. Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J 1997;16:6914–25.
12. Sidoti-De Fraisse C, Rincheval V, Risler Y, Mignotte B, Vayssiere JL. TNF-α activates at least two apoptotic signaling cascades. Oncogene 1998;17:1639–51.
13. Cappuzzo F, Magrini E, Ceresoli GL, et al. Akt phosphorylation and gefitinib efficacy in patients with advanced non-small-cell lung cancer. J Natl Cancer Inst 2004;96:1133–41.
14. Kulik G, Carson JP, Vomastek T, et al. Tumor necrosis factor α induces BID cleavage and bypasses antiapoptotic signals in prostate cancer LNCaP cells. Cancer Res 2001;61:2713–9.
15. Liu ZG, Hsu H, Goeddel DV, Karin M. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. Cell 1996;87:565–76.
16. Argast GM, Campbell JS, Brooling JT, Fausto N. Epidermal growth factor receptor transactivation mediates tumor necrosis factor-induced hepatocyte replication. J Biol Chem 2004;279:34530–6.
17. Chen WN, Woodbury RL, Kathmann LE, et al. Induced autocrine signaling through the epidermal growth factor receptor contributes to the response of mammary epithelial cells to tumor necrosis factor α. J Biol Chem 2004;279:18488–96.
18. Hirota K, Murata M, Itoh T, Yodoi J, Fukuda K. Redox-sensitive transactivation of epidermal growth factor receptor by tumor necrosis factor confers the NF-κ B activation. J Biol Chem 2001;276:25953–8.
19. Izumi H, Ono M, Ushiro S, Kohno K, Kung HF, Kuwano M. Cross talk of tumor necrosis factor-α and epidermal growth factor in human microvascular endothelial cells. Exp Cell Res 1994;214:654–62.
20. Wang D, Yang EB, Cheng LY. Modulation of EGF receptor by tumor necrosis factor-α in human hepatocellular carcinoma HepG2 cells. Anticancer Res 1996;16:3001–6.
21. Woodworth CD, Mcmullin E, Iglesias M, Plowman GD. Interleukin 1 α and tumor necrosis factor α stimulate autocrine amphiregulin expression and proliferation of human papillomavirus-immortalized and carcinoma-derived cervical epithelial cells. Proc Natl Acad Sci U S A 1995;92:2840–4.
22. Beg AA, Baltimore D. An essential role for NF-κB in preventing TNF-α-induced cell death. Science 1996;274:782–4.
23. Wang CY, Mayo MW, Baldwin AS, Jr. TNF-and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. Science 1996;274:784–7.
24. Wang CY, Guttridge DC, Mayo MW, Baldwin AS, Jr. NF-κB induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. Mol Cell Biol 1999;19:5923–9.
25. Mora AL, Corn RA, Stanic AK, et al. Antiapoptotic function of NF-κB in T lymphocytes is influenced by their differentiation status: roles of Fas, c-FLIP, and Bcl-xL. Cell Death Differ 2003;10:1032–44.
26. Kane LP, Shapiro VS, Stokoe D, Weiss A. Induction of NF-κB by the Akt/PKB kinase. Curr Biol 1999;9:601–4.
27. Rothe M, Pan MG, Henzel WJ, Ayres TM, Goeddel DV. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell 1995;83:1243–52.
28. Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS, Jr. NF-κB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 1998;281:1680–3.
29. Hoffmann M, Schmidt M, Wels W. Activation of EGF receptor family members suppresses the cytotoxic effects of tumor necrosis factor-α. Cancer Immunol Immunother 1998;47:167–75.
30. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39.
31. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.
32. Bianco R, Shin I, Ritter CA, et al. Loss of PTEN/MMAC1/TEP in EGF receptor-expressing tumor cells counteracts the antitumor action of EGFR tyrosine kinase inhibitors. Oncogene 2003;22:2812–22.
33. Ono M, Hirata A, Kometani T, et al. Sensitivity to gefitinib (Iressa, ZD1839) in non-small cell lung cancer cell lines correlates with dependence on the epidermal growth factor (EGF) receptor/extracellular signal-regulated kinase 1/2 and EGF receptor/Akt pathway for proliferation. Mol Cancer Ther 2004;3:465–72.
34. Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science 2004;305:1163–7.
35. Arao T, Fukumoto H, Takeda M, Tamura T, Saijo N, Nishio K. Small in-frame deletion in the epidermal growth factor receptor as a target for ZD6474. Cancer Res 2004;64:9101–4.
36. Prenzel N, Fischer OM, Streit S, Hart S, Ullrich A. The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. Endocr Relat Cancer 2001;8:11–31.
37. Rosenkranz S, Kazlauskas A. Evidence for distinct signaling properties and biological responses induced by the PDGF receptor α and β subtypes. Growth Factors 1999;16:201–16.
38. Uddin S, Fish EN, Sher DA, Gardziola C, White MF, Platanias LC. Activation of the phosphatidylinositol 3-kinase serine kinase by IFN-α. J Immunol 1997;158:2390–7.
39. Li L, Thomas RM, Suzuki H, De Brabander JK, Wang X, Harran PG. A small molecule Smac mimic potentiates TRAIL- and TNFα-mediated cell death. Science 2004;305:1471–4.
40. Ferreira CG, Van Der Valk P, Span SW, et al. Assessment of IAP (inhibitor of apoptosis) proteins as predictors of response to chemotherapy in advanced non-small-cell lung cancer patients. Ann Oncol 2001;12:799–805.
41. Vaziri SA, Grabowski DR, Tabata M, et al. c-IAP1 is overexpressed in HL-60 cells selected for doxorubicin resistance: effects on etoposide-induced apoptosis. Anticancer Res 2003;23:3657–61.
42. De Graaf AO, De Witte T, Jansen JH. Inhibitor of apoptosis proteins: new therapeutic targets in hematological cancer? Leukemia 2004;18:1751–9.
43. Nachmias B, Ashhab Y, Ben-Yehuda D. The inhibitor of apoptosis protein family (IAPs): an emerging therapeutic target in cancer. Semin Cancer Biol 2004;14:231–43.
44. Blick M, Sherwin SA, Rosenblum M, Gutterman J. Phase I study of recombinant tumor necrosis factor in cancer patients. Cancer Res 1987;47:2986–9.
45. Chapman PB, Lester TJ, Casper ES, et al. Clinical pharmacology of recombinant human tumor necrosis factor in patients with advanced cancer. J Clin Oncol 1987;5:1942–51.
46. Creaven PJ, Brenner DE, Cowens JW, et al. A phase I clinical trial of recombinant human tumor necrosis factor given daily for five days. Cancer Chemother Pharmacol 1989;23:186–91.
47. Feinberg B, Kurzrock R, Talpaz M, Blick M, Saks S, Gutterman JU. A phase I trial of intravenously-administered recombinant tumor necrosis factor-α in cancer patients. J Clin Oncol 1988;6:1328–34.
48. Gamm H, Lindemann A, Mertelsmann R, Herrmann F. Phase I trial of recombinant human tumour necrosis factor α in patients with advanced malignancy. Eur J Cancer 1991;27:856–63.
49. Spriggs DR, Sherman ML, Michie H, et al. Recombinant human tumor necrosis factor administered as a 24-hour intravenous infusion. A phase I and pharmacologic study. J Natl Cancer Inst 1988;80:1039–44.
50. Kuroda K, Miyata K, Fujita F, et al. Human tumor necrosis factor-α mutant RGD-V29 (F4614) shows potent antitumor activity and reduced toxicity against human tumor xenografted nude mice. Cancer Lett 2000;159:33–41.
51. Kuroda K, Miyata K, Tsutsumi Y, et al. Preferential activity of wild-type and mutant tumor necrosis factor-α against tumor-derived endothelial-like cells. Jpn J Cancer Res 2000;91:59–67.

Cancer Chemother Pharmacol (2005) 56 (Suppl 1): s90–s93
DOI 10.1007/s00280-005-0104-6

Kazuto Nishio · Tokuzo Arao · Tatsu Shimoyama · Yasuhiro Fujiwara · Tomohide Tamura · Nagahiro Saijo

Translational studies for target-based drugs

Published online: 5 November 2005

Abstract The biological background for the clinical and prognostic heterogeneity among tumors within the same histological subgroup is due to individual variations in the biology of tumors. The number of investigations looking at the application of novel technologies within the setting of clinical trials is increasing. The most promising way to improve cancer treatment is to build clinical research strategies on intricate biological evidence. New genomic technologies have been developed over recent years. These techniques are able to analyze thousands of genes and their expression profiles simultaneously. The purpose of this approach is to discover new cancer biomarkers, to improve diagnosis, predict clinical outcomes of disease and response to treatment, and to select new targets for novel agents with innovative mechanisms of action. Gene expression profiles are also used to assist in selecting biomarkers of pharmacodynamic effects of drugs in the clinical setting. Biomarker monitoring in surrogate tissues may allow researchers to assess "proof of principle" of new treatments. Clinical studies of biomarkers monitoring toxicity profiles have also been done. Such pharmacodynamic markers usually respond to treatment earlier than clinical response, and as such may be useful predictors of efficacy. Epidermal growth factor receptor (EGFR) mutation in lung cancer tissues is a strong predictive biomarker for EGFR-targeted protein tyrosine kinase inhibitors. Monitoring of EGFR mutation has been broadly performed in retrospective and prospective clinical studies. However, global standardization for the assay system is essential for such molecular correlative studies. A more sensitive assay for EGFR mutation is now under evaluation for small biopsy samples. Microdissection for tumor samples is also useful for the sensitive detection of EGFR mutation. Novel approaches for the detection of EGFR mutation in other clinical samples such as cytology, pleural effusion and circulating tumor cells are ongoing.

Keywords Biomarker · Proof of principle · Pharmacodynamic marker · EGFR mutation

This work was presented at the 20th Bristol-Myers Squibb Nagoya International Cancer Treatment Symposium, "New Concepts of Treatment Strategies for Hormone-Related Cancer", 11–12 March 2005, Nagoya, Japan.

K. Nishio (✉) · T. Arao · T. Shimoyama
Shien Lab, National Cancer Center Hospital,
Tsukiji 5–1–1, Chuo-ku, 104–0045 Tokyo, Japan
E-mail: knishio@gan2.res.ncc.go.jp
Tel.: +81-3-35422511
Fax: +81-3-35475185

T. Shimoyama · Y. Fujiwara · T. Tamura · N. Saijo
Medical Oncology, National Cancer Center Hospital, Tokyo, Japan

K. Nishio (✉) · T. Arao
Pharmacology Division,
National Cancer Center Research Institute, Tokyo, Japan

Correlative studies at the National Cancer Center Hospital

Molecular correlative studies are essential for the development of anticancer molecular-targeted drugs. One of the major purposes of a correlative study is "proof of principle" (POP). However, clinical POP studies for small molecules are often more difficult to complete than those for antibodies.

Since 2001, the National Cancer Center Hospital (Tokyo, Japan) has been operating as a laboratory for translational studies to develop molecular correlative studies. The laboratory members include medical oncologists, basic researchers, CRC research fellows, invited researchers from abroad, technicians and statisticians. The laboratory is located next to the phase I wards in the hospital, enabling more than ten molecular correlative studies to be simultaneously performed. New clinical samples can be quickly obtained from patients (including outpatients), prepared for storage and stored in the laboratory. The medical doctors

Table 1 Classification of biomarkers and their goals

Biomarker	Goal
Diagnostic markers	
Prognostic markers	
Predictive markers (patient selection)	Selection of patients most likely to benefit from given treatment
Pharmacodynamic markers	Dose finding and schedule
Response and efficacy markers	To measure or infer patient benefit/relate patient benefit to target inhibition
Toxicity prediction markers	

working in the laboratory are often research fellows supported by government grants as these individuals are often interested in this kind of research.

The location of the laboratory also gives frequent opportunities to medical oncologists to communicate with researchers. The significance of study endpoints, study design, technical and statistical information and feasibility are often discussed, especially among young medical oncologists and researchers. As a result, young oncologists and researchers often collaborate in the proposal of new molecular correlative studies.

The major activities of the laboratory are pharmacokinetics and pharmacodynamics studies for early clinical trials (phase I–II) and reverse translational studies. Essentially, "biomarker monitoring" using various biological technologies in these clinical studies are preformed. The selection and validation of biomarkers is a major endpoint for molecular correlative studies. Biomarkers are defined as described in Table 1. Tissue banking and quality control are two of the most important activities. Part of clinical sample testing is performed in collaboration with the Contract Research Organization (CRO) (Fig. 1).

Gene expression profiles

Gene expression array (DNA chips) has been widely used in clinical studies to predict response and in POP studies [3]. Many kinds of DNA chip are now available. Oligonucleotide arrays containing >40,000 genes have recently become popular. These chips can be used differentially depending on the requirements. Before the clinical use, however, an array's quality (linearity and reproducibility) should be determined in preclinical studies. At the National Cancer Center Hospital, the quality of each array is evaluated and expressed as the Pearson's product-moment coefficient of correlation. Based on the validated quality of the cDNA, protocols based on "experienced designs" are then established.

In clinical settings, sample quality and protocol feasibility are often major limitations in the design of new studies. To maintain the quality of clinical samples, a system for sample flow has been established. First, purity of the nucleotides must be carefully examined. Purification methods largely depend on the tumor types. For example, brain tumors contain large amounts of carbohydrate chains, lung cancer samples are sometimes very hard, and breast cancer biopsy samples are lipid rich. These sample characteristics influence the purification quality and efficiency.

After the gene expression profiles have been obtained for each sample, the data are analyzed by standardization, clustering, statistical analysis and validation methods. Statistical and biological validation are essential. Ideally, clinical cross-validation studies should be performed for independent clinical studies. On the other

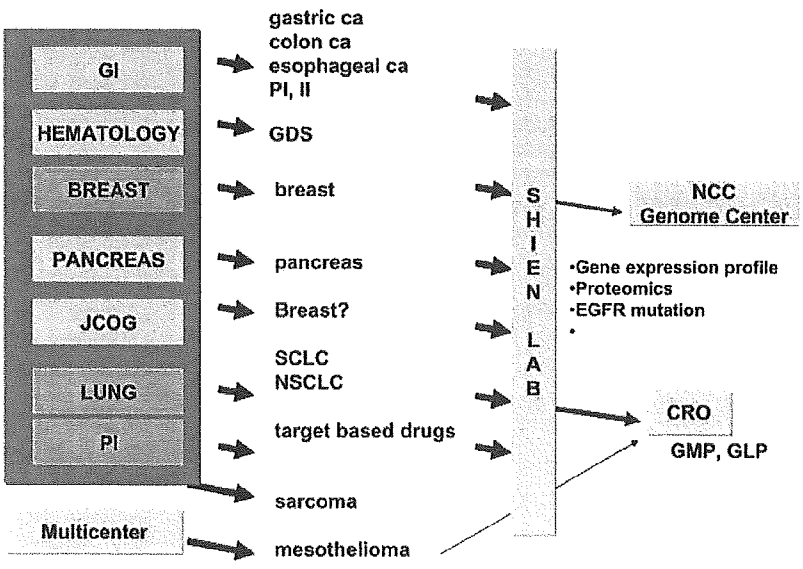

Fig. 1 Flow of clinical samples in molecular correlative studies at the National Cancer Center Hospital. (*GI* gastrointestinal, *JCOG* Japan Clinical Oncology Group, *PI* clinical phase I study, *PII* clinical phase II study, *GDS* gene delivery system, *SCLC* small cell lung cancer, *NSCLC* non-small cell lung cancer, *NCC* National Cancer Center, *CRO* Contact Research Organization, *GMP* Good Manufacturing Practice)

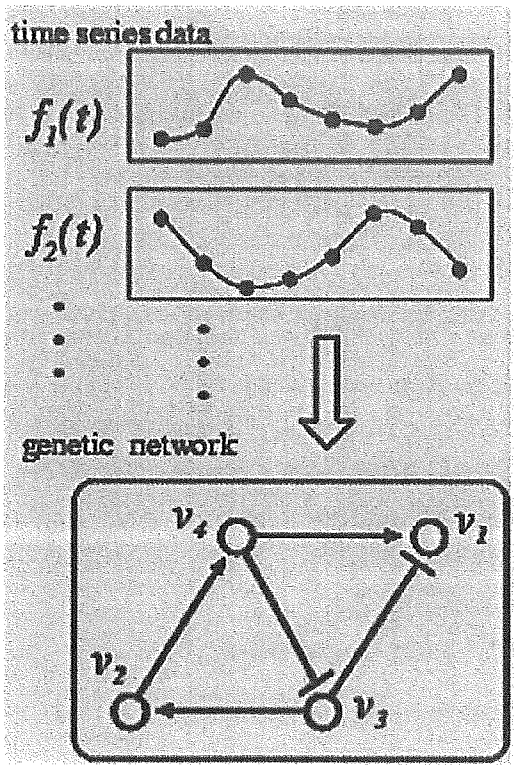

Fig. 2 Network analysis to determine transcriptional pathway and signal transduction pathway modulated by transcriptional regulators and multitarget tyrosine kinase inhibitors using gene expression profiling dataset

hand, biomarkers can be validated in the same clinical study by the "leave-one-out" method. The endpoint of these correlative studies is usually the selection of biomarkers for predicting response or toxicity. For such endpoints, the quality of the clinical study itself is also very important.

We have also used other endpoints in early clinical studies, such as comparing clinical samples obtained before and after the treatment. Analysis of gene alterations after treatment can be utilized to reveal pharmacodynamic effects. We have completed such correlative studies as part of a clinical assessment of multitarget tyrosine kinase inhibitors (TKI), farnesyl transferase inhibitor, and cytotoxic drugs [7].

For biological confirmation, we usually perform real-time RT-PCR and immunostaining. However, we recently discovered that "pathway analysis" is a powerful method for improving our understanding of the alteration of genes related to biological signal transduction pathways. To analyze transcription factors, "network analysis" can be used to identify their signaling pathways (Fig. 2).

Toxicogenomic project for breast cancer

As an approach of gene expression profiling in clinical samples, we monitored gene expression in breast cancer

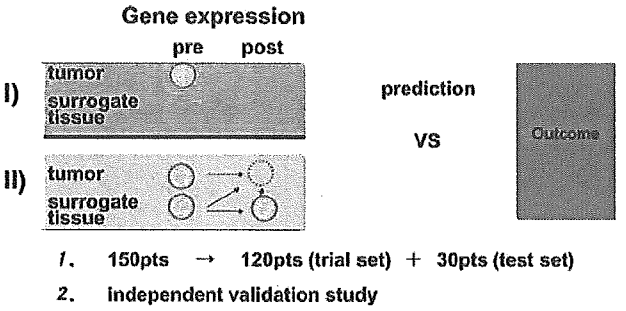

Fig. 3 Gene expression monitoring to distinguish the outcome of treatment for breast cancer patients

patients during treatment with FEC followed by weekly paclitaxel ± trastuzumab in the adjuvant setting. The purpose of this approach was to predict outcomes as well as to study the pharmacodynamic effects of each treatment. Gene expression profiles of peripheral blood mononuclear cells obtained pre- and posttreatment and of tumor biopsy samples obtained pretreatment were determined (Fig. 3). An algorithm to distinguish outcomes using the dataset of these three sampling points was created and expected to be more powerful than conventional outcome assessment techniques.

It seems quite an unusual approach to use normal cells in gene expression profiling in oncology; however, this has proved to be a useful way to monitor drug pharmacodynamic effects and to select biomarkers. Using this approach, we selected biomarkers to capture adverse effects of the treatments. Such "biomarker monitoring" is a rapidly growing field of research.

Biomarker monitoring for tyrosine kinase inhibitors

Recently, EGFR mutation has become an exciting topic in research on TKI [4, 6]. Mutation analysis is now essential for any correlative studies for TKI. Patients with tumors containing the EGFR mutation in different exons are thought to have different responses to TKI. A short, in-frame deletional mutant (E746-A750del) is one of the major mutant forms of EGFR in Japanese populations, and a determinant for EGFR-specific TKI such as gefitinib and ZD6474 (Fig. 4) [1, 8]. We investigated the biological and pharmacological functions of this mutated EGFR to determine whether tumors with deletional-EGFR status are responsive to ligand stimulation, whether mutated EGFR is constitutively active, and whether the downstream intracellular signaling pathway is altered. We concluded that deletional EGFR is constitutively active and that its downstream events are shifted to the AKT pathway (Fig. 5). In addition, a cell-free kinetic assay using mutant EGFR proteins demonstrated differential affinity to TKI among different EGFR mutants. Additional mutations after treatment are also generating interest with regard to their role in acquired resistance to TKI [2]. Thus, the mutation

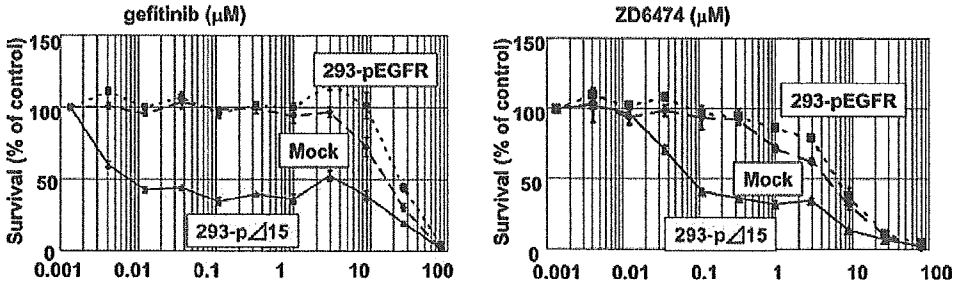

Fig. 4 In vitro sensitivity of 293 cells transfected with a deletional epidermal growth factor receptor (*EGFR*) gene (E746-A750) to tyrosine kinase inhibitors (gefitinib and ZD6474) determined by MTT assay. EGFR mutation (E746-A750 type deletion) increases sensitivity to tyrosine kinase inhibitors (gefitinib and ZD6474). HEK293 cells were transfected with empty vector (293-mock), wild-type EGFR (293 p-EGFR), and deletional EGFR (293-pΔ15). Reprinted with permission of the American Association for Cancer Research Inc., from Arao et al. [1]

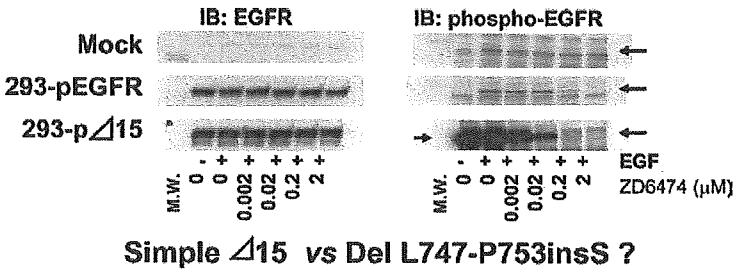

Fig. 5 Constitutive phosphorylation of mutant EGFR. Phosphorylation of EGFR was determined by immunoblotting in 293 cells transfected with Mock, wild-type EGFR, and deletional EGFR cDNA. Increased phosphorylation was observed in the 293-pΔ15 cells under no ligand stimulation. Reprinted with permission of the American Association for Cancer Research Inc., from Arao et al. [1]. (*EGF* epidermal growth factor receptor, *IB* immunoblotting)

status of EGFR is one of the determinants for the prediction of tumor response to EGFR-targeted TKI. On the other hand, the clinical impact of EGFR mutation on survival in patients treated with these TKI remains unclear. Therefore, molecular correlative study including EGFR mutation analysis is quite important for prospective studies. Various technologies for EGFR mutation assay have been developed and some of these assays have been validated in the clinical situation [5]. Gene mutation analysis in prospective studies of TKI using standardized technologies is very important.

Protein arrays

Proteomics technology has been developed and successfully used to identify biomarkers for target-based drugs in a few clinical studies. Additional approaches such as antibody arrays and "PowerBlots®", especially those using phospho-specific antibodies, should enable us to perform "kinome" analyses. Hence, these protein analysis technologies are now powerful tools for research on TKI.

Acknowledgements This work was supported by funds for the Third Term Comprehensive 10-Year Strategy for Cancer Control and a Grant-in-Aid for Scientific Research and for Health and Labour Science Research Grants, Research on Advanced Medical Technology, H14-Toxico-007.

References

1. Arao T, Fukumoto H, Takeda M, Tamura T, Saijo N, Nishio K (2004) Small in-frame deletion in the epidermal growth factor receptor as a target for ZD6474. Cancer Res 64:9101–9104
2. Koizumi F, Shimoyama T, Taguchi F, Saijo N, Nishio K (2005) Establishment of a human non-small cell lung cancer cell line resistant to gefitinib. Int J Cancer 116:36–44
3. Korfee S, Eberhardt W, Fujiwara Y, Nishio K (2005) The role of DNA-microarray in translational cancer research. Curr Pharmacogenomics (in press)
4. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 350:2129–2139
5. Nishio M, Ohyanagi F, Horiike A, Ishikawa Y, Satoh Y, Okumura S, Nakagawa K, Nishio K, Horai T (2005) Gefitinib treatment affects androgen levels in non-small-cell lung cancer patients. Br J Cancer 92:1877–1880
6. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 304:1497–1500
7. Shimoyama T, Yamamoto N, Hamano T, Tamura T, Nishio K (2005) Gene expression analysis to identify the pharmacodynamic effects of docetaxel on the Rho signal pathway in human lung cancer patients (abstract 2002). Proc Am Soc Clin Oncol 23:135s
8. Taguchi F, Koh Y, Koizumi F, Tamura T, Saijo N, Nishio K (2004) Anticancer effects of ZD6474, a VEGF receptor tyrosine kinase inhibitor, in gefitinib ("Iressa")-sensitive and resistant xenograft models. Cancer Sci 95:984–989

Lung Cancer (2005) 50, 393–399

ELSEVIER

LUNG CANCER

www.elsevier.com/locate/lungcan

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

Hideharu Kimura[a,b], Kazuo Kasahara[b], Masaru Sekijima[c], Tomohide Tamura[d], Kazuto Nishio[a,e,*]

[a] *Shien-Lab, National Cancer Center Hospital Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045, Japan*
[b] *Respiratory Medicine, Kanazawa University Hospital, Takara-machi13-1, Kanazawa, Ishikawa, Japan*
[c] *Mitsubishi Chemical Safety Institute, Ibaraki, Japan*
[d] *Medical Oncology, National Cancer Center Hospital, Japan*
[e] *Pharmacology Division, National Cancer Center Research Institute, Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045, Japan*

Received 15 March 2005; received in revised form 7 July 2005; accepted 15 July 2005

KEYWORDS
Gefitinib;
EGFR;
MIP-1β;
Cytokine;
Lung cancer;
Skin toxicity

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.

* Corresponding author. Tel.: +81 3 3542 2511x6143; fax: +81 3 3547 5185.
E-mail address: knishio@gan2.res.ncc.go.jp (K. Nishio).

0169-5002/$ – see front matter
doi:10.1016/j.lungcan.2005.07.012

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 ≧ Grade1
Skin	8	13	5	2	0	71.4
Hepatitis	22	4	1	1	0	21.4
Pneumonitis	25	0	0	3	0	1.1
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)	23.01 (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[a]	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).
[a] Significant difference between pre and post.

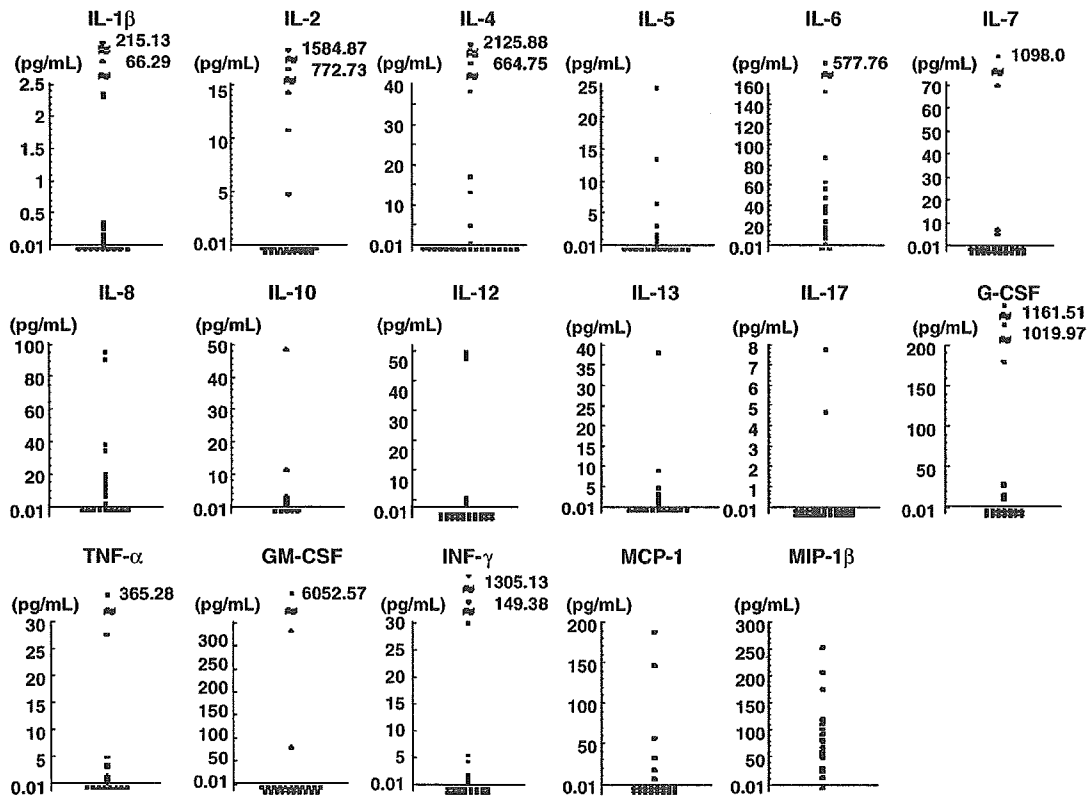

Fig. 1 Plasma concentrations of seventeen cytokines before the commencement of gefitinib in 24 patients. The plots under the line of 0.01 indicate levels lower than the measurement sensitivities.

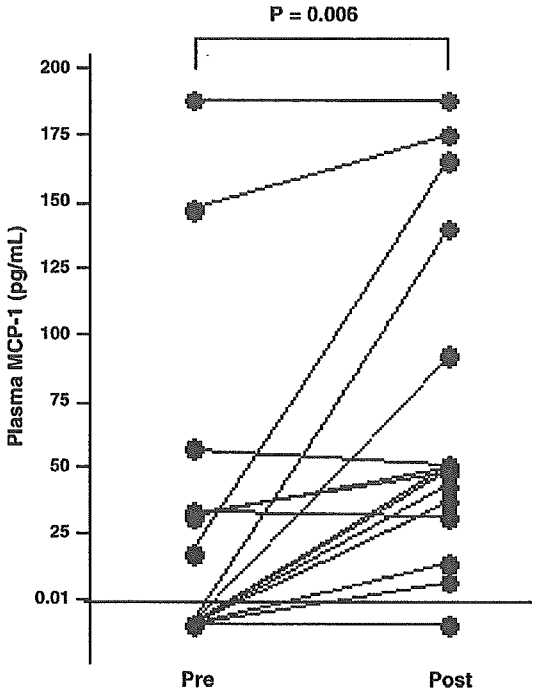

Fig. 2 Plasma concentrations of MCP-1 in 23 patients before and after gefitinib treatment. The differences between the values before and after treatment were significant ($p < 0.05$, paired t-test).

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

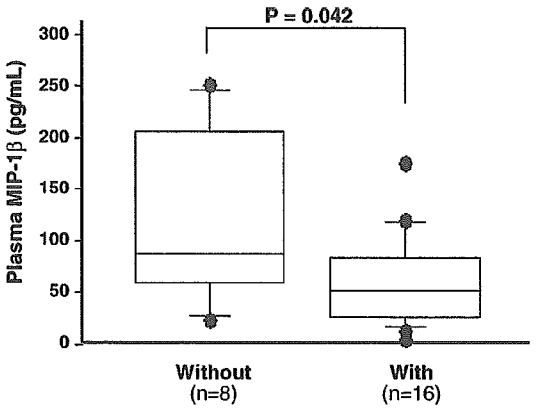

Fig. 3 Box-plots comparing the plasma concentrations of MIP-1β between patients with and without dermatitis; these samples were collected before the initiation of gefitinib. The patients were separated into two groups according to whether or not they developed dermatitis. Patients with skin toxicity 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.

Acknowledgements

H. Kimura received support from an Awardee of the Research Resident Fellowship from the Foundation for Promotion of Cancer Research (Japan) for the 3rd Term Comprehensive 10-Year-Strategy for Cancer Control.

References

[1] Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol 1995;19:183–232.
[2] Franklin WA, Veve R, Hirsch FR, Helfrich BA, Bunn Jr PA. Epidermal growth factor receptor family in lung cancer and premalignancy. Semin Oncol 2002;29:3–14.
[3] Ciardiello F, Caputo R, Bianco R, Damiano V, Pomatico G, De Placido S, et al. Antitumor effect and potentiation of cytotoxic drugs activity in human cancer 15 cells by ZD1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. Clin Cancer Res 2000;6:2053–63.
[4] Moasser MM, Basso A, Averbuch SD, Rosen N. The tyrosine kinase inhibitor ZD1839 ("Iressa") inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells. Cancer Res 2001;61:7184–8.
[5] Koizumi F, Kanzawa F, Ueda Y, Koh Y, Tsukiyama S, Taguchi F, et al. Synergistic interaction between the EGFR tyrosine kinase inhibitor gefitinib ("Iressa") and the DNA topoisomerase I inhibitor CPT-11 (irinotecan) in human colorectal cancer cells. Int J Cancer 2004;108:464–72.
[6] Fukuoka M, Yano S, Giaccone G, Tamura T, Nakagawa K, Douillard JY, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. J Clin Oncol 2003;21:2237–46.
[7] Kris MG, Natale RB, Herbst RS, Lynch Jr TJ, Prager D, Belani CP, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. J Am Med Assoc 2003;290:2149–58.
[8] Dawson NA, Guo C, Zak R, Dorsey B, Smoot J, Wong J, et al. A phase II trial of gefitinib (Iressa, ZD1839) in stage IV and recurrent renal cell carcinoma. Clin Cancer Res 2004;10:7812–9.
[9] van Doorn R, Kirtschig G, Scheffer E, Stoof T, Giaccone G. Follicular and epidermal alterations in patients treated with ZD1839 (Iressa), an inhibitor of the epidermal growth factor receptor. Br J Dermatol 2002;147:598–601.
[10] Jacot W, Bessis D, Jorda E, Ychou M, Fabbro M, Pujol JL, et al. Acneiform eruption induced by epidermal growth factor receptor inhibitors in patients with solid tumours. Br J Dermatol 2004;151:238–41.
[11] Saltz LB, Meropol NJ, Loehrer PJ, Needle MN, Kopit J, Mayer RJ. Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. J Clin Oncol 2004;22:1201–8.
[12] Govindan R. Cetuximab in advanced non-small cell lung cancer. Clin Cancer Res 2004;10:4241S–4S.
[13] Soulieres D, Senzer NN, Vokes EE, Hidalgo M, Agarwala SS, Siu LL. Multicenter phase II study of erlotinib, an oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with recurrent or metastatic squamous cell cancer of the head and neck. J Clin Oncol 2004;22:77–85.
[14] Pérez-Soler R, Chachoua A, Hammond LA, Rowinsky EK, Huberman M, Karp D, et al. Determinations of tumor response and survival with erlotinib in patients with non-small cell lung cancer. J Clin Oncol 2004;22:3238–47.

[15] Koh YC, Yuan D. The effect of NK cell activation by tumor cells on antigen-specific antibody responses. J Immunol 1997;139:4745–52.
[16] Lejeune FJ, Ruegg C, Lienard D. Clinical applications of TNF-alpha in cancer. Curr Opin Immunol 1998;10:57380.
[17] McKeown DJ, Brown DJ, Kelly A, Wallace AM, McMillan DC. The relationship between circulating concentrations of C-reactive protein, inflammatory cytokines and cytokine receptors in patients with non-small-cell lung cancer. Br J Cancer 2004;91:1993–5.
[18] Neuner A, Schindel M, Wildenberg U, Muley T, Lahm H, Fischer JR. Prognostic significance of cytokine modulation in non-small cell lung cancer. Int J Cancer 2002;101:287–92.
[19] De Vita F, Orditura M, Auriemma A, Infusino S, Roscigno A, Catalano G. Serum levels of interleukin-6 as a prognostic factor in advanced non-small cell lung cancer. Oncol Rep 1998;5:649–52.
[20] Orditura M, De Vita F, Catalano G, Infusino S, Lieto E, Martinelli E, et al. Elevated serum levels of interleukin-8 in advanced non-small cell lung cancer patients: relationship with prognosis. J Interferon Cytokine Res 2002;22:1129–35.
[21] Hidalgo GE, Zhong L, Doherty DE, Hirschowitz EA. Plasma PGE-2 levels and altered cytokine profiles in adherent peripheral blood mononuclear cells in non-small cell lung cancer (NSCLC). Mol Cancer 2002;1:5.
[22] Steube K, Meyer C, Drexler H. Secretion of functional hematopoietic growth factors by human carcinoma cell lines. Int J Cancer 1998;78:120–4.
[23] Bronte V, Chappell D, Apolloni E. Unopposed production of granulocyte-macrophage colony stimulating factor by tumor inhibits CD8+ T cell responses by dysregulating antigen-presenting cell maturation. J Immunol 1999;162:5728–37.
[24] Chopra V, Dinh T, Hannigan EV. Angiogenin, interleukins, and growth factor levels in serum of patients with ovarian cancer. Correlation with angiogenesis. Cancer J Sci Am 1996;2:279–85.
[25] Chopra V, Dinh T, Hannigan E. Circulating serum levels of cytokines and angiogenic factors in patients with cervical cancer. Cancer Invest 1998;16:152–9.
[26] Pekarek L, Weichselbaum R, Beckett M, Nachman J, Schreiber H. Footprinting of individual tumors and their variants by constitutive cytokine expression patterns. Cancer Res 1993;53:1978–81.
[27] Hertl M, Merk HF. Lymphocyte activation in cutaneous drug reactions. J Invest Dermatol 1995;105:95S–8S.
[28] Hari Y, Urwyler A, Hurni M, Yawalkar N, Dahinden C, Wendland T, et al. Distinct serum cytokine levels in drug- and measles-induced exanthema. Int Arch Allergy Immunol 1999;120:225–9.
[29] Torres MJ, Corzo JL, Leyva L, Mayorga C, Garcia-Martin FJ, Antunez C, et al. Differences in the immunological responses in drug- and virus-induced cutaneous reactions in children. Blood Cells Mol Dis 2003;30:124–31.
[30] Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000;92:205–16.
[31] Busam KJ, Capodieci P, Motzer R, Kiehn T, Phelan D, Halpern AC. Cutaneous side-effects in cancer patients treated with the antiepidermal growth factor receptor antibody C225. Br J Dermatol 2001;144:1169–76.
[32] Lee MW, Seo CW, Kim SW, Yang HJ, Lee HW, Choi JH, et al. Cutaneous side effects in non-small cell lung cancer patients treated with Iressa (ZD1839), an inhibitor of epidermal growth factor. Acta Derm Venereol 2004;84:23–6.
[33] Nanney LB, Maglid M, Stoscheck CM, King Jr LE. Comparison of epidermal growth factor binding and receptor distribution in normal human epidermis and epidermal appendages. J Invest Dermatol 1984;83:385–93.
[34] Nanney LB, Stoscheck CM, King Jr LE, Underwood RA, Holbrook KA. Immunolocalization of epidermal growth factor receptors in normal developing human skin. J Invest Dermatol 1990;94:742–8.
[35] Herbst RS, LoRusso PM, Purdom M, Ward D. Dermatologic side effects associated with gefitinib therapy: clinical experience and management. Clin Lung Cancer 2003;4:366–9.
[36] Jost M, Kari C, Rodeck U. The EGF receptor, an essential regulator of multiple epidermal functions. Eur J Dermatol 2000;10:505–10.
[37] Premack BA, Schall TJ. Chemokine receptors: gateways to inflammation and infection. Nat Med 1996;2:1174–8.
[38] Bernardini G, Hedrick J, Sozzani S, Luini W, Spinetti G, Weiss M, et al. Identification of the CC chemokines TARC and macrophage inflammatory protein-1 beta as novel functional ligands for the CCR8 receptor. Eur J Immunol 1998;28:582–8.
[39] Matsukawa A, Hogaboam CM, Lukacs NW, Kunkel SL. Cytokines and innate immunity. Rev Immunogenet 2000;2:339–58.

0The Role of DNA-Microarray in Translational Cancer Research

Sönke Korfee[1,2,*], Wilfried Eberhardt[2], Yasuhiro Fujiwara [3] Kazuto Nishio[1]

FINAL

[1]*Shien Lab, National Cancer Center Hospital Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045, Japan,* [2]*Department of Internal Medicine (Cancer Research), West German Cancer Centre, Hufeland Strasse 55,45122 Essen, Germany,* [3]*Breast and Medical Oncology Division, National Cancer Center Hospital Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045, Japan*

Abstract: The overall prognosis for the majority of cancer patients remains poor. Current conventional strategies in clinical cancer research are unable to adequately answer a large number of important unsolved questions. Although some patients achieve substantial benefits from classical cytotoxic chemotherapy, others will not. The mechanisms behind this phenomenon are still not identified in detail. Furthermore, the activity of promising novel molecular targeting anticancer agents like tyrosinekinase inhibitors is currently not predictable within the individual patient. The biological background for this clinical and prognostic heterogeneity in behavior is more or less the large individual variation in the biological nature of tumors within the same classified histological subgroup. The overall usefulness of conventional histopathological classifications to adequately predict patient prognosis or response to chemotherapy is limited. The most promising way to solve this issue is to found clinical research strategies on basic biological evidence. New genomic technologies have been developed within recent years. These techniques are able to analyze thousands of genes and their expression profiles simultaneously. An increasing number of investigations has reported applications of these novel technologies within clinical trials settings. The aim of this approach is to identify new subsets of cancer patients, to improve prediction of their clinical outcome or response to treatment and select new targets for innovative therapeutic drugs based on the findings from gene expression profiles. Results of these gene expression profile studies could potentially lead to more individually tailored systemic cancer therapy. In the recent years, a remarkable number of studies based on these techniques have already been reported. Although the published results are clearly impressive and highly promising, a lot of work remains to be done. Moreover, there is a strong need for an increase in reliability and reproducibility of such gene expression profiling techniques and thus introduction of reproducible quality control in the performance of these assays. Although a large number of issues remain to be clarified prior to a more general application of genomic profiling techniques in clinical cancer research, this strategy will eventually turn out as a promising approach to improve successful management of cancer patients.

INTRODUCTION

The overall prognosis for the majority of cancer patients is still unsatisfactory. Hardly any stage IV lung cancer patient will be alive five years following initial diagnosis [Mountain 1997]. Even new generation cytotoxic agents with higher efficacy and more favorable toxicity profiles like paclitaxel, docetaxel and gemcitabine have not brought an identifiable breakthrough in cancer therapy [Schiller 2002]. A large group of tumor entities is primarily resistant or will develop secondary resistance to cytotoxic chemotherapy. On the other hand, there is a definite subset of patients with proven benefit from cancer chemotherapy. The basic mechanisms behind this clinical phenomenon are not clearly identified. Adjuvant chemotherapy following definitive local treatment of early disease (e.g complete resection) represents another important issue. In earlier stages there is currently no reliable method to predict those patients who will gain significant benefit from adjuvant treatment. The current situation regarding the use of novel molecular targeting drugs is of striking parallelity. Activity of these agents is at the moment not predictable in the individual patient. The background of this lies in the remarkable individual variety of biological nature and clinical behavior of tumors even within the same pathological entity. Thus, the impact of classical histological subclassifications to adequately predict patient prognosis or response to chemotherapy is limited. In contrast, more information on molecular tumor biology may improve cancer treatment strategies in the future. This strategy could be one important step to individualize cancer management. New genomic technologies have been developed within the recent years. These techniques have the capability to analyze the expression and activity of thousands of different genes simultaneously. An increasing number of investigations has applied these genomic techniques as an adjunct to clinical studies with the purpose to discover new sub-classes of tumors or predict outcome of therapy on the basis of these gene expression profiles. Although a number of studies have been published during the last years with impressive and clinically relevant results, a lot of work remains to be performed. One major challenge will be to find the appropriate statistical method for correctly analyzing the large data sets to get valid and reliable scientific results. Currently, another major problem is the lack of comparability between results from different investigations. Several different genomic techniques (cDNA-microarray, filter-array, short and long oligonucleotide arrays) and statistical methods (supervised and unsupervised analysis) have been used in recent studies. International standardizations of gene profiling based studies are needed for a proper interpretation of results in the future. Despite several remaining issues in applying genomic techniques to clinical cancer research, these methods still belong to the most promising tools for improving treatment results in the

*Address correspondence to this author at the Shien Lab, National Cancer Center Hospital, Pharmacology Div, National Cancer Center Res Inst, Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045, Japan; Fax: +81 3 3547 5185; E-mail: knishio@gan2.res.ncc.go.jp

1570-1603/05 $50.00+.00