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Gefitinib (IRESSA) sensitive lung cancer cell lines show phosphorylation of Akt without ligand stimulation

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Abstract

Background: Phase III trials evaluating the efficacy of gefitinib (IRESSA) in non-small cell lung cancer (NSCLC) lend support to the need for improved patient selection in terms of gefitinib use. Mutation of the epidermal growth factor receptor (*EGFR*) gene is reported to be associated with clinical responsiveness to gefitinib. However, gefitinib-sensitive and prolonged stable-disease-defined tumors without *EGFR* gene mutation have also been reported.

Methods: To identify other key factors involved in gefitinib sensitivity, we analyzed the protein expression of molecules within the EGFR family, PI3K-Akt and Ras/MEK/Erk pathways and examined the sensitivity to gefitinib using the MTT cell proliferation assay in 23 lung cancer cell lines.

Results: We identified one highly sensitive cell line (PC9), eight cell lines displaying intermediate-sensitivity, and 14 resistant cell lines. Only PC9 and PC14 (intermediate-sensitivity) displayed an *EGFR* gene mutation including amplification. Eight out of the nine cell lines showing sensitivity had Akt phosphorylation without ligand stimulation, while only three out of the 14 resistant lines displayed this characteristic ($P = 0.0059$). Furthermore, the ratio of phosphor-Akt/total Akt in sensitive cells was higher than that observed in resistant cells ($P = 0.0016$). Akt phosphorylation was partially inhibited by gefitinib in all sensitive cell lines.

Conclusion: These results suggest that Akt phosphorylation without ligand stimulation may play a key signaling role in gefitinib sensitivity, especially intermediate-sensitivity. In addition, expression analyses of the EGFR family, *EGFR* gene mutation, and FISH (fluorescence *in situ* hybridization) analyses showed that the phosphorylated state of EGFR and Akt might be a useful clinical marker of Akt activation without ligand stimulation, in addition to *EGFR* gene mutation and amplification, particularly in adenocarcinomas.

Background

Gefitinib(IRESSA) [4-(3-chloro-4-fluoroanilino)-7-methoxy-6-(3-morpholinopropoxy)-quinazoline] is an orally active, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor that inhibits EGFR signaling [1,2]. IDEAL (IRESSA Dose Evaluation in Advanced Lung cancer) 1 and 2 were randomized Phase II trials in patients with non-small cell lung cancer (NSCLC) refractory to platinum-based chemotherapy. These trials demonstrated that gefitinib was active and generally well tolerated [3,4]. However, ISEL (IRESSA Survival Evaluation in Lung cancer), a Phase III trial evaluating the efficacy of gefitinib compared to best supportive care for refractory or recurrent NSCLC, failed to demonstrate a significant survival benefit, except in an Eastern subpopulation and in those who had never smoked [5]. These data indicate that improved patient selection and combination strategies are probably required to maximize the benefits of using this targeted therapy.

Gefitinib exerts its antitumor activity through the inhibition of EGFR tyrosine kinase; however, this activity does not significantly correlate with the level of EGFR expression by the tumor cell [6]. Recent reports have shown that there are differences between gefitinib responders and non-responders in the frequency of activating mutations in the *EGFR* gene, which suggests that such mutations might be predictive markers for sensitivity to gefitinib [7,8]. *EGFR* gene amplification, as detected by fluorescence in situ hybridization (FISH), was also reported to be a predictive marker [9]. However, there are known to be gefitinib-sensitive and intermediate-sensitive tumors that have no activating mutations in the *EGFR* gene, including gene amplification. Thus, it is likely that other factors in lung cancer cells may sensitize cells to gefitinib in addition to *EGFR* gene mutation and the amplification detected by FISH. In order to identify additional key molecules involved in gefitinib resistance, we examined the sensitivity to gefitinib of 23 lung cancer cell lines using the 3- [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay and identified sensitive, intermediate-sensitive, and resistant lung cancer cell lines. A few reports have suggested that small cell lung cancer(SCLC) is responsive to gefitinib [10,11]. Tanno *et al* reported that MAPK, a downstream effector of the EGFR was inhibited by gefitinib in SCLC cell lines that expressed the EGFR even at a very low level[11]. SCLC cell lines were included in our series. We also analyzed the genomic status of the *EGFR* gene mutation and *EGFR* gene amplification, as well as the protein expression level of key molecules in the EGFR family (EGFR, Her2, Her3), PI3K-Akt (Akt and phosphatase and tensin homolog [PTEN]) and Ras/MEK/Erk pathways (p44/42 mitogen-activated protein [MAP] kinase, p38 MAP kinase) which act downstream of EGFR. We correlated the cytotoxic activity of gefitinib in our 23 lung tumor cell lines to the corresponding expression patterns of these proteins.

Methods

Cell Lines

Twenty-three lung cancer cell lines were used in this study. They comprised: 10 adenocarcinoma cell lines (ABC-1, A549, PC3, PC7, RERF-LCMS, RERF-LCKJ, LCD, LCOK, PC9, and PC14), eight squamous-cell carcinoma cell lines (QG56, EBC-1, LK-2, LC-1/sq, PC1, RERF-LCAI, PC10, and SQ5), and five small-cell carcinoma cell lines (NCI-H69, SBC3, NCI-N231, Lu135, and MS-1). The Lu135 cell line was provided by Y. Shimosato and T. Terasaki, and the LCD and LCOK cell lines by S. Hirohashi (National Cancer Center Research Institute, Tokyo, Japan). The NCI-N231, A549, and NCI-H69 cell lines were obtained from the American Type Culture Collection (Rockville, MD) [12,13]. The PC1, PC3, PC7, PC9, PC10, PC14, and QG56 cell lines were obtained from IBL (Gunma, Japan). The RERF-LCKJ, SQ5, LC-1/sq, RERF-LCAI, and MS-1 cell lines were obtained from RIKEN Cell Bank (Ibaragi, Japan). The ABC-1, RERF-LCMS, LK-2, EBC-1, and SBC3 cell lines were obtained from the Health Science Research Resources Bank (Osaka, Japan).

In order to determine the activation of the members downstream of EGFR without ligand stimulation, all cell lines were separately cultured in serum-containing (+) and serum-free (-) conditions for 24 h.

Drugs and Growth-Inhibition Assay

Gefitinib was provided by AstraZeneca and dissolved in dimethyl sulfoxide (DMSO) for *in vitro* studies. We used the colorimetric MTT assay to examine the activity of gefitinib against all 23 lung cancer cell lines as previously reported [14]. Cell suspensions (200 μl, 10^5 cells/ml) were seeded into 96-well microtiter plates and 10 μl of drug solution added, at various concentrations. After incubation for 72 h at 37°C, 20 μl of MTT solution (5 mg/ml in phosphate buffered saline [PBS]) was added to each well and incubation then continued for a further 4 h at 37°C. The IC_{50} value was defined as the concentration of gefitinib needed for a 50% reduction in absorbance (560 nm) based on cell growth curves.

Western Blot Analysis

Western blot analysis was performed as previously described [15]. The membranes were first incubated overnight at 4°C with antibody specific for the following primary antibodies: PTEN (Santa Cruz Biotechnology, Santa Cruz, CA) and Akt, phospho-Akt (Ser473), p42/44 MAP kinase and phospho-p44/42 MAP kinase (Thr202/Tyr204), p38 MAP kinase and phospho-p38 MAP kinase (Thr180/Tyr182), β-actin, EGFR, and phospho-EGFR (Y1068), Her2 and Her3 [all from Cell Signaling Technol-

ogy, Beverly, MA]. The membranes were then incubated with peroxidase-conjugated secondary antibodies and protein was detected with enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham, Buckinghamshire, UK) [16,17]. These images were quantified by measuring signal intensity using National Institute of Health (NIH) Image (ImageJ1.32j). The ratios of phospho-EGFR/EGFR, Her2/β-actin, Her3/β-actin, phospho-Akt/Akt, PTEN/β-actin, phospho-p44/42 MAP kinase/p44/42 MAP kinase, and phospho-p38 MAP kinase/p38 MAP kinase were calculated from cells grown in both serum-containing (+) and serum-free (-) conditions.

Polymerase Chain Reaction Single Strand Conformation Polymorphism Analyses and Sequencing of Genomic DNA

Genomic DNA samples were obtained from each cell line by proteinase K treatment and phenol chloroform extraction using standard protocols [17]. From each genomic DNA sample, exons 18, 19 and 21 of the *EGFR* gene and exon 1 and 2 of the *K-Ras* gene were amplified separately with the relevant polymerase chain reaction (PCR) primers [7,18] using the Gene Amp XL PCR kit (Perkin Elmer/Roche, Branchburg, NJ). PCR conditions for genomic DNA analysis were as follows: 40 cycles at 94°C for 40 seconds, 60°C for 30 seconds, and 68°C for 90 seconds, followed by 68°C for 8 minutes. Fluorescein-isothiocyanate (FITC)-labeled PCR products were denatured, cooled on ice, and loaded on neutral 6% polyacrylamide gels with or without 5% (vol/vol) glycerol, as described previously [13]. After electrophoresis, the gels were analyzed with the FluorImager 595 (Molecular Dynamics Inc., Sunnyvale, CA). Each DNA sample was examined with three primer pair combinations, with the M13 sequence (TGTAAAACGACGACGGCCAGT) added in each case to the appropriate primer. PCR analysis was performed as described above, with the resulting products being purified and sequenced by fluorescence-based automated sequencing (Perkin Elmer/Applied Biosystems, Foster City, CA).

FISH Analysis

Cells were fixed on slides in Carnoy's fluid (60% ethanol; 30% chloroform; 10% acetic acid) and incubated for 30 min at 60°C and 30 min at 37°C in 2 × sodium chloride sodium citrate solution (SSC)/0.1%Tween20 followed by ethanol dehydration. The LSI EGFR/CEP7 SpectrumGreen Probe (Vysis Downers Grove, IL) was applied to the slides [9] which were then incubated for 5 min at 75°C for the codenaturation of probe and chromosomal DNA. Hybridization proceeded overnight at 37°C, after which the slides were washed in 50% formamide/2 × SSC for 15 min, 2 × SSC for 10 min and 2 × SSC/0.1%Tween29 for 5 min at 47°C. The chromatin was counterstained with 4'6'-diamidino-2-phenylindole (DAPI) (Roche). The frequency of tumor cells with specific copy numbers of the *EGFR* gene (red signal) and chromosome 7 (green signal) were estimated under a BX61 Olympus fluorescence microscope in a minimum of 200 nuclei. A gene amplification was defined as gene/chromosome per cell ratio >2 or >15 copies of EGFR per cell in >10% of analyzed cells. *EGFR* gene copy numbers were counted and were classified into six FISH strata by Hirsh's criteria [9,19].

Exposure to Gefitinib

Lung cancer cell lines were serum-starved for 24 h and treated with various concentrations of gefitinib (0, 0.1, 1 and 10 μM/ml) for 2 h before exposure to 10 ng/ml epidermal growth factor (EGF; [BA-53, Santa Cruz Biotechnology]) for 5 min in order to evaluate the effect of gefitinib on the PI3K-Akt and Ras/MEK/Erk pathways. These images were quantified by measuring signal intensity using NIH Image (ImageJ1.32j).

Statistical Analysis

The Chi-square test was used to evaluate the relationship between gefitinib sensitivity and activation of the molecules in the EGFR family, PI3K-Akt and Ras/MEK/Erk pathways. The cut-off value of signal intensity measured by NIH Image was defined by Receiever Operating Characteristics (ROC)curve. The expression ratio of phospho-EGFR/EGFR, Her2/β-actin, Her3/β-actin, phospho-Akt/Akt, phospho-p44/42 MAP kinase/p44/42 MAP kinase, phospho-p38 MAP kinase/p38 MAP kinase and PTEN/β-actin was compared between gefitinib-resistant and gefitinib-sensitive cell lines using the Mann-Whitney test. In addition, the correlations between PTEN expression and phosphorylation of Akt were examined by Spearman's rank order coefficient across all 23 cell lines. $P < 0.05$ was considered to be statistically significant.

Results

Effect of Gefitinib on Cell Growth In Vitro

The IC_{50} values of gefitinib for the 23 lung cancer cell lines, as determined by MTT assay, are summarized in Fig. 1. Only the PC9 cell line had an IC_{50} of <1 μmol/L (highly-sensitive), 14 cell lineshad an IC_{50} of >10 μmol/L (resistant), and the remaining eight had an IC_{50} of 1 to 10 μmol/L (intermediate-sensitive).

Expression and Phosphorylation Status of EGFR, Her2, Her3, Akt, p44/42, p38 MAP kinase, and PTEN in Gefitinib-sensitive Versus Gefitinib-resistant Cell Lines

The protein expression levels and phosphorylation status of EGFR, Her2, Her3, Akt, p44/42, p38 MAP kinase, and PTEN were analyzed in all 23 lung cancer cell lines by measuring the signal intensity using NIH Image (ImageJ1.32j) (Figs. 2, 3, 4 and 5). To evaluate activation without ligand stimulation, the cell lines were cultured in serum-containing (+) and serum-free (-) conditions. The ratios of phospho-EGFR/EGFR, Her2/β-actin, Her3/β-

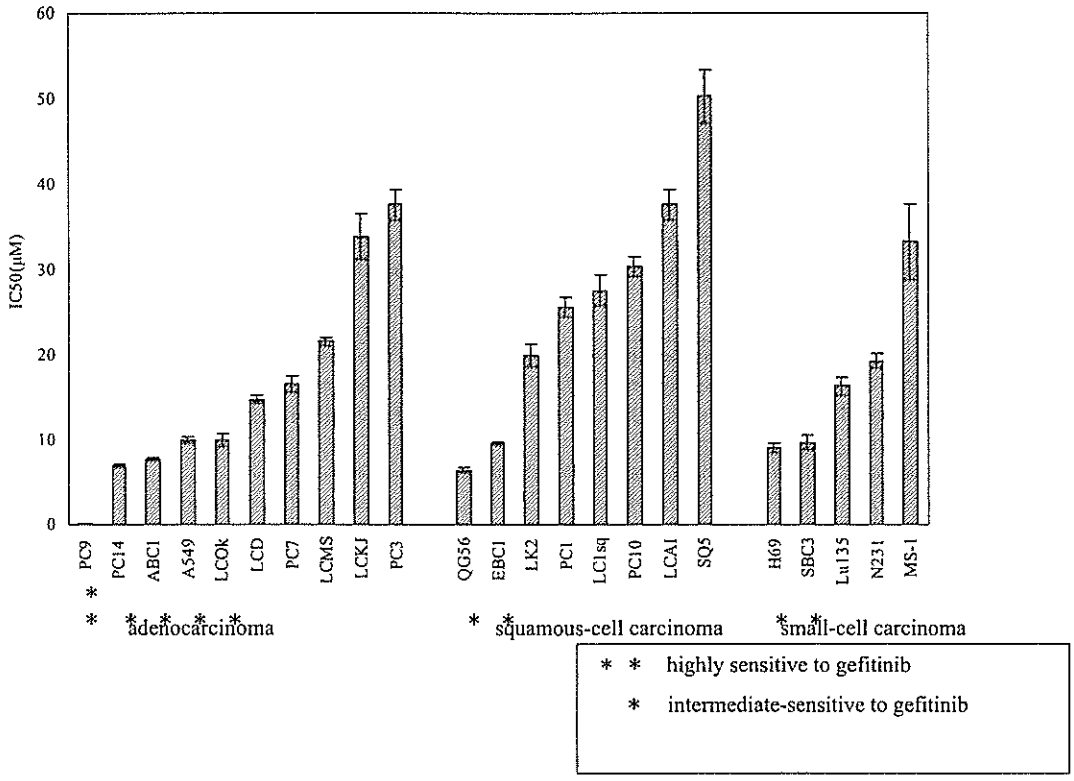


Figure 1
IC_{50} values for 23 lung cancer cell lines using the MTT assay. Cell lines were classified as: sensitive ($IC_{50} \leq 1$ μM), intermediate-sensitive (1 μM < $IC_{50} \leq 10$ μM), and resistant (10 μM < IC_{50}) to gefitinib. The bars represent the IC_{50} and standard deviation (SD) obtained in three indpendent MTT assays. *Point*: mean of three independent experiments, bar: ± SD.

actin, phospho-Akt/Akt, PTEN/β-actin, phospho-p44/42 MAP kinase/p44/42 MAP kinase, and phospho-p38 MAP kinase/p38 MAP kinase were calculated in cells grown under both conditions. Across the entire cohort of 23 lung cancer cell lines, those showing sensitivity to gefitinib exhibited greater phosphorylation of Akt and EGFR without ligand stimulation than gefitinib-resistant cell lines, according to the Mann-Whitney test (P = 0.0016, P = 0.0274, respectively) (Figs. 3 and 5). Furthermore, the ratio of phospho-Akt/total Akt in intermediate-sensitive cells was higher than that observed in resistant cells (P = 0.003). There was no statistical difference in the phosphorylation of p44/42 MAP kinase, p38 MAP kinase without ligand stimulation, and expression of PTEN, Her2 and Her3 (Mann-Whitney test: PTEN: P = 0.8; p-p44/42: P = 0.8; p-p38: P = 0.45; Her2:P = 0.052; Her3:P = 0.08, respectively) (Fig. 3, 4 and 5). As shown in Table 1, gefitinib-sensitive cells frequently expressed phospho-Akt, phospho-EGFR and Her2 with ligand stimulation (P = 0.0059, P = 0.0319; P = 0.0344, Chi-square test). In particular, cell lines characterized by phosphorylation of Akt without ligand stimulation had a greater than 29 times probability of being sensitive to gefitinib than other cells. Interestingly, sensitive cells, especially in the adenocarcinoma cell lines, had more Akt phosphorylation with EGFR phosphorylation than resistant cells in serum-containing conditions (Chi-square test: p = 0.034: total, p = 0.0114: in adenocarcinoma) (Table 1). In eight squamous cell carcinoma cell lines, PTEN expression seemed to be inversely correlated with phosphorylation of Akt (Spearman's rank order coefficient: r = -0.76, P = 0.04) (Fig. 3). However, in the other histological types of cells, PTEN

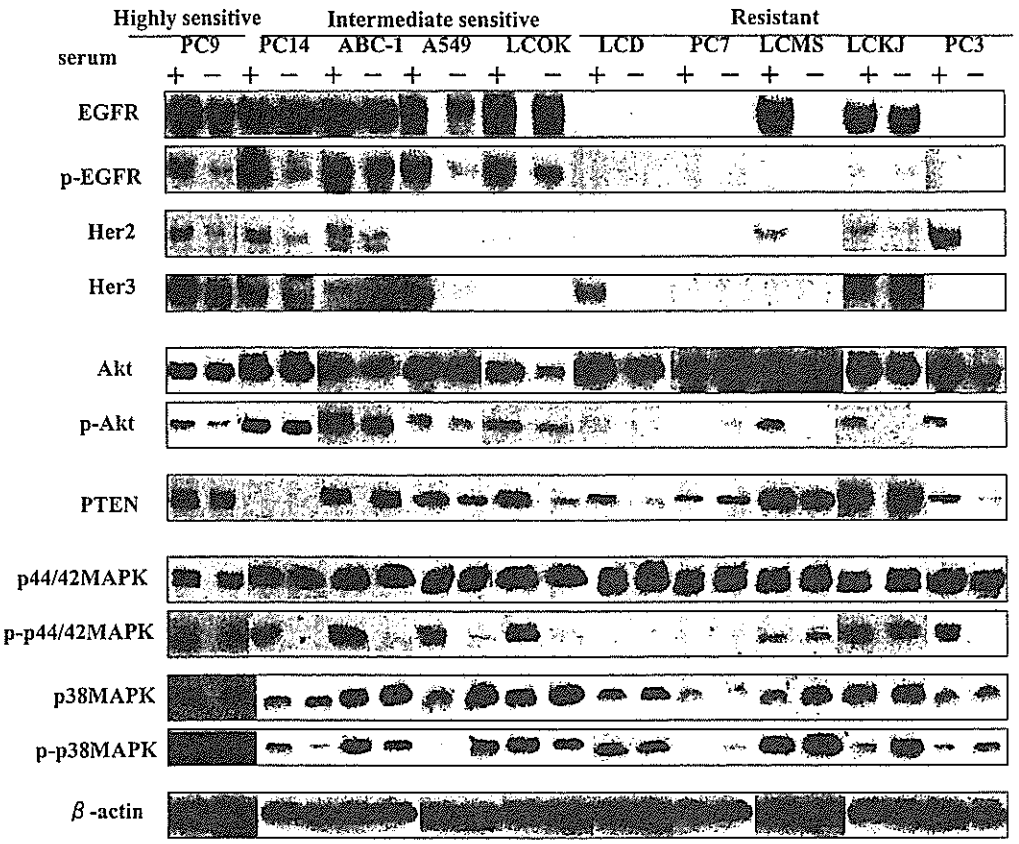


Figure 2
Western blot analyses: protein expression and phosphorylation of epidermal growth factor receptor (EGFR), Akt, PTEN, Her2, Her3, p44/42 mitogen activated protein kinase (MAPK), and p38 MAPK in adenocarcinoma cell lines cultured for 24 h with (+) or without (-) serum.

expression did not correlate with phosphorylation of Akt (Spearman's rank order coefficient: adenocarcinomas: r = -0.08, P = 0.17, small-cell carcinomas: r = 0.1, P = 0.84).

Mutation Analyses of the EGFR Gene and K-Ras Gene

Correlations have been reported between *EGFR* mutation and clinical responsiveness to gefitinib [7,8]. Therefore, exons 18, 19, and 21 in the *EGFR* gene were sequenced from 23 lung cancer cell lines, in order to assess mutations in the tyrosine kinase domain. The PC9 cell line, which was sensitive to gefitinib, had an in-frame deletion within the EGFR gene, removing amino acids 746 through 750 (delE746-A750) within the activation loop of the tyrosine kinase that flanks the ATP cleft. The other lung cancer cell lines did not display any *EGFR* gene mutations in these regions. A549(intermediate-sensitive) and LCKJ(resistant) cell lines had a point mutation(*G12S*) in codon 12 evaluated by *K-ras* gene mutational analyses.

FISH Analysis

Genomic gains of the *EGFR* gene were examined by FISH analysis. PC9 (highly-sensitive cell) with *EGFR* mutation, and PC14 (intermediate-sensitive cell) both had *EGFR* gene amplification (Fig. 6) [9,19]. There were high levels of expression and phosphorylation of EGFR in PC9 and PC14.

Effect of Gefitinib on the Phosphorylation of EGFR, Akt, p44/42 MAP kinase, and p38 MAP kinase

The effect of gefitinib was investigated on the phosphorylation of EGFR, Akt, p44/42 MAP kinase, and p38 MAP kinase, in serum-starved, gefitinib-pretreated lung cancer

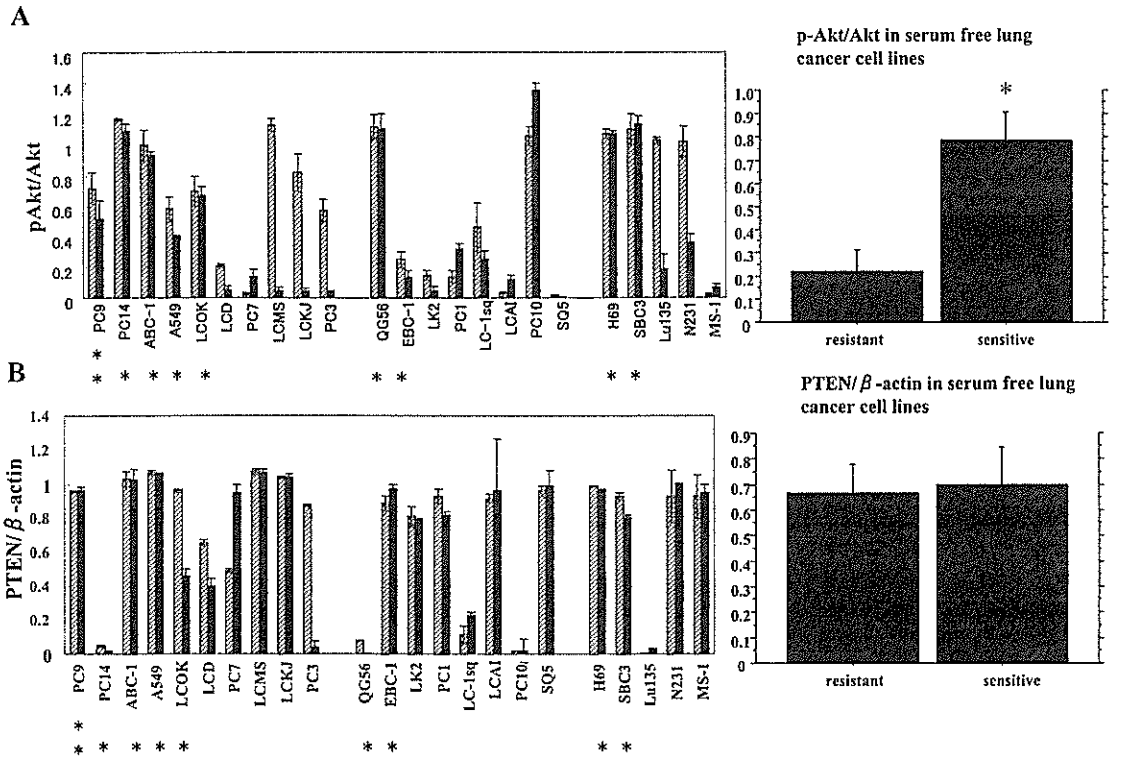


Figure 3

Summary of expression of PTEN and phosphorylation of Akt in 23 lung cancer cell lines grown under serum-containing (cross bars) and serum-free (black bars) conditions. (A) The ratio of phosphorylated Akt signal/total Akt signal (pAkt/Akt); (B) the ratio of PTEN signal/β-actin signal; The density of expression of each of the molecules was measured by NIH Image. *Point*: mean of three independent experiment; bars, ± standard deviation (SD). The expression ratio of the molecules in serum-free conditions was compared between gefitinib-resistant and gefitinib-sensitive cell lines using the Mann-Whitney test; *point*, mean, bars, ± standard error (SE). *$P < 0.05$ was considered to be statistically significant.

cells (Figs. 7 and 8). Akt and EGFR were phosphorylated without ligand stimulation in the highly gefitinib-sensitive cell line PC9, which has an EGFR gene mutation, as well as intermediate-sensitive cell line. Akt and EGFR phosphorylation were inhibited by gefitinib in sensitive cell lines (Figs. 7 and 8). With EGF stimulation, phosphorylation of Akt increased further in A549 and PC9 cells, but not in PC14 and ABC-1 cells. In the resistant cell lines, Akt was phosphorylated through gefitinib treatment, although EGFR phosphorylation was inhibited (Figs. 7 and 8). In PC9 cells, phosphorylation of p44/42 MAP kinase was inhibited at low concentrations of gefitinib. However, in cells showing intermediate sensitivity to gefitinib, phosphorylation of p44/42 MAP kinase was not clearly inhibited, either in the presence or absence of EGF. Phosphorylation of p38 MAP kinase was not clearly inhibited in anyof the lung cancer cell lines.

Eight of the nine lines showing sensitivity to gefitinib had Akt phosphorylation without ligand stimulation, while only three of the 14 resistant lines displayed this characteristic. Furthermore, the ratio of phospho-Akt to total Akt in sensitive cells was higher than that observed in resistant cells. Akt phosphorylation was partially inhibited by gefitinib in highly and intermediate-sensitive cell lines. These results suggest that Akt activation without ligand stimulation may play a key signaling role in gefitinib sensitivity.

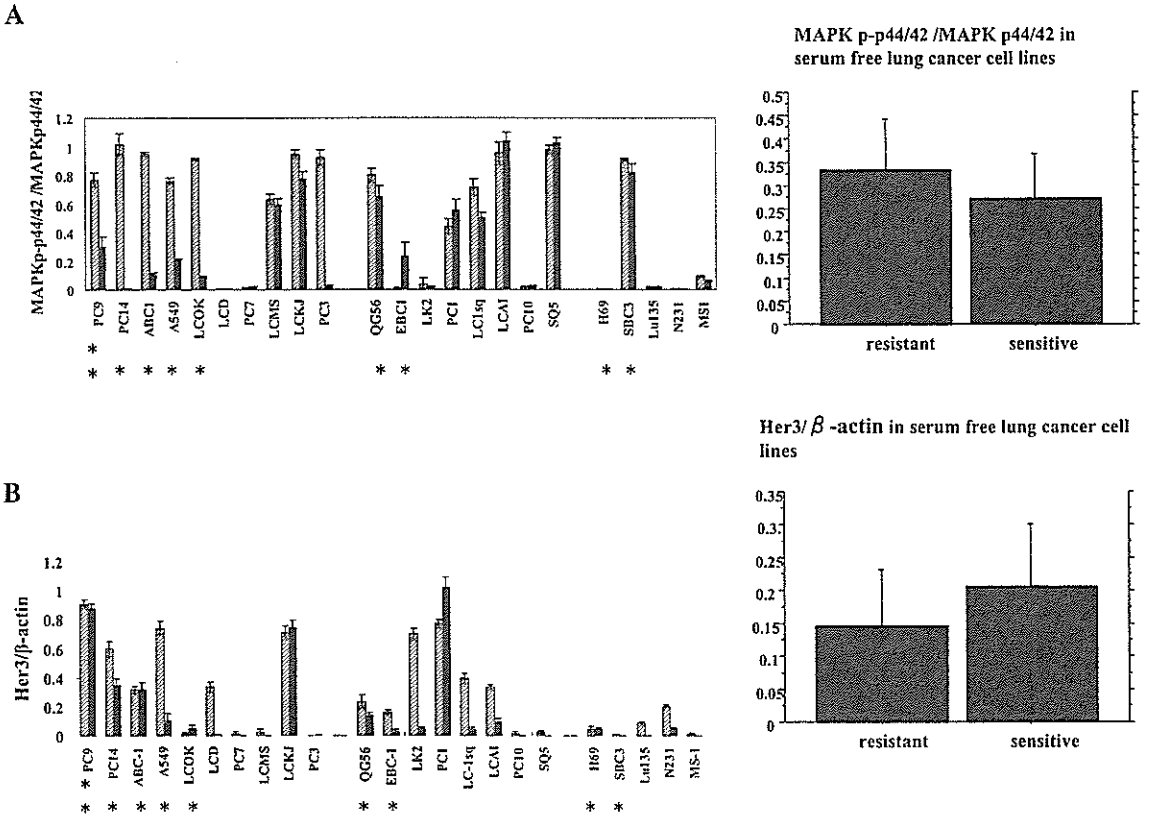


Figure 4
Summary of expression of Her3 and phosphorylation of p44/42 MAPK in 23 lung cancer cell lines grown under serum-containing (cross bars) and serum-free (black bars) conditions. (A) the ratio of phosphorylated p44/42 MAPK signal/total p44/42MAPK signal (MAPKp-p44/42/MAPKp44/42); (B) the ratio of Her3 signal/β-actin signal; The density of expression of each of the molecules was measured by NIH Image. *Point*: mean of three independent experiment; bars, ± standard deviation (SD). The expression ratio of the molecules in serum-free conditions was compared between gefitinib-resistant and gefitinib-sensitive cell lines using the Mann-Whitney test; *point*, mean, bars, ± standard error (SE). *$P < 0.05$ was considered to be statistically significant.

Discussion

Clinically, gefitinib-sensitive tumors have been observed that contain no evidence of activating mutations in the *EGFR* gene [20]. We examined the sensitivity of 23 lung cancer cell lines to gefitinib using the MTT cell proliferation assay and identified one highly gefitinib-sensitive lung cancer cell line (PC9), eight intermediate-sensitive lung cancer cell lines, and 14 resistant lung cancer cell lines. The IC_{50} value of the PC9 cell line was about one-sixth of the clinical dose. Only the PC9 cell line displayed a mutational event in the *EGFR* gene: a 15 bp deletion in exon 19. The IC_{50} values in the intermediate-sensitive cell lines ranged between 1 and 10 μM, which was similar to a previous report [21]. In our study, the IC_{50} value in A549 cells was 10 μM, which has been previously reported in a xenograft model as sensitive to gefitinib [22,23]. These values are higher than the maximum serum concentration of gefitinib observed in patients (~1 μM). Nevertheless, *in vitro* studies occasionally do not correlate with *in vivo* work. However, these differences in this study mainly seem to depend on the exposure time to gefitinib. In the xenograft study, A549 was sensitive in gefitinib-intake for 35 days. In our study, Akt phosphorylation was partially inhibited by gefitinib (<1 μM) in intermediate-sensitive cell lines, whereas their IC_{50} value ranged between 1 and 10 μm.

In this study, cancer cell lines showing sensitivity to gefitinib exhibited more phosphorylation of Akt and EGFR without ligand stimulationthan gefitinib-resistant cell

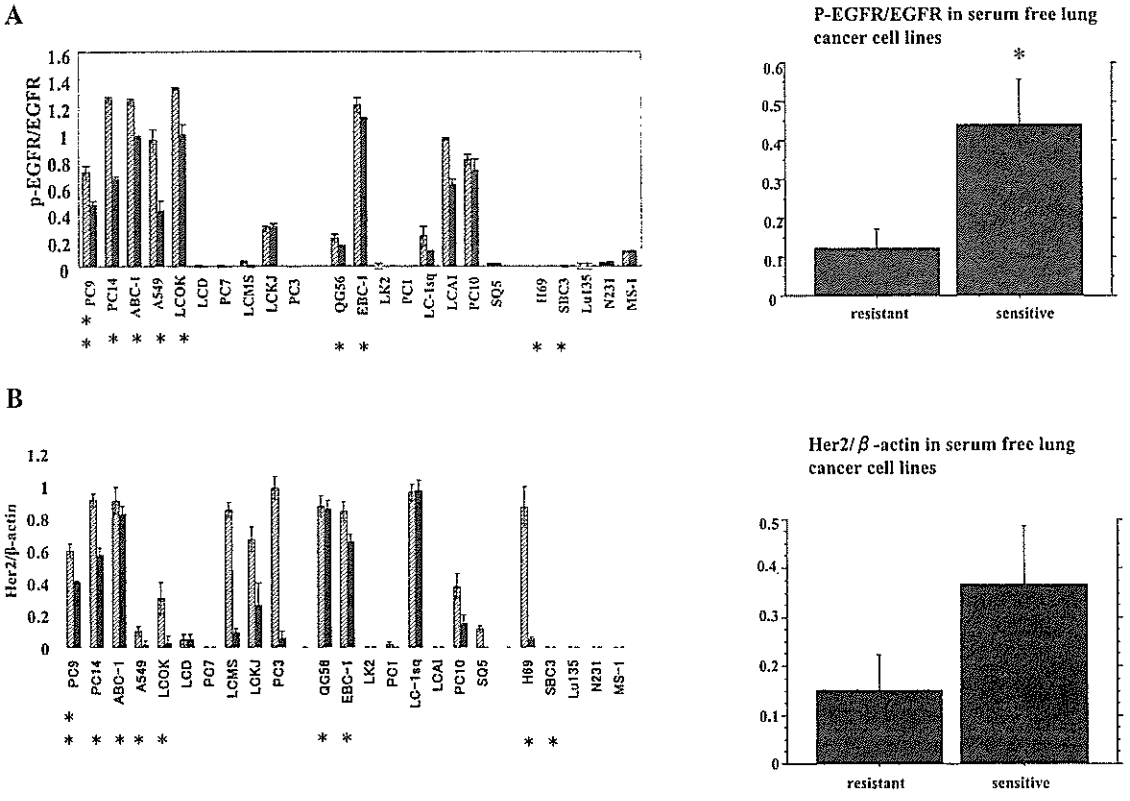
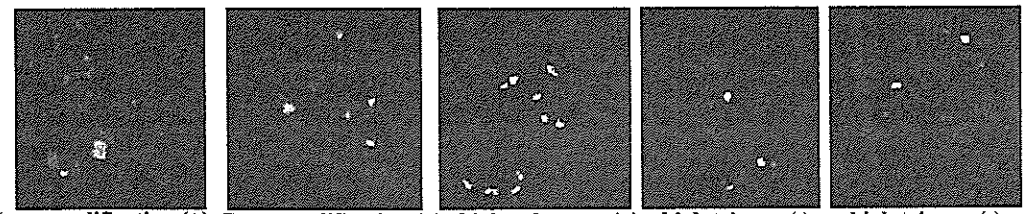


Figure 5
Summary of expression of Her2 and phosphorylation of EGFR in 23 lung cancer cell lines grown under serum-containing (cross bars) and serum-free (black bars) conditions. (A) the ratio of phosphorylated EGFR signal/EGFR signal; (B) the ratio of Her2 signal/β-actin signal. The density of expression of each of the molecules was measured by NIH Image. *Point*: mean of three independent experiment; bars, ± standard deviation (SD). The expression ratio of the molecules in serum-free conditions was compared between gefitinib-resistant and gefitinib-sensitive cell lines using the Mann-Whitney test; *point*, mean, bars, ± standard error (SE). *$P < 0.05$ was considered to be statistically significant.

Table 1: Phosphorylation and expression of the molecules and their relationship to gefitinib sensitivity

Expression of the molecules: (+) positive; (-) negative	*P*
Phosphorylated Akt/Akt (+)	0.0059**
PTEN/β-actin (+)	0.361
phospho-p44/42/p44/42 (+)	0.852
phospho-p38/p38 (+)	>0.99
phospho-EGFR/EGFR (+)	0.0319**
Her2/β-actin (+)	0.0344**
Her3/β-actin(+)	0.5582
phospho-EGFR(+) phospho-Akt(+) (vs phospho-EGFR(-) or phospho-Akt(-))	0.0344**

The relationship between gefitinib-sensitivity and the expression of these molecules was investigated by the Chi-square test. **$P < 0.05$ was considered to be statistically significant.



	Highly sensitive	Intermediate-sensitive		Resistant	
	PC9	PC14	ABC1	PC7	LCKJ
FISH(+-)	Gene amplification (+)	Gene amplification (+)	high polysomy (+)	high trisomy(-)	high trisomy(-)
EGFR mutation	positive	negative	negative	negative	negative
EGFR expresion	high	high	high	negative	high
Phosphorylated EGFR	high	high	high	defective	low

Figure 6
Fluorescence *in situ* hybridization (FISH) analyses of lung cancer cell lines, and EGFR mutations and expression. EGFR gene copy numbers were counted and were classified into six FISH strata [9,19]. EGFR gene amplification was detected in PC9 and PC14 cells. In the ABC1 cell line, the EGFR gene copy number was five in over 40% of cells (high polysomy:FISH negative); in the PC7 and LCKJ cell lines, the EGFR gene copy number was three in over 40% of cells (high trisomy:FISH negative).

lines (Mann-Whitney test:$P = 0.0016$, $P = 0.0274$, respectively). Sensitive cells frequently had phospho-Akt and phospho-EGFR without ligand stimulation ($P = 0.0059$, $P = 0.0319$; Chi-square test). This is the first report suggesting that unstimulated phosphorylation of Akt seems to have a strong correlation with gefitinib sensitivity, especially, intermediate sensitivity. Unstimulated phosphorylation of Akt seems to be mainly due to constitutive activation of the Akt signaling pathway. Cappuzzo *et al* have reported that patients with phospho-Akt-positive tumors who received gefitinib had a better response rate in terms of stable disease, disease control rate, and time to disease progression than patients with phospho-Akt-negative tumors [24]. Our results support these clinical findings. In our study, Akt phosphorylation was inhibited by gefitinib in all these cell lines. In this situation, there remains a question whether Akt is really so central in determining the sensitivity to gefitinib or if it is just a downstream molecule that is sensing activation of other upstream molecules. Amann *et al* reported that the NSCLC cell line H1819, which does not have an *EGFR* mutation, but shows high expression levels of EGFR, ErbB2, and ErbB3, showed intermediate sensitivity to tyrosine kinase inhibitors [25]. They also reported that, in this cell line, Akt was constitutively phosphorylated, but remained prone to inhibition by an EGFR-directed tyrosine kinase inhibitor. They suggested that, in addition to *EGFR* gene mutation, other factors, such as high expression levels of ErbB family members, might constitutively activate Akt and sensitize cells to EGFR inhibitors [25]. The EGFR family (EGFR, Her2 and Her3) operates a complex the signal transduction of its downstream with makes the formation of each receptor homo- or heterodimerzation and transducecascade through PI3K-Akt and Ras-Erk-MAPK pathways. Over expression of Her2 has been shown to promote the constitutive phosphorylation of EGFR and to delay and prolong the phosphorylation of EGFR [26]. In our study, there were statistically significant differences in Her2 expression between gefitinib-sensitive and resistant cells ($P = 0.0344$; Chi-square test). Unstimulated phosphorylation of Akt might be a hallmark of sensing activation of other upstream molecules.

In highly- and intermediate-sensitive cells, Akt was phosphorylated without ligand stimulation. Clinical markers of Akt activation without ligand stimulation should be

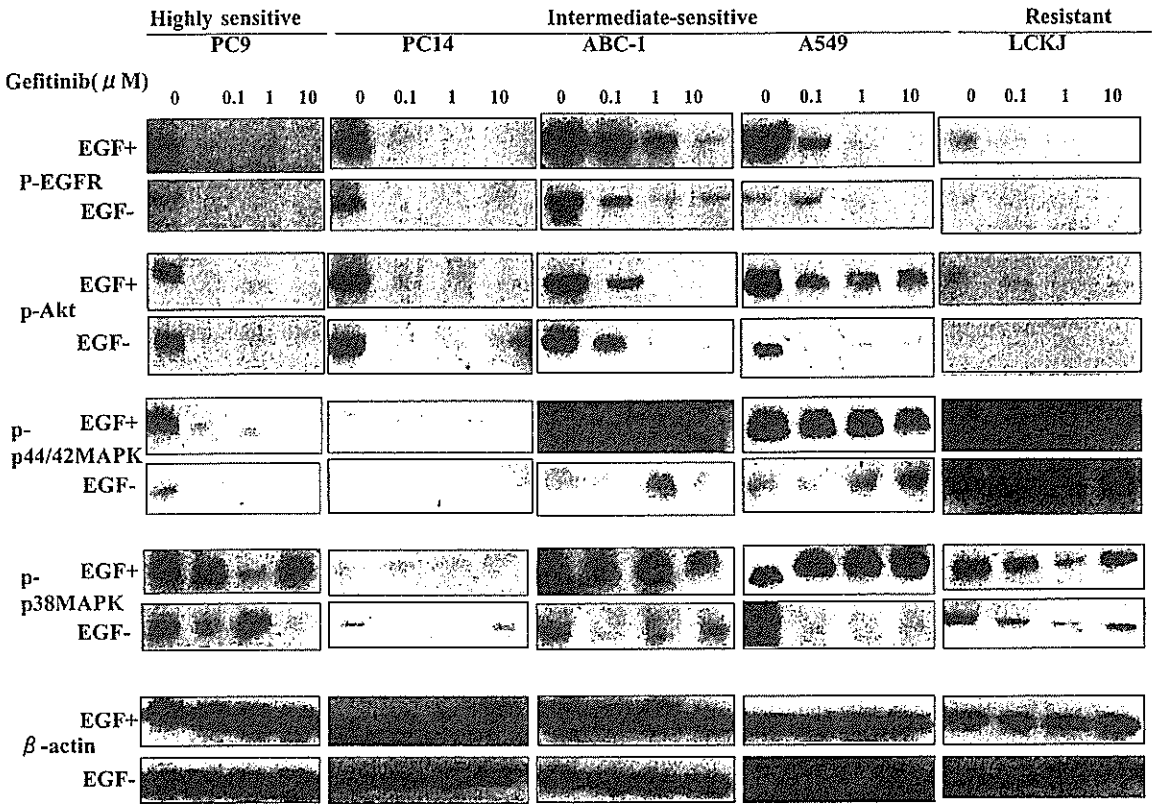


Figure 7
Lung cancer cell lines: Effect of gefitinib on phosphorylated EGFR(p-EGFR), phosphorylated Akt (p-Akt) and the molecules in the Ras/MEK/Erk pathway with or without EGF.

looked for. The eight cell lines with Akt phosphorylation without ligand stimulation consisted of: five adenocarcinomas, one squamous cell carcinoma, and two small-cell carcinomas. All five adenocarcinomas had EGFR phosphorylation (Figs. 2, 3 and 5). In all the adenocarcinoma lines, the phosphorylation state of EGFR was predictive of Akt phosphorylation without ligands stimulation (Figs. 2, 3 and 5). Both PC9 (gefitinib-sensitive cell line) with EGFR mutation, and PC14 (intermediate-sensitive cell line) had EGFR gene amplification. These lines had the EGFR and Akt phosphorylation without ligand stimulation. Overall, these results suggested that EGFR and Akt activations without ligand stimulation might be partially due to EGFR mutation including amplification. One squamous cell carcinoma had the loss of PTEN protein. Two small-cell carcinoma cell lines with Akt phosphorylation without ligand stimulation, H69 and SBC3, exhibited intermediate sensitivity to gefitinib (Fig. 3). However, they had little EGFR expression and phosphorylation. It is possible that gefitinib may block signal transduction via different receptors including the EGFR family or that SCLC cells may have small amounts of functional EGFR that cannot be detected by Western blotting. H69 had a moderate amount of Her2 expression.

EGF stimulation further increased the phosphorylation of Akt in A549 and PC9 cells, but not in PC14 and ABC-1 cells. A549 and PC9 had EGF responsiveness as well as EGFR and Akt phosphorylation without ligand stimulation. In PC9 cells, the phosphorylation of p44/42 MAP

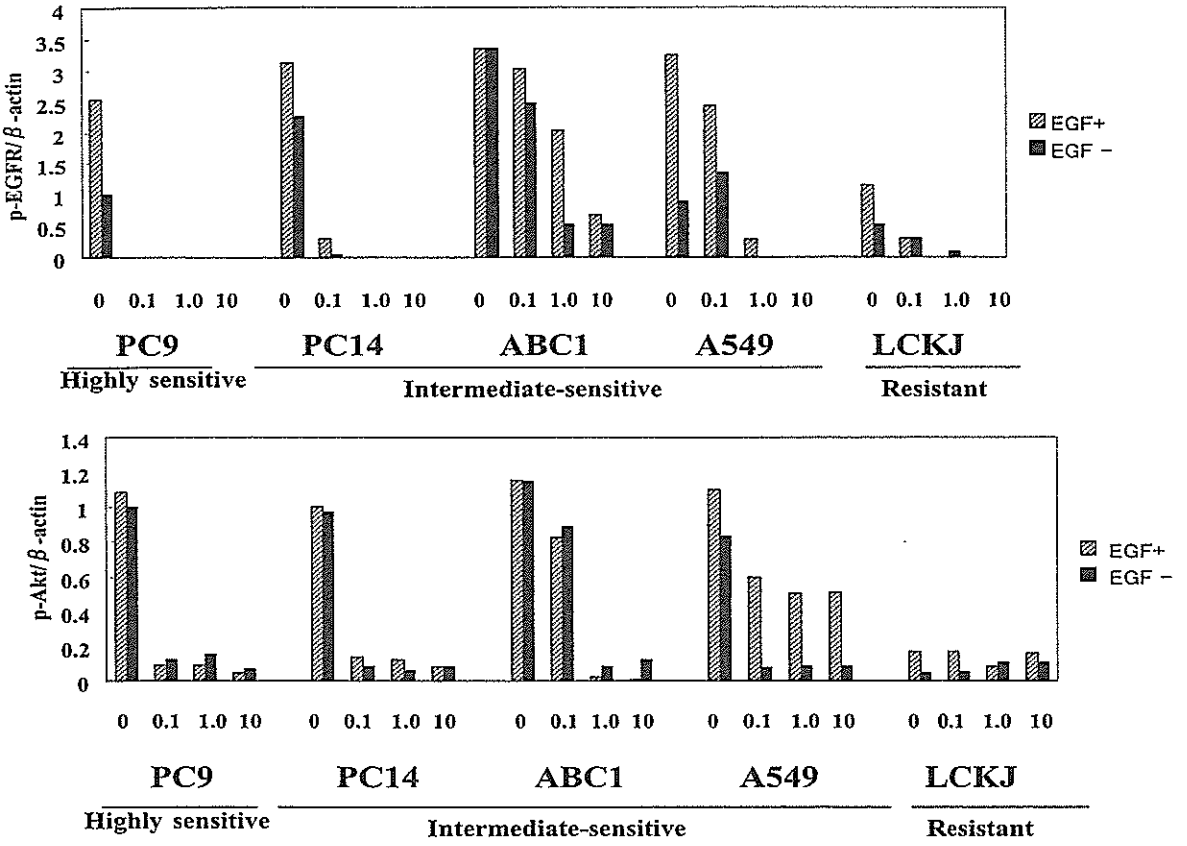


Figure 8
Summary of the effect of gefitinib on the molecules downstream of EGFR (Fig.7.). These images were quantified by measuring signal intensity using NIH Image (ImageJ1.32j).

kinase was inhibited at low concentrations of gefitinib. In cell lines with intermediate sensitivity to gefitinib, the phosphorylation of p44/42 MAP kinase was not clearly inhibited, either with or without EGF. These phenomena may be due to differences in activating mechanisms. Lung cancer cells with phosphorylation of p44/42 MAP kinase had no *K-ras* gene mutation other than LCKJ. Han et al reported that only 18.1% of patients with p-Erk positive tumors harbored *K-ras gene* mutation, and identification of other molecular mechanisms leading to p-Erk activation and gefitinib resistance was mandatory [18]. There was no correlation between gefitinib sensitivity, including intermediate-sensitivity, and the status of the *K-ras* gene in our study.

Conclusion

Our report indicates that sensitivity to gefitinib is related to thephosphorylation of Akt without ligand stimulation. The phosphorylated state of EGFR and Akt might be clinical markers of Akt activation without ligand stimulation and increase specificity of gefitinib sensitivity and, therefore, may prove to be useful prognostic tests of tumor responsiveness, in addition to EGFR gene mutation and amplification. These findings seem to apply especially to adenocarcinomas. Furthermore, EGFR phosphorylation may be an attractive candidate for bioimaging for use in the design of EGFR-targeted therapies.

Competing interests

There are no financial or other interests with regard to the submitted manuscript that might be construed as a conflict of interest.

Authors' contributions

RN, AG and SK designed this study analyzed and interpreted the data. RN, YK, SK, YM, KM, KK, MS, TO and AY

carried out the cell culture and the sensitivity test. RN, KK, MS and MC carried out analyses of the molecules'expression. and *EGFR* gene status.

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Docetaxel in Combination with Either Cisplatin or Gemcitabine in Unresectable Non-small Cell Lung Carcinoma: A Randomized Phase II Study by the Japan Lung Cancer Cooperative Clinical Study Group

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Purpose: To evaluate whether cisplatin-free chemotherapy (docetaxel and gemcitabine [DG]) provides a comparable alternative to cisplatin-based chemotherapy (docetaxel and cisplatin [DC]) as first-line treatment for patients with advanced non-small cell lung cancer (NSCLC).

Patients and Methods: Patients (n = 133) with stage IIIB to IV NSCLC were randomly assigned to receive DG (docetaxel 60 mg/m^2, day 8 + gemcitabine 800 mg/m^2, days 1 and 8, every 3 weeks; n = 65) or DC (docetaxel 60 mg/m^2, day 1 + cisplatin 80 mg/m^2, day 1, every 3 weeks; n = 68). The primary end point of the study was overall response rate. No prophylactic use of human recombinant granulocyte colony stimulating factor was allowed.

Results: The planned patient number was 150. However, an unexpectedly high incidence of grade 3 interstitial lung disease (11.1%) was identified in the DG arm, so the study was closed early. The overall response rates of the DG and DC arms were 27% and 23.5%, respectively, which demonstrated that the DG treatment was not inferior to the DC arm. Gastrointestinal toxicities were less frequent in the DG arm than in DC arm. Interstitial lung disease was exclusively observed in seven of 63 patients in the DG arm (11.1%). Median survival and 1-year survival rate were comparable between the two arms (median survival, DG 13.7 months versus DC 11.4 months; 1-year survival, DG 56.6% versus DC 47.7%).

Conclusion: The DG regimen has a response rate and survival rate comparable to those of the DC regimen and can therefore be considered from an efficacy point of view to be comparable. However, the DG regimen may have induced pulmonary toxicity in 11% of the patients exposed and therefore should be used cautiously among patients with advanced NSCLC.

Key Words: Docetaxel, Gemcitabine, Cisplatin, Combination chemotherapy, First-line, Advanced non-small cell lung cancer, Randomized phase II Study.

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Lung cancer ranks among the most commonly occurring malignancies and currently is the leading cause of cancer-related deaths in industrialized countries all around the world. In Japan, lung cancer is the most common cause of cancer-related deaths in men and the second most common cause of cancer-related deaths in women, with an incidence of 44 per 100,000 individuals. Approximately 55,000 Japanese died from this disease in 2001. Non-small cell lung cancer (NSCLC) represents approximately 80% to 85% of cases of lung cancer. The prognosis of patients with this cancer is poor: two-thirds are inoperable, and the overall 5-year survival is less than 15%. These inoperable patients are potential candidates for systemic chemotherapy. Meta-analyses of trials comparing systemic chemotherapy with best supportive therapy in advanced NSCLC concluded that chemotherapy could prolong survival by a modest but statistically significant period.[1–5]

In the1990s, several new anti-cancer agents such as irinotecan (CPT-11), docetaxel (DTX), paclitaxel (PTX), gemcitabine (GEM), and vinorelbine (VNR) demonstrated promising antitumor activity against NSCLC, with documented responses ranging from 13% to 27%.[6] Among these agents, DTX a semisynthetic taxoid derived from the European yew Taxus baccata, is used for treating patients with advanced NSCLC, either in combination with other chemotherapeutic agents or as a single agent.[7–10] DTX is often used in combination with platinum-based chemotherapeutic agents. A four-arm randomized study, ECOG 1594, evaluated DTX + cisplatin (CDDP), GEM + CDDP, and PTX + carboplatin (CBDCA) against a control arm of PTX +

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This study was presented in part at ESMO, October 2004. Poster #635

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CDDP, to reveal comparable clinical outcome of the three experimental arms with the control.[11] Another randomized study, TAX326, showed a superior response rate and survival benefit of DTX + CDDP compared with VNR + CDDP, but no superiority of DTX + CBDCA over the control arm (VNR + CDDP).[12] In addition, in the TAX-JP301study in Japan, DTX (60 mg/m^2, day1) + CDDP (80 mg/m^2, day 1) was proven to have a response rate and survival benefit superior to a control arm of vindesine (VDS) + CDDP.[13] Therefore, the DTX + CDDP regimen is now considered a standard for the treatment of advanced NSCLC.

GEM is a difluorinated analogue of deoxycytidine resembling cytalabine and an active agent for NSCLC, either in combination or as a single agent.[14–16] Phase II and III studies of DTX + GEM have demonstrated equivalent responses to DTX + CDDP.[17–19] A phase I/II study of DTX + GEM gave promising results in Japan with a recommended regimen of GEM 800 mg/m^2 on days 1 and 8 plus docetaxel 60 mg/m^2 on day 8. The overall response rate was 43.9%, and the median survival time was 11.8 months.[20]

The purpose of the present study was to assess the efficacy of the DTX + GEM (DG) regimen and to determine whether it is comparable to the DTX + CDDP (DC) regimen. The primary end point was response rate, and the secondary end points were 1-year survival rate and toxicity.

PATIENTS AND METHODS

Eligibility Criteria

The eligibility criteria for study entry included histologically or cytologically confirmed stage IIIB or IV NSCLC, no prior therapy, the presence of a measurable lesion by Response Evaluation Criteria In Solid Tumors (RECIST), age 20 to 75 years, an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 or 1, adequate baseline organ function (defined as 4,000/μL $\leq$ WBC$\leq$ 12,000/μL, neutrophils $\geq$ 2000/μL, platelets $\geq$ 100,000/μL, hemoglobin $\geq$ 9.5 g/dL, serum transaminase levels $\leq$ 2.5 times the upper limit of normal, bilirubin $\leq$ upper limit of normal, creatinine $\leq$ upper limit of normal), creatinine clearance $\geq$60 mL/min, PaO_2 $\geq$70 Torr, a life expectancy of at least 3 months, and written informed consent. The study was conducted according to the Helsinki Declaration and was approved by the ethics committees of the participating centers. Patients with the following criteria were excluded from the study: interstitial pneumonia (pulmonary fibrosis) manifest on chest radiograph and pulmonary symptom (non-productive cough or dyspnea on exertion), uncontrolled complications of heart or liver, diabetes mellitus, bleeding, peripheral neuropathy of grade 2 or worse, symptomatic brain metastases, active concomitant malignancy, pregnancy, breast feeding, myocardial infarction within 3 months, or other conditions rendering the patient unsuitable for this study.

Treatment Plan

Patients were randomly allocated to receive DC or DG stratified by study center, disease stage (IIIB or IV) and sex. Patients were randomly assigned to receive at least two cycles of DC or DG every 21 days (Fig. 1). Patients in the control arm (DC) received DTX 60 mg/m^2 IV (in 250 mL of 5% glucose over 1 hour), and CDDP 80 g/m^2 (in 500 mL of normal saline over 1 hour) IV on day 1. Although docetaxel is usually administered as a 75-mg/m^2 dose, Okamoto et al.[21] demonstrated that a response rate of 42% could be achieved when 60 mg/m^2 DTX and 80 mg/m^2 CDDP were administered to patients with stage IV NSCLC. Patients on the DG arm received DTX 60 mg/m^2 IV (in 250 mL of 5% glucose over 1 hour) on day 8, and GEM 800 mg/m^2 IV (in 100 mL of 5% glucose over 30 minutes) on days 1 and 8. Dexamethasone, 5-hydroxytryptamine-3 receptor antagonists, diphenhydramine, and ranitidine were given before chemotherapy for antiemetic and hypersensitivity prophylaxis. Patients received at least two courses of treatment unless disease progression or unacceptable toxicity was documented. Responders or patients without disease progression continued treatment until the appearance of progressive disease or major toxicity. Recombinant human granulocyte colony-stimulating factor (G-CSF) was administered when National Cancer Institute Common Toxicity Criteria grade 3 to 4 leukopenia or neutropenia occurred. However, G-CSF was not used prohylactically.

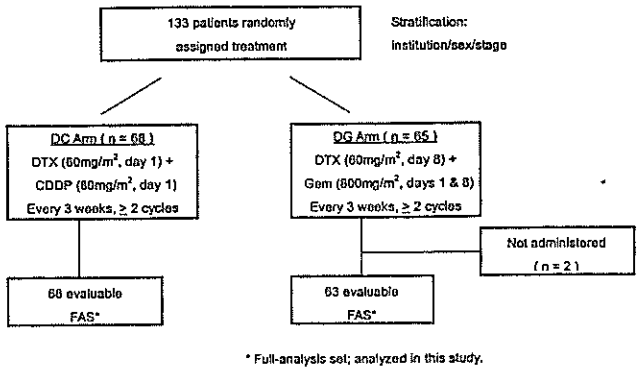

FIGURE 1. Outline of the study.

Treatment of day 8 GEM in the DG arm was delayed until recovery (no longer than 1 week) if the neutrophil count decreased $<$1,500/μL and/or the platelet count was $<$100,000/μL and if non-hematological toxicities were $\geq$ grade 2, excluding those caused by nausea/vomiting and alopecia. If recovery was delayed longer than 1 week, the dose of GEM was omitted. Two weeks' delay of initiation of the subsequent course was allowed; otherwise, the patient was withdrawn from the study. Treatment was discontinued in the event of grade 3 neuropathy or if infection developed. Dose reduction of any of the drugs was not allowed during the subsequent treatment cycle in both arms. Use of thoracic or other radiotherapy after completion of chemotherapy and use of second-line chemotherapy were left to the discretion of treating clinicians.

Patient Evaluation

Patients were assessed at baseline and at each administration of chemotherapy, then every 4 weeks for the first year and thereafter at 8-week intervals. Assessments at baseline and during treatment included history and physical ex-

amination (including weight and height), ECOG performance status, blood chemistry, and full blood count. Minimal baseline imaging consisted of chest radiograph; thoracic, abdominal, and brain CT scans; and isotope bone scan. Toxicities were assessed according to National Cancer Institute Common Toxicity Criteria after each cycle (version 2.0, revised 1994). Patients were evaluated for response at the start of each cycle by CT scan, which was repeated every 4 weeks. RECIST criteria were used to define response.[22] An extramural review was conducted to validate the eligibility of the patients, staging, responses, and toxicities. Disease status and any additional anticancer treatment were reported at each follow-up visit.

Statistical Considerations

The primary end point was response rate, and noninferiority was the basis of the hypothesis to be tested. Parity could be concluded if the lower boundary of the 90% confidence interval of the difference of the response rates between two treatment arms was greater than 20%.[23] The expected response rate for each arm was 33%. The planned sample size of 69 patients per treatment group provided the study with 80% power to detect a difference of response rate of two arms with a type I error of 0.05 (one-sided). We compared Kaplan-Meier curves for overall survival and progression-free survival using the standard log-rank test. Tumor responses in both groups were compared using Fisher's exact test. Other categorical data, such as treatment data and incidence of adverse events, were compared between treatment groups using the χ^2 test. All analyses were performed on an intention-to-treat basis except for the analyses of response and toxicity. All P values are two-sided.

RESULTS

Patient Characteristics

From May 2002 until October 2003, 133 previously untreated patients were recruited for the study from 24 centers of the Japan Lung Cancer Cooperative Clinical Study Group and were randomly assigned to treatment in the trial (Fig. 1). The planned patient number was 150 (75 in each arm). However, an unexpected high incidence of grade 3 interstitial lung disease (ILD) was identified exclusively in DG arm by the Adverse Event Reporting system. The principal investigator stopped the enrollment into the trial on September 30, 2003. The Safety Committee reviewed the investigator's report and recommended that the Japan Lung Cancer Cooperative Clinical Study Group terminate the study immediately because of lung injury in the DG arm.

Two patients in the DG arm did not receive any protocol treatment. One patient suffered from uncontrollable atrial fibrillation, and the investigator decided against this patient receiving protocol treatment. The other patient had a massive hematemesis from a gastric cancer that was discovered after enrollment (second primary). Because two patients were deemed ineligible, 131 patients were evaluated for survival, response, and toxicity. The characteristics of eligible patients are listed in Table 1.

TABLE 1. Patient Characteristics

	Total (n = 131)	DC arm (n = 68)	DG arm (n = 63)	
Sex				
M	86 (65.6)	45 (66.2)	41 (65.1)	NS
F	45 (34.4)	23 (33.8)	22 (34.9)	
Age				
Median (range)	63 (31-75)	65 (31-75)	61 (49-75)	
< 65 yr	74 (56.5)	33 (48.5)	41 (65.1)	P = 0.0832
> 65 yr	57 (43.5)	35 (51.5)	22 (34.9)	
Performance status				
0	61 (46.6)	32 (47.1)	29 (46.0)	NS
1	70 (53.4)	36 (52.9)	34 (54.0)	
Stage				
IIIB	34 (26.0)	18 (26.5)	16 (25.4)	NS
IV	97 (74.0)	50 (73.5)	47 (74.6)	
Histological type				
Adeno carcinoma	87 (66.4)	46 (67.6)	41 (65.1)	
Squamous	36 (27.5)	18 (26.5)	18 (28.6)	NS
Others	8 (6.1)	4 (5.9)	4 (6.3)	
Ineligible after treatment	2 (1.5)	1 (1.5)	1 (1.5)	

χ^2 test (Yates).
Values are n (%) or n (range).

Treatment Delivery

The median number of treatment cycles delivered was 2.0 for the DC arm and 3.0 for the DG arm (Table 2). Fifty-two patients (76%) in the DC arm and 54 patients (86%) in the DG arm received at least two cycles of chemotherapy. The main reasons for treatment discontinuation before the second cycle in the DC and DG arms, respectively, were disease progression (8.8% vs 7.9%), adverse event (8.8% vs 4.8%), and adverse event with withdrawal of consent (5.9% vs 1.6%) Table 2.

Response

There were 16 partial responses with an overall response rate of 23.5% (95% CI, 13.5–33.6%) in the DC arm. The DG arm had 17 partial responses, with an overall response rate of 27.0% (95% CI, 16.0–37.9%) (Table 3). Because the difference of the response rate between two treatment arms was 3.5% (90% confidence interval, −9.0 to 16.0%) and the lower bound of the 90% confidence interval

TABLE 2. Courses administered

Course	DC arm (n = 68) (%)	DG arm (n = 63) (%)
1	23.5	14.3
2	27.9	31.7
3	14.7	23.8
4	27.9	17.5
>5	5.9	12.7
Median	Two courses	Three courses

TABLE 3. Tumor response

	Total (n = 131)	DC arm (n = 68) (%)	DG arm (n = 63) (%)
CR	0	0	0
PR	33 (25.2)	16 (23.5)	17 (27.0)
SD	78 (59.5)	42 (61.8)	36 (57.1)
PD	16 (12.2)	9 (13.2)	7 (11.1)
NE	4 (3.1)	1 (1.5)	3 (4.8)

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, no evidence of disease.

95% confidence interval of response rate: DC 13.5%–33.6%; DG 16.0%–37.9%.

90% confidence interval of difference (χ^2 = 3.5%) of response rate −9.0% to 16.0%.

was not lower than −20%, it was demonstrated that the DG regimen was not inferior to the DC regimen.

Survival

The median overall survival was 11.4 months for the DC arm and 13.7 months for the DG arm.

The hazard ratio was 0.822 (95% CI, 0.531–1.271). One-year survival rate was 47.7% versus 56.6% in the DC and DG arms, respectively (Fig 2).

Toxicity

No differences were seen among the two treatment groups with regard to grade 3 to 4 neutropenia, febrile neutropenia, leukocytopenia, thrombocytopenia, or anemia (Table 4). Grade 3 lung toxicity occurred exclusively in the DG arm. Seven patients in the DG arm (one woman [4.5%] and eight men [14.6%]) had grade 3 ILD, and all patients were treated successfully with the intravenous administration of high-dose methylpredonisolone and oxygen therapy (Table 5). The occurrence of Grade 3 pneumonitis in the first four patients occurred between April and June 2003; patients recovered to grade 1 or 2 with appropriate treatment. Another two cases of Grade 3 pneumonitis occurred in September 2003, at which time the Safety Committee recommended early termination of the trial because of lung injury in the DG arm. Therefore, this trial was terminated early, on October 13, 2003. Another patient developed grade 3 pneumonitis after three courses of chemotherapy in November 2003.

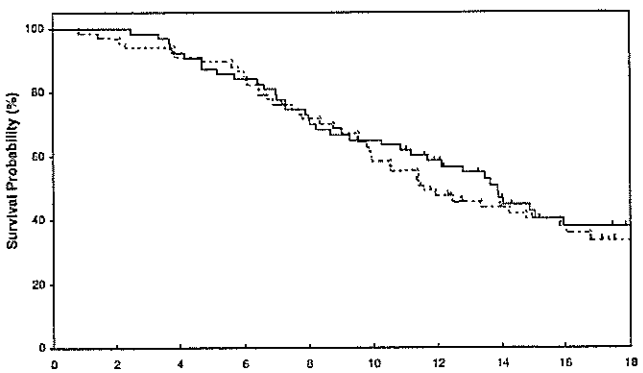

FIGURE 2. Survival curves according to regimen.

TABLE 4. Grade 3 or 4 hematological toxicities

	Total (n = 131)	DC arm (n = 68)	DG arm (n = 63)
Neutropenia (Grade 3, 4)	64.1 (32.8, 31.3)	63.2 (25.0, 38.2)	65.1 (41.3, 23.8)
Febrile neutropenia (Grade 3, 4)	12.2 (9.9, 2.3)	10.3 (7.4, 2.9)	14.3 (12.7, 1.6)
Leukocytopenia (Grade 3, 4)	41.2 (35.9, 5.3)	42.6 (33.8, 8,8)	39.7 (38.1, 1.6)
Thrombocytopenia (Grade 3, 4)	3.8 (3.8, 0.0)	1.5 (1.5, 0.0)	6.3 (6.3, 0.0)
Anemia (Grade 3, 4)	4.6 (3.8, 0.8)	7.4 (5.9, 1.5)	1.6 (1.6, 0.0)

Values are expressed as percentages. P = not significant (Fisher's exact test).

TABLE 5. Grade 3 or 4 non-hematological toxicities

	Total (n = 131)	DC arm (n = 68)	DG arm (n = 63)	
Lung injury (Grade 3, 4)	5.3 (5.3, 0.0)	0.0 (0.0, 0.0)	11.1 (11.1, 0.0)	P = 0.0050*
Hepatic dysfunction (Grade 3, 4)	3.1 (2.3, 0.8)	4.4 (2.9, 1.5)	1.6 (1.6, 0.0)	NS
Diarrhea (Grade 3, 4)	4.6 (4.6, 0.0)	8.8 (8.8, 0.0)	0.0 (0.0, 0.0)	P = 0.0284
Nausea/vomiting (Grade 3, 4)	13.7 (13.7, 0.0)	20.6 (20.6, 0.0)	6.3 (6.3, 0.0)	P = 0.0222
Appetite loss (Grade 3, 4)	19,8 (18.3, 1.5)	29.4 (26.5, 2.9)	9.5 (9.5, 0.0)	P = 0.0046
Fever not related to neutropenia (Grade 3, 4)	6.9 (6.9, 0.0)	2.9 (2.9, 0.0)	11.1 (11.1, 0.0)	NS
Edema (Grade 3, 4)	0.8 (0.8, 0.0)	0.0 (0.0, 0.0)	1.6 (1.6, 0.0)	NS

Values are expressed as percentages.
NS, not significant.
P values obtained using Fisher's exact test.

Interstitial pneumonitis occurred after two courses of chemotherapy in six of seven patients; most cases occurred in the third course of therapy. All the patients presented with the acute onset of dyspnea, sometimes associated with cough or low-grade fever. High-resolution CT scans typically showed bilateral diffuse ground glass opacities and mild thickening of interstitial septa. No infectious agents were identified in the blood, sputum, or broncho-alveolar lavages in any of these patients, and there was no response to antibiotics.

A higher percentage of patients in the DC arm experienced grade 3 or 4 diarrhea, nausea and vomiting, and appetite loss compared with patients in the DG arm. No treatment-related death was observed in either treatment arm.

Second-Line Treatment

After discontinuation or completion of the study, the number of patients who received second-line therapy and the nature of the second-line therapy were well balanced between

the groups. In total, 102 patients (77.9%) went on to receive additional chemotherapy, including gefitinib (DC, 72.1%; DG, 84.1%), and 38 patients (29.0%) received radiotherapy (DC, 27.9%; DG, 30.2%). The most common second-line chemotherapy was gefitinib monotherapy, and there was no difference in the proportion of patients who received gefitinib between the two arms (DC, 32.4%; DG, 30.2%).

DISCUSSION

In the present article, we describe the randomized phase II trial of DTX+ CDDP (DC) and DTX + GEM (DG) in the treatment of chemotherapy-naïve patients with advanced NSCLC. The response rates were comparable in each arm (DC; 23.5%, DG; 27.0%), which demonstrates that the DG regimen was not inferior to the DC regimen with respect to response rate. This was also the case for the median survival time and 1-year survival rate (13.7 months and 56.6%, respectively, for the DG arm versus 11.4 months and 47.7%, respectively, for the DC arm). These observations might suggest that non–platinum-based chemotherapy doublets incorporating newer anticancer drugs have activity similar to that of platinum-based doublets in terms of overall survival and response rate. The reason why the survival rate of our trial seems to be better than often reported for this population of patients (i.e., median survival of 8-10 months) may be the use of second-line treatment with gefitinib. An international randomized phase II trial among patients with advanced or metastatic NSCLC after platinum-based chemotherapy demonstrated a response rate of 28% in the Japanese population.[24] As 32.4% of patients in DC arm and 30.2% of patients in DG arm received gefitinib, this may explain the apparently better overall survival in our study.

Three other studies have evaluated the combination of DTX and GEM in randomized trials. In their study of 441 patients, Georgoulias et al.[19] reported that DC (DTX 100 mg/m^2, day 1, CDDP 80 mg/m^2, day 1) versus DG (DTX 100 mg/m^2, day 8; GEM 1100 mg/m^2, days 1 and 8) had similar efficacy (response rate, 32.4% vs 30.2%) and survival data (median survival time, 10 vs 9.5 months; 1-year survival rate, 42% vs 38%). Compared with the DC arm, the DG arm had a more favorable toxicity profile. However, in their study, all patients received prophylactic recombinant G-CSF. They also conducted another randomized phase III trial (413 patients) comparing DG with CDDP plus VNR (VC) with prophylactic G-CSF support.[25] Overall response rates were 30% and 39.2% (P = 0.053) for the DG and VC arms, respectively. Median survival time was 9.0 and 9.7 months (P = 0.965) for the DG and VC arms, respectively. Quality of life was improved for the DG but not for the VC patients. The DG regimen had a better toxicity profile. Pujol et al.[26] also demonstrated that a non–cisplatin-based regimen was as effective as a cisplatin-based regimen. Their randomized phase III study compared the efficacy, including progression-free survival (PFS), and safety of DG regimen (DTX 85 mg/m^2, day 8 plus GEM 1000 mg/m^2, days 1 and 8) versus VC regimen (VNR 30 mg/m^2, days 1, 8, and 15 plus CDDP 100 mg/m^2, day 1). A total of 311 patients were enrolled. Objective response rates did not differ significantly (31% for DG, 35.9% for VC). Neither PFS nor overall survival differed significantly between the two arms (median PFS 4.2 and 4 months; median survival 11.1 and 9.6 months for DG and VC, respectively). The VC arm experienced a higher number of serious adverse events except pulmonary events and a lower compliance with the protocol.

Several systematic reviews comparing cisplatin-based and cisplatin-free chemotherapy doublet regimens in advanced NSCLC have been published. Platinum-free doublet regimens based on third-generation drugs (i.e., VNR, DTX, PTX, and GEM) yield a better efficacy/toxicity ratio and are expected to offer the patients improved survival without decreasing their quality of life.[27–29] Recently, D'Addario et al.[30] performed a meta-analysis to compare the activity, efficacy, and toxicity of platinum-based versus non–platinum-based chemotherapy among patients with advanced NSCLC. A 62% increase in the odds ratio (OR) for response was attributed to platinum-based therapy (OR, 1.62; 95% CI, 1.46-1.8; P< 0.0001). No statistically significant increase in 1-year survival was found when platinum therapies were compared with third-generation–based combination regimens (OR, 1.11; 95% CI, 0.96-1.28; P= 0.17). The toxicity of platinum-based regimens was significantly higher for hematological toxicity, nephrotoxicity, and nausea and vomiting. Thus, the role of cisplatin may be challenged by well-tolerated third-generation combination regimens. These modern combination regimens are valid options for patients with advanced NSCLC.

The present randomized study was terminated early because seven patients in the DG arm had grade 3 ILD. Fortunately all patients were successfully treated with oxygen and steroid treatment. The incidence rate of 11% in our study was rather high, and ILD was only observed in the DG regimen. Matsui et al.[31] reported a result of a phase I/II study comparing regimen schedules of GEM and DTX in Japanese patients with stage IIIB/IV chemotherapy-naïve NSCLC. Grade 3 ILD was observed in three of 51 patients (5.9%) who received GEM 1000 mg/m^2, days 1 and 8 and DTX 50-60 mg/m^2, day 1. The Japan Clinical Oncology Group conducted a randomized study to compare DTX (D) with DTX plus GEM (DG) for second-line treatment of NSCLC.[32] One hundred thirty patients were randomized to receive either D (DTX 60 mg/m^2, day 1) or DG (DTX 60 mg/m^2, day 8, GEM 800 mg/m^2, days 1, 8), repeated every 21 days until disease progression. Their trial was terminated early with an unexpected high incidence of ILD (17%) and three treatment-related deaths (5%) because of ILD only in DG arm. There was no baseline risk factor for predicting ILD, except for male gender. In our study, the incidence of ILD was apparently greater among men (14.6%) than women (4.5%). However, there was no statistical difference between men and women. The incidence of ILD was, however, reported to be 0% to 5.2% in other studies conducted on the same treatment schedule in Europe[17,19,25,26,33] The predisposing factor for ILD might be related to ethnicity, as a high incidence of ILD was observed in Japanese patients treated with gefitinib. The incidence of ILD was apparently high among Japanese patients (3.2%–10%) compared with Euro-Americans (less than

1%).[34–37] The West Japan Thoracic Oncology Group analyzed 1,976 patients receiving gefitinib retrospectively.[34] The incidence of ILD was 3.2%, and the death rate resulting from ILD was 1.3%. In this case, multivariate analyses also revealed that risk factors included being male, individuals who smoked, and complication of interstitial fibrosis among Japanese patients.

ILD was also observed in a relatively high incidence when DTX and GEM were administered weekly. Popa et al.[17] reported a phase II trial of DG among patients with chemotherapy-naïve, advanced NSCLC. Thirty-two patients were treated with GEM (1000 mg/m^2) and DTX (40 mg/m^2) on days 1 and 8 every 21 days. Six patients (18.7%) experienced grade 3 pneumonitis that was at least possibly related to the combination of DG. There was another phase I study that reported a high incidence of ILD when chemotherapy-naïve patients with advanced NSCLC were treated with DG.[38] Escalated dose of GEM and DTX were given on a weekly basis for 3 consecutive weeks in cycles of 4 weeks. At the dose of DTX 40 mg/m^2 per week and GEM 1000 mg/m^2 per week, the study was prematurely closed because of a high incidence of severe ILD. Six patients (23%) developed ILD, which was fatal in two. No risk factors were identified contributing to ILD. These reports seem to indicate that the weekly administration of DTX and GEM in a relatively high dose may be associated with a high incidence of pulmonary adverse events.

As far as radiation is concerned, Popa et al.[17] suggested that there might be a predisposition to ILD among patients who had previously received radiation therapy because five of six patients in the study who developed treatment-related ILD had previously received radiation therapy of the chest and mediastinum. However, seven patients who developed ILD in our study had no history of prior thoracic radiation. In the case of the Georgoulias et al.,[25] 8 of 209 patients had previous radiotherapy, and two patients developed grade 2 ILD. Although only 2 of 155 patients had a history of thoracic radiotherapy in Pujol et al.'s trial, eight patients experienced ILD.[26] Therefore, the exact mechanism by which it induces severe ILD is not known.

In conclusion, the DG regimen had a response rate and 1-year survival rate comparable to that of the DC regimen, and gastrointestinal toxicities were less frequent in the DG regimen than in the DC regimen. The DG regimen used in the present schedule and dose may induce pulmonary toxicity and should be carefully administered in patients with advanced NSCLC.

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