gemcitabine, or irinotecan is probably acceptable as the current standard first-line chemotherapy.

First-line single agent with gefitinib is active, but produces unacceptably frequent ILD in the Japanese population. Being female, as well as adenocarcinoma, those who never smoked, and *EGFR* mutation were associated with response to gefitinib. Patients who responded to gefitinib did not experience ILD during gefitinib chemotherapy. Further research via genetics and image analysis is needed to avoid ILD and identify a subgroup of patients that benefit from gefitinib treatment. If this is realized, single agent treatment with gefitinib could be an option as first-line chemotherapy in selected patients with advanced NSCLC. Furthermore, randomized trials are warranted to compare first-line single agent treatment with gefitinib followed by second-line platinum-based chemotherapy with first-line platinum-based chemotherapy followed by second- or third-line gefitinib treatment.

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Authors' Disclosures of Potential Conflicts of Interest

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

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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

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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.

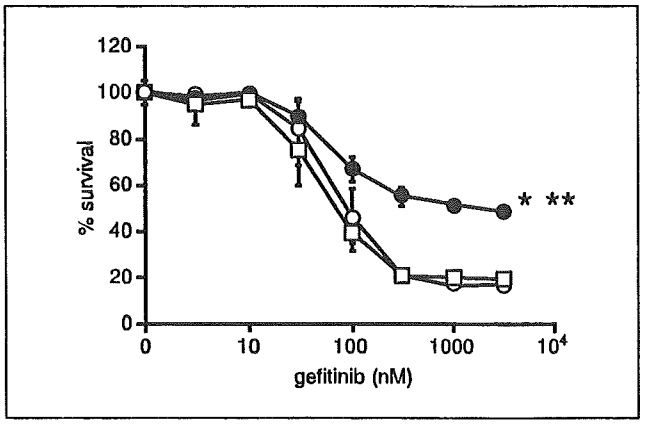

Fig. 1. Cytotoxic effects of gefitinib in a gefitinib-resistant NSCLC cell line. The cells (2×10^3 per well) were seeded onto a 96-well plate and preincubated overnight, then continuously exposed to the indicated concentrations of gefitinib for 4 or 5 days. The growth inhibition rate was analyzed with 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

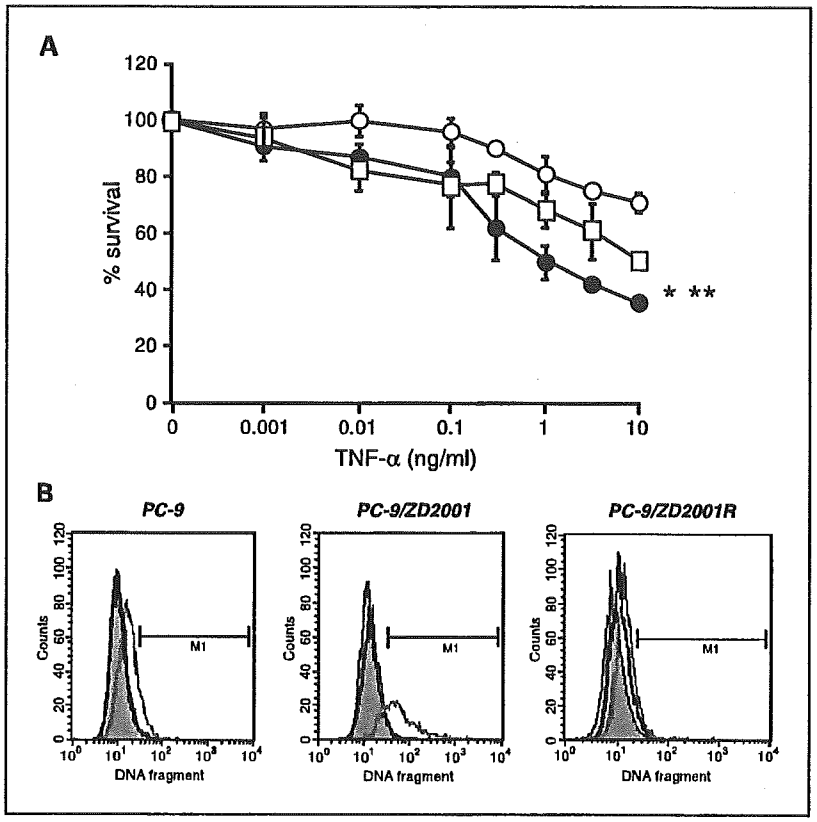

Fig. 2. Gefitinib-resistant cells acquired sensitivity to TNF-α. *A*, the cells were continuously treated with the indicated concentrations of TNF-α for 4 or 5 days. The growth inhibition rate was analyzed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in Materials 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

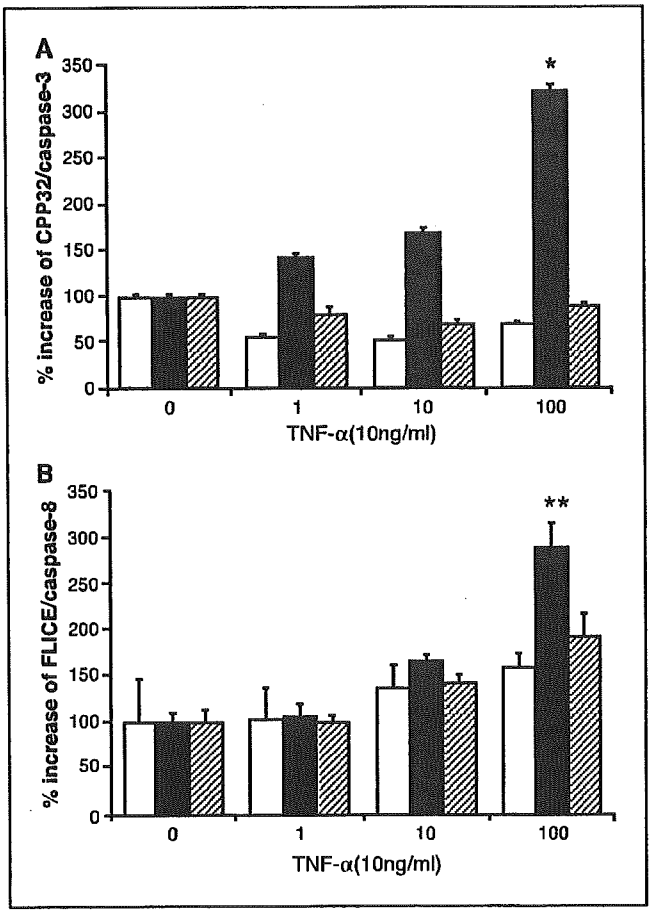

Fig. 3. TNF-α-mediated activation of CPP32/caspase-3 and FLICE/caspase-8 in PC-9, PC-9/ZD2001, and PC-9/ZD2001R cells. Activation of CPP32/caspase-3 and FLICE/caspase-8 was measured as described in Materials and Methods. The cells were exposed to the indicated concentrations of TNF-α for 12 hours; after 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

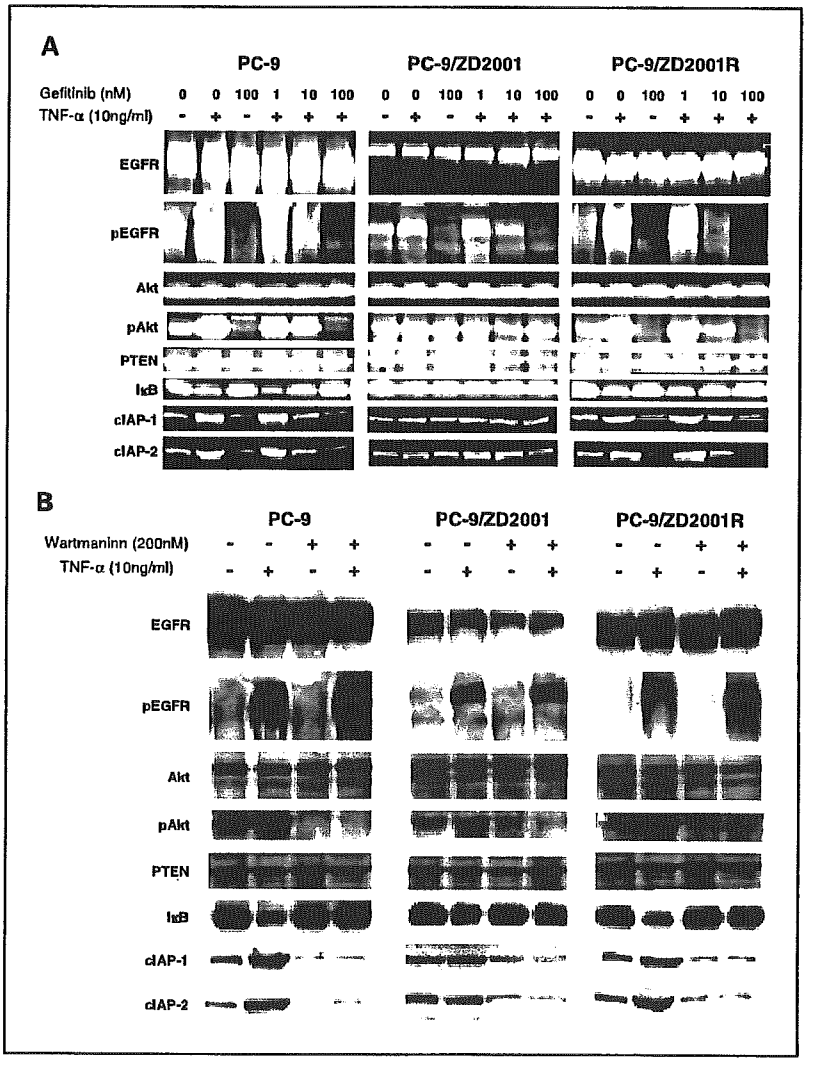

Fig. 4. Inhibitory effect of gefitinib on TNF-α-induced phosphorylation of Akt1 and degradation of IκB. Cells were treated with TNF-α with or without gefitinib (*A*) or wortmannin (*B*) simultaneously for 30 minutes at 37°C. Cell lysates were prepared and equivalent amounts of protein from each cell 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*).

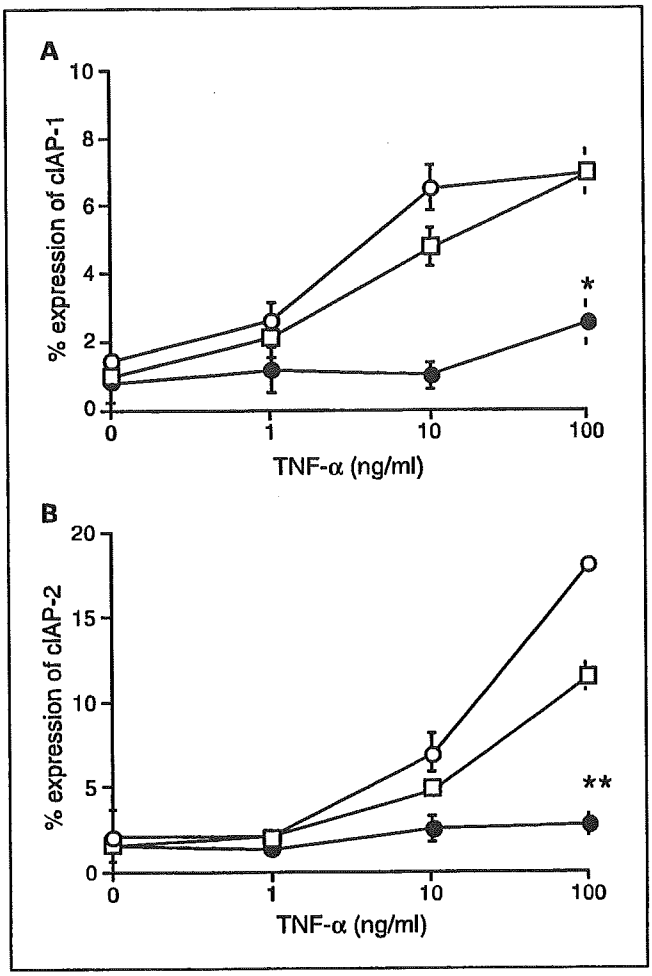

Fig. 5. TNF-α induced c-IAP1 and c-IAP2 mRNA expression in PC-9 and PC-9/ZD2001R cells but not in PC-9/ZD2001 cells. The cells were exposed to the indicated concentrations of TNF-α for 12 hours; after which mRNA was isolated with the guanidium isothiocyanate method. Induction of c-IAP1 (*A*) and c-IAP2 (*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.

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Translational studies for target-based drugs

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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

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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

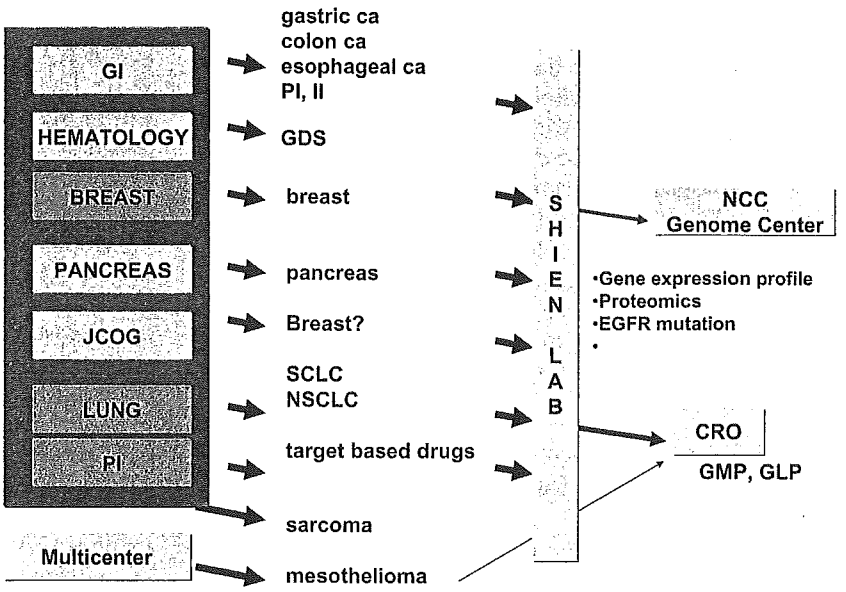


Fig. 1 Flow of clinical samples in molecular correlative studies at the National Cancer Center Hospital. (*GI* gastrointestinal, *JCOG* Japan Clinical Oncology Group, *PI* clinical phase I study, *PII* clinical phase II study, *GDS* gene delivery system, *SCLC* small cell lung cancer, *NSCLC* non-small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

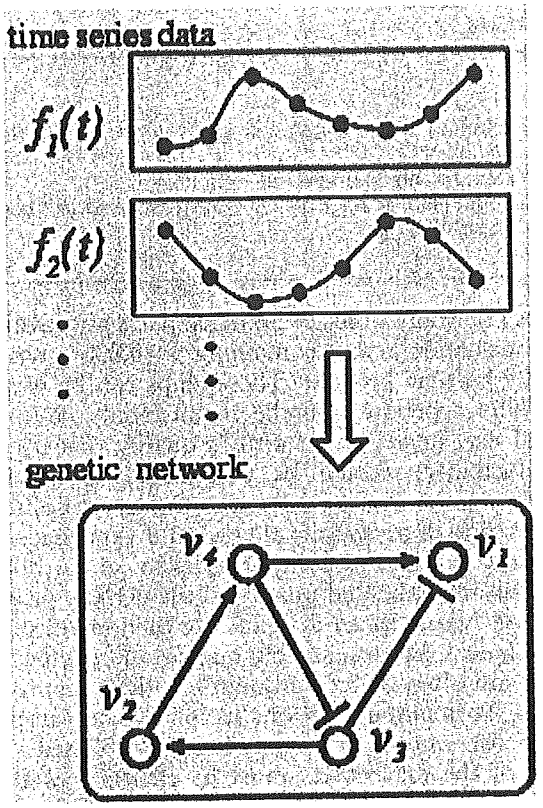


Fig. 2 Network analysis to determine transcriptional pathway and signal transduction pathway modulated by transcriptional regulators and multitarget tyrosine kinase inhibitors using gene expression profiling dataset

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

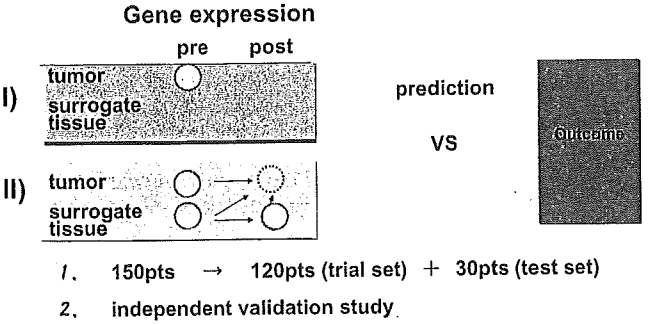


Fig. 3 Gene expression monitoring to distinguish the outcome of treatment for breast cancer patients

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

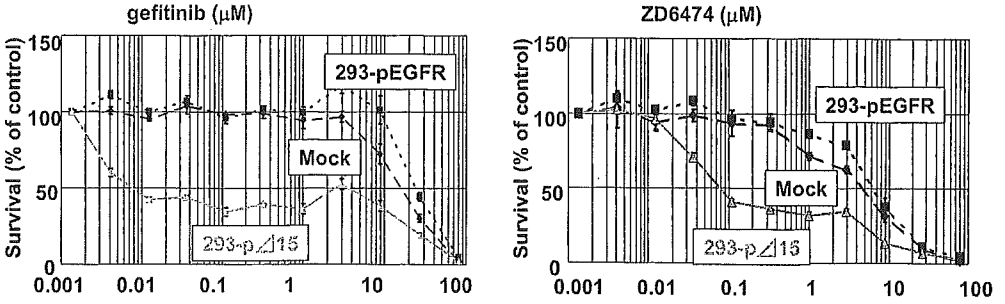
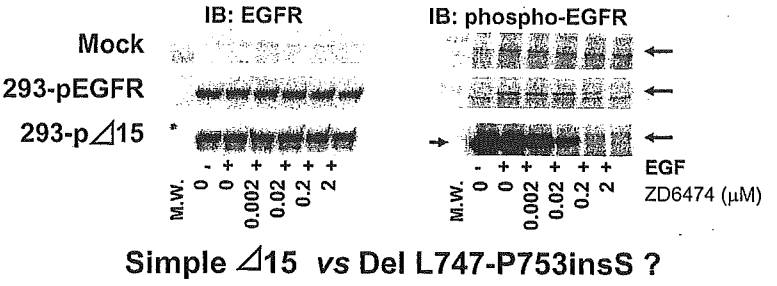


Fig. 4 In vitro sensitivity of 293 cells transfected with a deletional epidermal growth factor receptor (*EGFR*) gene (E746-A750) to tyrosine kinase inhibitors (gefitinib and ZD6474) determined by MTT assay. EGFR mutation (E746-A750 type deletion) increases sensitivity to tyrosine kinase inhibitors (gefitinib 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]



Simple Δ15 *vs* Del L747-P753insS ?

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.

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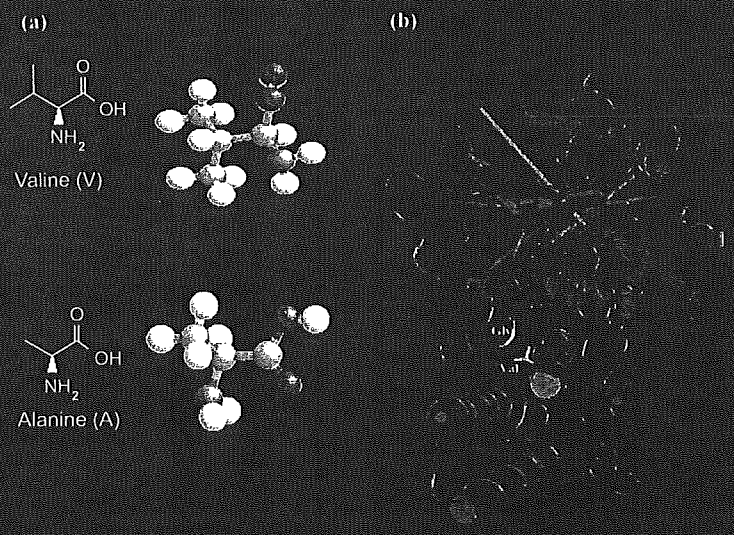

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The Role of DNA-Microarray in Translational Cancer Research

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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 tyrosinkinase 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

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future. In the following overview, we will try to focus on the current role of genomic techniques in translational cancer research. In the first part of this paper, we will summarize the most important published studies for frequent tumor entities. Furthermore, the individual methods and their clinical relevance will be critically reviewed. In the second part of the manuscript, we will deal with issues of reliability and reproducibility of reported genomic array data.

BREAST CANCER

In 2000, a Norwegian – American Cooperative Group performed an analysis of breast cancer cell lines and tumor tissue based on DNA microarrays [Perou 2000]. They could classify the tumors into subtypes distinguished by their different gene expression profiles using the "hierarchical clustering[1]" methodology based on 1753 genes independent from the histological classification. The tumors were subdivided into an ER+(estrogen-receptor-positive)/luminal like, a basal-epithelium-like group, an ERB-B2-(erythroblastic leukemia viral oncogene homolog 2) group and a normal-breast-like group. The same group published within the following year, a further investigation on this subject [Sorlie 2001]. This time, in a larger number of tumors, at least six subtypes could be differentiated on the basis of their gene expression patterns using an intrinsic set of 457 genes (Table **1**). The previous (ER+)/luminal-like group could be subdivided into three prognostic groups. In addition, a list of 256 genes related to clinical outcome was developed using a special supervised data-analysis (SAM: significance analysis of microarray, this technique will be explained more in detail in the second part of this article) derived from a clinical data set of 76 carcinomas, in which sufficient data were available. This 256-gene-"predictor" was correlated with clinical outcome in a cohort of 49 patients with locally-advanced breast cancer uniformly treated in a prospective clinical investigation. The basal-like subtype was associated with poorer prognosis regarding overall as well as relapse-free survival (Table **1**). Interestingly, significant differences could be observed in the clinical outcome between two different estrogen–receptor-positive groups. West and co-workers could demonstrate the potential usefulness of DNA microarray analysis to discriminate different breast cancer patients on the basis of their ER (estrogen-receptor) status (Table **2**) [West 2001]. A 100-gene "predictor" to estimate estrogen receptor status was developed analyzing a set of 38 samples (the "training set"). Then in a second step, the "predictor" was validated in a set of nine independent tissue samples (the "test set") (Table **3**). In some samples, the "predictor" could adequately predict clinical ER-status. Five samples with conflicting results of immunohistochemistry and immunoblotting data regarding the ER-status were included within this "test set". In some of these samples, the predictive probability of the "predictor" was found to be lower. This could reflect the heterogeneity of expression within the individual tumor. Furthermore, impressive data regarding an analysis of estrogen-receptor status by calculating gene expression profiles were published by a

Table 1. Breast Cancer Microarray Classification by Sorlie - Based on a Intrinsic Set of 457 Genes

Correlation of microarray classification with overall survival prognosis (Sorlie 2001) (n=49; p<0,01)	
Subtype	Prognosis
ER+/luminal like Typ A	good
ER+/luminal like Typ B	intermediate
ER+/luminal like Typ C	intermediate
Basal like	poor
ERB-B2	poor
Normal like	intermediate

The estrogen receptor positive ER+/luminal like group is subdivided into three subtypes. Correlation with overall survival reveals a poor prognosis for the Basal like and ERB-B2 group. Interestingly different prognosis for patients was found within the three estrogen receptor positive (ER+) groups.

Table 2. Class Prediction Studies Regarding ER-Status in Breast Cancer

Author	Patients	Technique	Statistical method	Number of genes of predictor	Training set	Test set	correct prediction (%)
West (2001)	48	cDNA microarray	Bayesian regression	100	38	9	100
Gruvberger (2001)	58	cDNA microarray	Artificial Neural Network	100	47	11	100

"Predictors" for estrogen receptor status based on microarray data were established by to different groups in 2001. Both "predictors" include 100 genes. After developing the "predictor" in a set of samples and corresponding clinical data (Trainig-set) both groups could validate their "predictor" in independent set of samples and clinical data (Test set) with high accuracy.

[1] **Hieachical clustering:** A hierarchical clustering is a sequence of partitions in which each partition is nested into the next partition in the sequence

Table 3. Top 5 Ranked Genes for Prediction of ER-status

Rank	West 2001	Gruvberger 2001
1	Trefoil factor 1 (ps2)	Estrogen Receptor 1
2	Estrogen receptor	Trefoil factor 3
3	Cytochrome P450	GATA Bindind protein 3
4	Trefoil factor 3	ESTs
5	Estrogen like growth factor	Calgranulin A

West and Gruvberger established in 2001 independently "predictors" for estrogen-receptor status in breast cancer based on microarray data. The five genes with strongest correlation of expression and ER-status of the 100 gene"predictors" by West and Gruvberger are listed in this table. Both "predictors" show similarities. Beside the estrogen receptor itself the trefoil factor 3 is find within the five top ranked genes in both studies.

Swedish group within the same year (Table **2**) [Gruvberger 2001]. A 100-gene "predictor" for ER status was developed in a "trainings set" of 47 tumors based on microarray data using "artificial neural network[2]" (Table **3**). The "predictor" was validated in 11 independent samples of a "test set". All 11 tumors could be classified correctly by this 100-gene marker. Interestingly, even without the top level discriminator genes, including ER itself, the "artificial neural network" could adequately predict ER-status.

Adjuvant cancer therapy is well-established in the treatment of breast cancer. Chemotherapy or hormonal therapy is able to reduce the risk of disease dissemination in one-third of the patients, but a large number of patients will have already been cured without the application of adjuvant therapy. Taking into account that the application of adjuvant chemotherapy carries a well-defined morbidity and mortality risk, the proper selection of patients with a clear and established benefit from adjuvant chemotherapy would be extremely helpful. Based on the hypothesis that patients with a poor prognosis following surgery would mostly benefit from adjuvant therapy, a Dutch group performed a translational study testing the predictive impact of DNA Microarray data on overall survival prognosis in young females with primary-lymph–node-negative breast cancer [van 't Veer 2002]. In this study, tumor tissue from 117 young patients with primary lymph-node-negative breast cancer was analyzed by DNA microarray technique. A 70-gene prognosis "predictor" ("poor prognosis signature") for patients with a short interval to the development of distant metastasis was established by supervised classification of the gene expression profiling. This "poor prognosis signature" included genes regulating cell cycle, invasion, metastasis and angiogenesis. A second correlative study was performed by the same group to confirm the predictive power of this 70-gene prognostic marker in a larger and less homogeneous group of patients [van de Vijver 2002]. A series of 295 consecutive patients with stage I and II breast cancer who underwent surgery, were included into this study. All patients had to be younger than 53 years. In this investigation, patients with-lymph-node-negative (151 pts) and lymph-node-positive (144 pts) disease were analyzed. A group of 180 patients with "poor prognosis signature" could be separated from 115 patients with "good-prognosis-signature" (Table **4**). The overall 10-year survival rate was found to be 54.6 percent within the "poor" and 94.5 percent in the "good prognosis group". The probability of remaining free from distant metastases within 10 years was found to be 50.6 percent in the group with "poor-prognosis-signature" and 85.2 percent in the group with "good–prognosis-signature". In comparison to the "good prognosis group", the estimated hazard ratio for developing distant metastasis in the group with "poor-prognosis-signature" was 5.1 (95% confidence interval, 2.9 to 9.0; P <0.001). This microarray based prognosis profile was identified as a strong independent factor in predicting disease outcome. Interestingly, the prognostic profile did not depend on lymph–node status, well-known to be one of the classical prognostic factors. On

Table 4. Overall Survival and Distant Metastasis Free Survival Probability According to Prognosis Signature (Van't Vijver 2002)

Group	No. of patients	Overall survival (%)		free of distant metastasis (%)	
		5YR	10YR	5YR	10YR
Poor prognosis signature	180	74.1	54.6	60.5	50.6
Good prognosis signature	115	97.4	94.5	94.7	85.2

A 70 gene prognostic marker ("predictor") was tested by van't Vijver in a series of 295 consecutive patients with stage I and II breast cancer who underwent surgery. They could distinguish 180 patients with poor prognosis (Poor prognosis signature) from 115 patients with good prognosis (Good prognosis signature) regarding to overall survival and distant metastasis-free survival.

[2] **Artificial neural network:** A network of many very simple processors "units" or "neurons"), earch possibly having a (small amount of) local memory. The units are connected by unidirectional communication channels ("connections", which carry numeric (as opposed to symbolic) data. The units operate only on their local data and on the inputs they receive *via* the connections.

the other hand, the positive-estrogen–receptor status, the other classical predictor, was strongly correlated with a "good prognosis signature" profile. In spite of these impressive results, there has been important criticism towards these findings: An insufficient analysis of intratumoral representativeness of the investigated tumor samples and the measurement of tumor size is critically discussed [Kunkler 2003, Kopans 2003]. However, this pioneer investigation could prove, that a prediction of clinical outcome based on microarray data may in principle be possible.

LUNG CANCER

Lung cancer is still the leading cause of cancer-related deaths in the industrialized world [Jemal 2002]. Physicians treating patients with this disease often face difficult decisions to be made within all clinical disease stages. For example, the issue of adjuvant chemotherapy following complete resection is not conclusively solved with conflicting results published [Scagliotti 2003; International Adjuvant Lung Cancer Trial Cooperative Group 2003, LeChevalier 2003*]. A reliable method to predict patient prognosis following surgical treatment of early stage lung cancer could be most helpful to estimate the benefit of adjuvant chemotherapy within the individual patient. Classical histopathology examination is definitely insufficient for this decision making. Beer published in 2002, a translational research study correlating gene expression profile data with overall survival in patients with early stages lung adenocarcinoma after surgery [Beer 2002]. Tumor tissue of 86 primary adenocarcinomas of the lung, including 67 stage I and 19 stage III tumors, was investigated. As an internal control, 10 non-neoplastic lung tissue samples were analyzed. Using "hierarchical clustering" methodology, three different patient groups with association of cluster and stage (P=0,030) or tumor differentiation (P=0,01) could be differentiated. All ten non-neoplastic tissue samples could be clustered within the same patient group. In addition, the authors could derive a 50-gene-risk-index by identifying survival related genes using univariate Cox analysis (Table 5). When calculating the 50-gene-risk-index and grouping the patients based on the results, significant differences in overall survival between the individual groups could be identified. Grouped "high-risk" and "low-risk" stage I adenocarcinomas differed significantly between each other (p=0.003), whereas low and high risk stage III tumors did not. The robustness of the 50-gene-risk-index in predicting overall survival in early stage lung adenocarcinoma was tested in an independent data set of 84 tumor samples and related to overall survival. A high and a low risk group could be separated (P=0.003). Interestingly, among the 62 stage I tumors in this analyzed population, high and low risk groups could be observed differing significantly (P=0.006) in their overall survival duration. In conclusion, the authors postulated, that the identification of a high risk group within stage I lung cancer patients would lead to the consideration of a postoperative adjuvant intervention for this group. In 2003, a Japanese group published another important cDNA microarray based study regarding lung cancer [Kikuchi 2003]. A set of 37 tumor tissue samples of non-small cell lung cancer patients were analyzed. To avoid investigation on irrelevant tissue, they only analyzed cancer cells selected by laser capture microdissection. The most frequent NSCLC-subtypes adenocarcinoma and squamous cell carcinoma could be easily distinguished by applying a clustering algorithm to the expression data results. To explore gene expression in post-chemotherapeutic lung cancer tissue, a small pilot-study using RNA filter-array was performed in our institution [Ohira 2002]. Lung and normal tissue from three patients who underwent neoadjuvant therapy prior to surgery were collected, following the end of chemotherapy. Gene expression data obtained by a 588 gene filter arrays were

Table 5. Selected Examples of the 50 Gene Risk Index of Beer (2002)

Gene name	P	Coefficient	Comment
	(normal versus tumor-t-test)	β	
Caspase 4	0,56	0,0022	apoptosis-related cysteine protease
LAMB 1	0,14	0,0027	Laminin β 1
BMP 2	0,54	0,0044	Bone morhogenetic protein 2
CDC 6	1,31E-05	0,0124	cell division cycle 6
Serpine 1	2,89E-03	0,0008	Serine (or cysteine) proteinase inhibitor (clade E)
ERBB2	0,04	0,0013	v-erb-b2 (Receptor)
PDE7A	0,12	- 0,0187	Phosphodiesterase 7°
PLGL	0,04	- 0,0011	Plasminogen like

The 50-gene-risk index was validated in an independent set of 84 tumor samples and corresponding A positive coefficient β is associated with poorer outcome. A 50 gene risk index ("predictor") for lung adenocarcinomas was established in a microarray based correlation study (Beer 2002). Selected examples for interesting genes of this risk index were shown in this table. The coefficient β shows the relation of gene expression and outcome. A positive coefficient β is associated with poorer outcome. This 50 survival data. Among the 62 stage I tumors including this set they could identify a high and a low risk group which differ significant in survival.

* Le Chevalier, T. for the IALT Investigators (2003): Results of the Randomized International Adjuvant Lung Cancer Trial (IALT): cisplatin-based chemotherapy (CT) *vs* no CT in 1867 patients (pts) with resected non-small cell lung cancer (NSCLC). *Proc. Am. Soc. Clin. Oncol.* 22, page 2, (abstr 6).

analyzed by "hierarchical clustering" method. Remarkably, normal tissue and tumor tissue from the same patient showed more similarities and clustered nearer than normal and normal tissue or tumor and tumor samples from different patients (Fig. (1)). On the other hand, groups of genes significantly differed in expression profiles between normal and malignant tissue. Especially, angiogenesis and invasion related genes were upregulated in the tumor samples (Fig. (2)). These results suggest that molecules involved in angiogenesis are suitable targets for novel drugs administered following chemotherapy. This early study is one example of how genomic techniques could help to discover new candidates for target based therapeutics in the future.

GASTROINTESTINAL CANCER

Gastric cancer is still the fourth leading cause of cancer in the world [Parkin 2001]. Due to the lack of sufficient systemic control induced by current anti-neoplastic agents,

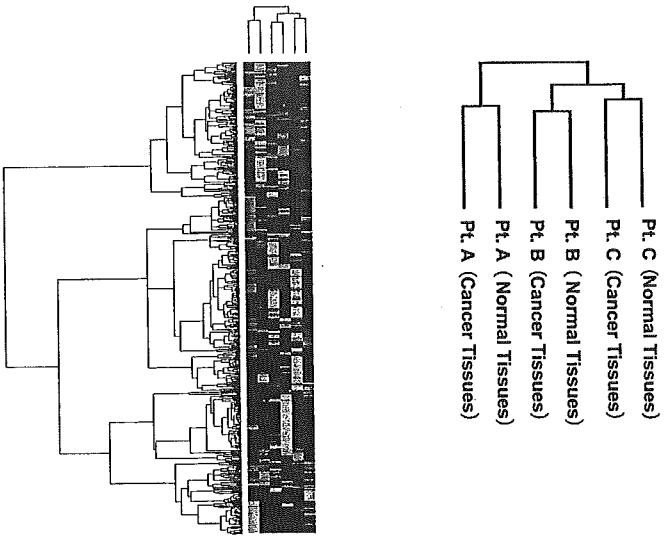

Fig (1). Clustering of gene expressions of tissues 3 from lung cancer patients (Ohira 2002). Tumor tissue and normal lung tissue was collected while surgery after neoadjuvant chemotherapy. Tumor tissue and normal tissue from the same patient show more similarities and clustered nearer than normal and normal or tumor and tumor tissue from different patients.

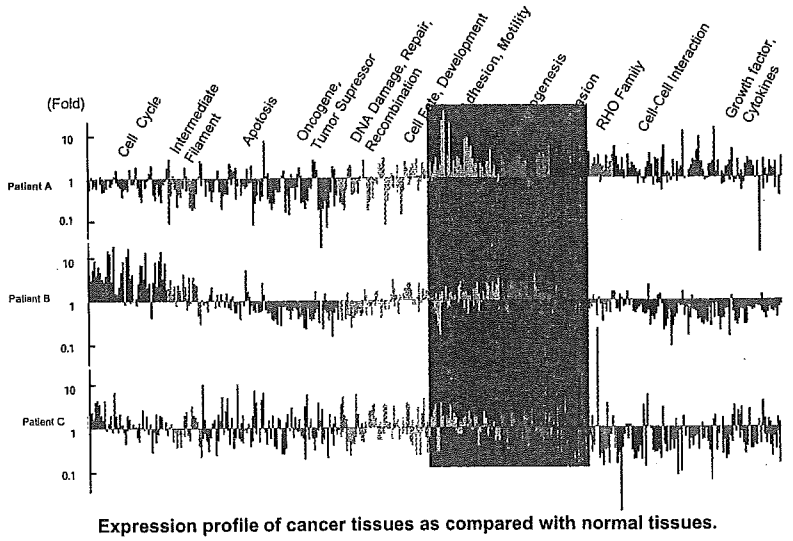

Fig. (2). Histogram of gene expression profile of lung cancer tissue. Expression profile of cancer tissues as compared with normal tissues.
Case B; increased expression of the genes related to cell cycle regulator, intermediate filaments, adhesion motility and angiogenesis in the tumor tissues. Expression of the other gene group were decreased in tumor tissue. Case C; increased expression of genes related with cell cycle adhesion were observed in the tumor tissue. Decreased expression of growth factor and cytokine related genes were also observed in tumor of Case C. Taken together, the expression profile of lung carcinoma could be characterized by the increased expression of the genes related with adhesion motility and angiogenesis.