In never/former smokers, both the *EGFR* mutation rate and the response rate were significantly higher than in current smokers. We speculate that *EGFR* mutations occur equally throughout the entire population, regardless of smoking history, and account for smoking-unrelated carcinogenesis. Because many other genetic alterations, like *KRAS* mutations, occur and induce lung adenocarcinoma more frequently in smokers, the *EGFR* mutation rate seems to be relatively lower in smokers with lung adenocarcinoma.

The response rate of 53% and the *EGFR* mutation rate of 59% observed in this study were higher than previously reported rates. These results can partially be attributed to the fact that the physicians tended to select patients with characteristics known to be predictive for gefitinib sensitivity: women, never-smokers, and patients with adenocarcinoma. Consequently, this cohort was not necessarily representative of unselected NSCLC populations in Japan. However, other recent studies have also shown relatively high frequencies (32% to 55%) of *EGFR* mutations in Japanese or East Asian patients with lung adenocarcinoma who underwent surgical resection.[7,9-11,13] The reason why such somatic mutations occur selectively in East Asian people remains unknown. Environmental or genetic factors common among East Asian populations should be investigated to answer this question.

Recently, no significant survival benefit of gefitinib was reportedly observed in the initial analysis of the IRESSA Survival Evaluation in Lung Cancer (ISEL) trial, a phase III trial comparing gefitinib monotherapy to a placebo as a second- or third-line treatment for patients with advanced NSCLC.[31] Because subgroup analyses of the trial suggested survival benefits in never smokers or Asian patients, the selection of patients is thought to be crucial when considering gefitinib treatment. Because the present study showed that the *EGFR* mutation status is a major determinant of gefitinib sensitivity, mutational analyses in patients with advanced NSCLC should be considered before deciding on a course of treatment.

In this study, we performed LCM and direct sequencing using methanol-fixed surgical specimens to obtain high-quality data. If we had analyzed only bulk tumor samples without LCM, nine of the 39 patients with *EGFR* mutations would have been misjudged as having wild-type *EGFR*. Thus such procedures with LCM are presently recommended for the detection of *EGFR* mutations. However, obtaining appropriate tumor samples is often difficult in patients with advanced NSCLC, and performing LCM and direct sequencing in all patients is not practical. Thus more practical methods for detecting the major *EGFR* mutations using small tumor samples contaminated with normal tissue should be developed and validated.

Other than *EGFR* mutations, some candidate predictive biomarkers have been studied. The *EGFR* copy number is the leading candidate, and it can also be detected by FISH. Practicality and accuracy should be assessed comparing FISH and quantitative real-time PCR. The impact of *ERBB2* mutations on clinical outcome remains to be investigated because we could not detect any mutations in *ERBB2* in the present study. Protein expression analyses by IHC are easier to perform than the genetic analyses, but their significance is still controversial. Further studies are required to evaluate the predictive values of these biomarkers and to determine whether they are independent predictors of gefitinib sensitivity or surrogate markers of *EGFR* mutations.

In conclusion, this study indicates that *EGFR* mutations and increased copy numbers predict better clinical outcome in patients with NSCLC treated with gefitinib. Further research and clinical trials are needed to incorporate these markers into clinical practice appropriately.

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Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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ORIGINAL REPORT

Epidermal Growth Factor Receptor Mutations and Gene Amplification in Non–Small-Cell Lung Cancer: Molecular Analysis of the IDEAL/INTACT Gefitinib Trials

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A B S T R A C T

Purpose
Most cases of non–small-cell lung cancer (NSCLC) with dramatic responses to gefitinib have specific activating mutations in the epidermal growth factor receptor (*EGFR*), but the predictive value of these mutations has not been defined in large clinical trials. The goal of this study was to determine the contribution of molecular alterations in *EGFR* to response and survival within the phase II (IDEAL) and phase III (INTACT) trials of gefitinib.

Patients and Methods
We analyzed the frequency of *EGFR* mutations in lung cancer specimens from both the IDEAL and INTACT trials and compared it with *EGFR* gene amplification, another genetic abnormality in NSCLC.

Results
EGFR mutations correlated with previously identified clinical features of gefitinib response, including adenocarcinoma histology, absence of smoking history, female sex, and Asian ethnicity. No such association was seen in patients whose tumors had *EGFR* amplification, suggesting that these molecular markers identify different biologic subsets of NSCLC. In the IDEAL trials, responses to gefitinib were seen in six of 13 tumors (46%) with an *EGFR* mutation, two of seven tumors (29%) with amplification, and five of 56 tumors (9%) with neither mutation nor amplification ($P = .001$ for either *EGFR* mutation or amplification *v* neither abnormality). Analysis of the INTACT trials did not show a statistically significant difference in response to gefitinib plus chemotherapy according to *EGFR* genotype.

Conclusion
EGFR mutations and, to a lesser extent, amplification appear to identify distinct subsets of NSCLC with an increased response to gefitinib. The combination of gefitinib with chemotherapy does not improve survival in patients with these molecular markers.

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Lung cancer remains the highest cause of cancer-related mortality in the United States and Western Europe, and while transient responses to aggressive chemotherapy are observed in approximately 30% of patients, the impact on patient survival has been modest.[1] The success of imatinib (Gleevec; Novartis, Basel, Switzerland) in the treatment of chronic myeloid leukemia and gastrointestinal stromal tumor has provided compelling evidence for the effectiveness of tyrosine kinase inhibitors in the treatment of

some types of cancer.[2-4] Imatinib targets the kinase pocket of *ABL*, which is activated by the characteristic *BCR-ABL* translocation of CML, and that of C-KIT, which is activated by mutations in gastrointestinal stromal tumors. In these and in a few other tumor types, the dramatic effect of imatinib is thought to result from its targeting a critical genetic lesion on which tumor cells have become dependent for their survival, so-called oncogene addiction.[5]

Extrapolation of these therapeutic approaches to common epithelial cancers has been limited by the absence of comparable insight about critical genetic lesions. Small molecule kinase inhibitors have been developed against growth factor receptors frequently expressed in epithelial cancers, the first of which, gefitinib (Iressa; AstraZeneca, Wilmington, DE), targets the epidermal growth factor receptor (EGFR).[6] Gefitinib was tested in chemotherapy-refractory non–small-cell lung cancer (NSCLC) patients, on the basis of their frequent expression of *EGFR* and their poor response to standard therapies. A phase II trial of two doses of gefitinib monotherapy in refractory NSCLC in the United States reported an overall 10% partial response (PR) rate (IDEAL-2), with a 19% PR observed in a companion European/Japanese study (IDEAL-1).[7,8] Two subsequent phase III trials randomized previously untreated patients with advanced NSCLC to standard platinum-based chemotherapy, with or without the addition of gefitinib at two doses (INTACT-1, cisplatin and gemcitabine ± gefitinib; INTACT-2, carboplatin and paclitaxel ± gefitinib).[9,10] These trials reported no difference in response rate, time to progression (TTP), or 1-year or overall survival (OS) with the addition of gefitinib to standard chemotherapy. These findings were nearly identical to the results of the TALENT and TRIBUTE studies of similar design to INTACT but using the *EGFR*-tyrosine kinase inhibitor (TKI), erlotinib.[11,12] Thus, despite randomized clinical studies involving nearly 4,000 patients with advanced disease there was no discernable improvement in outcome following the addition of *EGFR*-TKI to standard cytotoxic chemotherapy.

While initial trials of gefitinib failed to show activity in most cases of NSCLC, a subset of cases that did respond had rapid and dramatic tumor shrinkage. These responses were more common in women, East Asians, and in nonsmokers, and their tumors were primarily adenocarcinomas, often with areas of bronchoalveolar histology. Expression levels of EGFR did not correlate with gefitinib response in the IDEAL trials.[13] We, and others, have recently reported that the majority of tumors with dramatic responses harbor mutations in the *EGFR* kinase domain that were not found in nonresponsive cases.[14-16] These mutations were detected in approximately 10% of NSCLC cases in North America and 30% of patients in Asia.[14-28] *EGFR* mutations associated with gefitinib response include amino acid substitutions and in-frame deletions clustered around the ATP binding pocket, which also serves as the drug binding site. A small number of different mutations account for most cases, suggesting that they confer specific enzymatic properties. Indeed, reconstitution of these mutations in vitro reveals that they mediate dramatically increased anti-apoptotic signals following binding of the EGF ligand to the receptor, compared with wild-type EGFR.[29-31] Suppression of these survival signals, either by gefitinib or by direct targeting of the mutant *EGFR* transcript using small interfering RNA, leads to rapid apoptosis, consistent with the oncogene addiction model.[29]

Studies linking *EGFR* mutations to gefitinib response have involved retrospective analysis of patients with dramatic responses to the drug. To obtain a more comprehensive view of the molecular determinants of gefitinib response, analysis of unselected specimens from large clinical trials is essential. We describe here a molecular analysis of *EGFR* in tumor specimens collected within the IDEAL and INTACT trials, to gain further insight into the clinical responses associated with gefitinib treatment of NSCLC.

Clinical Material

The IDEAL-1 and IDEAL-2 studies of gefitinib monotherapy (250 mg/d and 500 mg/d, respectively) in advanced NSCLC patients, who had received prior chemotherapy, enrolled 425 patients. Tumor samples were not mandatory and were obtained at the time of randomization or up to several years before study entry. Paraffin-embedded tumor blocks were available from 155 patients for analysis of *EGFR* mutation and gene amplification. The INTACT-1 and INTACT-2 studies comparing chemotherapy to chemotherapy plus gefitinib (250 mg/d or 500 mg/d, respectively) in previously untreated NSCLC enrolled 2,130 patients. Paraffin-embedded diagnostic tumor blocks were available from 666 patients for analysis of *EGFR* gene sequence and amplification.

DNA Extraction and EGFR sequencing

Hematoxylin and eosin-stained sections of formalin-fixed paraffin-embedded tissue were reviewed by a pathologist to identify regions of tissue comprising at least 50% tumor cells. Cases where tumor cells comprised less than 50% of the tissue, or where the amount of tumor tissue was limited, were excluded from further analysis (36 IDEAL cases and 142 INTACT cases). Genomic DNA was isolated using the Gentra purification system according to the manufacturer's instructions. Polymerase chain reaction (PCR) conditions for the amplification of *EGFR* are available on request.

PCR amplification of p53, Kras, and PTEN

Exons 1 and 2 of *Kras*, exons 5 to 8 of *p53*, and exons 1 to 9 of *PTEN* were amplified from all available patients determined to have an *EGFR* mutation. Primers and PCR conditions are available on request.

Mutational Analysis

PCR amplicons generated from specimens collected within the IDEAL and INTACT trials were purified using exonuclease I (United States Biochemical, Cleveland, OH) and shrimp alkaline phosphatase (United States Biochemical, Cleveland, OH) followed by dilution in water before sequencing. Bidirectional capillary sequencing was performed using BigDye Terminator

v1.1 chemistry (Applied Biosystems, Foster City, CA) in combination with an ABI3100 instrument. Electropherograms were aligned and reviewed using Sequence Navigator software (Applied Biosystems). All mutations were confirmed by analysis of at least two independent PCR amplifications.

Quantative Real-Time PCR

EGFR copy number was determined by TaqMan real-time quantitative PCR with TaqMan Universal PCR mastermix and an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City CA). The primers (5′-3′) and fluorogenic probe used for *EGFR* were CAATTGCCAGTTAACGTCTTCCTT (sense primer), TTTCTCACCTTCTGGGATCCA (antisense primer), and TCTCTCTGTCATAGGGAC (probe). For the control gene on chromosome 4, *PCDH7*, these were GCTGCAATCTCCTCCCTGAA (sense primer), TGCCTTTTCTCACCTGCATTC (antisense primer), and CCACTGCTCCGACATG (probe). For *COG5*, a control gene located on chromosome 7, primers and probe were TGGAAGATGATGCACAAGATATATTCA (sense primer), CCAACTAACAGGTCAAATTAAACAAACA (antisense primer), and CCAAAAAAGCCAGATTATGA (probe), respectively.

Molecular Characterization of EGFR in Specimens From IDEAL and INTACT Trials

All available tumor specimens (n = 821) from the IDEAL-1, IDEAL-2, INTACT-1, and INTACT-2 studies were subjected to analysis. Pathology review of paraffin-embedded sections was performed for all of these cases, and tumors were considered adequate for analysis if microdissection resulted in more than 50% tumor cell content (n = 643). Nucleotide sequencing of the kinase domain of *EGFR* (exons 18 to 21) was performed using nested PCR amplification of individual exons. The unequal signal observed for genetic variants in some cases raised the possibility of selective allelic amplification, which was confirmed using quantitative real-time PCR analysis. For IDEAL-1 and IDEAL-2, 119 tumor samples were available for molecular analysis, representing 28% of all cases entered in the trials. Of these, 79 samples (66%) were successfully sequenced and 90 samples (76%) were successfully subjected to amplification analysis. For INTACT-1 and INTACT-2, 524 samples were retrieved for analysis from 2,130 clinical cases (25%), of which 312 samples (59%) were successfully sequenced and 453 samples (86%) had successful gene copy number quantification. Objective responses (OR, including partial or complete responses) to gefitinib were achieved in 15% of cases (12 out of 79) from the IDEAL trials for which tumor material was analyzed, consistent with the 10% to 19% OR previously reported for the clinical cohorts. For the INTACT cases available for molecular analysis, the OR to chemotherapy alone and chemotherapy plus gefitinib was 40% and 57%, respectively, compared with 29% to 45% and 30% to 50% reported for the entire cohort in the clinical trials. The specimens analyzed were, therefore, representative of the clinical studies as represented by a comparison of demographic factors between the populations with assessable sample to the overall trial populations (Tables 1 and 2).

EGFR mutations were found in 14 out of 79 cases (18%) from the IDEAL studies (Table 3). These included overlapping in-frame deletions within exon 19 (n = 11), representing four distinct nucleotide deletions encompassing the LREA (LeuArgGluAla) motif within the kinase domain; the recurrent L858R missense mutation in exon 21 (two cases); and a novel insertion of a single amino acid residue within exon 20 (n = 1). For the INTACT studies, *EGFR* mutations were detected in 32 out of 312 cases (10%), with comparable distribution among the three treatment arms as well as among the different chemotherapy regimens used in the two INTACT studies. Mutations were in-frame deletions in exon 19 (n = 22), the L858R missense variant (n = 6), and other novel missense mutations (n = 4). For both IDEAL and INTACT, the overall frequency of mutations detected is consistent with reported *EGFR* mutation rates in NSCLC.[14-28]

Nucleotide sequencing tracings showed most mutations to be present at the expected ratio for heterozygous mutations. In some cases, however, apparent allelic imbalance raised the possibility of differential gene amplification. To explore this possibility, we used quantitative real time-PCR analysis to analyze gene copy numbers for *EGFR*, located at chromosome 7p, using two sets of controls: a marker at chromosome 7q and another on chromosome 4. Amplification of the *EGFR* locus was observed in seven of 90 IDEAL cases (8%) and 33 of 453 INTACT cases (7%). Amplification levels ranged from four-fold to more than 1,000-fold, with a median of eight-fold. Of 14 cases with gene amplification for which mutational status was available, 10 cases (80%) had amplification of wild-type *EGFR*. Of interest, *EGFR* amplification accounted for only a small subset of cases with high levels of protein expression as measured by immunohistochemistry, pointing to other mechanisms that regulate *EGFR* expression (Fig 1).

Clinical Correlates of EGFR Mutation Versus Amplification

Clinical responses to gefitinib have been observed more commonly in NSCLC with adenocarcinoma or bronchoalveolar histology, arising in nonsmokers, women, and patients of East Asian ethnicity.[7,8] As shown in previous studies, these clinical features are well correlated with the presence of *EGFR* mutations.[14-28] Consistent with this, in the IDEAL and INTACT trials, *EGFR* mutations were more frequent in adenocarcinomas (37 of 213; 17%) than in tumors with other histologies (nine of 178; 5%; $P = .0001$); in tumors from women (23 of 124; 19%) than men (23 of 267; 9%; $P = .006$); nonsmokers (14 of 55; 26%) than smokers (22 of 284; 8%; $P = .0004$); and Asians (five of 27; 19%) than non-Asians (41 of 364; 11%; $P = .346$; Table 4).

Table 1. Comparison of Patients With Assessable Sample to Overall Population-IDEAL Trials

Variable	Population With Samples Assessable for Mutation (n = 79)		Population With Samples Assessable for Amplification (n = 90)		Overall Population-IDEAL I and II (n = 425)	
	No.	%	No.	%	No.	%
Gefitinib dose, mg						
250	38	48.1	47	52.2	205	48.2
500	41	51.9	43	47.8	220	51.8
Age group, years						
18-64	55	69.6	62	68.9	275	64.7
65-74	22	27.9	26	28.9	129	30.4
≥ 75	2	2.5	2	2.2	21	4.9
Gender						
Female	29	36.7	35	38.9	154	36.2
Male	50	63.3	55	61.1	271	63.8
Race						
Asian	20	25.3	26	28.9	107	25.2
Non-Asian	59	74.7	64	71.1	318	74.8
Histology						
Adenocarcinoma	49	62.0	55	61.1	274	64.5
Other	30	38.0	35	38.9	151	35.5
Smoking status						
Never	24	30.4	30	33.3	120	28.2
Ever	53	67.1	57	63.3	290	68.2
Unknown	2	2.5	3	3.3	15	3.5
Objective tumor response						
CR	0	0.0	0	0.0	1	0.2
PR/PRNM	12	15.2	14	15.5	60	14.1
SD	22	27.8	24	26.7	133	31.3
PD	40	50.6	48	53.3	199	46.9
Unknown	5	6.3	4	4.5	32	7.5

Abbreviations: CR, complete response; PR/PRNM, partial response/partial response non-measurable; SD, stable disease; PD, progressive disease.

In contrast, there was no correlation between these gefitinib-responsive demographic groups and cases with *EGFR* amplification (Table 4). The frequency of *EGFR* amplification was 19 of 275 (7%) in adenocarcinomas and 21 of 267 (8%) in other histologies; five of 79 (6%) in nonsmokers and 31 of 381 (8%) in smokers; two of 39 (5%) in Asians and 38 of 504 (8%) in non-Asians (P value not significant). Lung cancers in women were somewhat more likely to have *EGFR* amplification (21 of 192, 11%) than those arising in men (19 of 351, 5%; $P = .02$). Taken together, there was no significant increase in the prevalence of *EGFR* amplification in cases with clinical features that are characteristic of strong responses to gefitinib. In addition, we observed that *EGFR* mutations were more prevalent in patients diagnosed before the age of 64 (37 of 259; 14%) compared with those diagnosed at a later age (nine of 132; 7%; $P = .03$). This was in contrast to *EGFR* amplification, which was more frequent in older patients (17 of 92; 18%) than in younger patients (23 of 351; 7%; $P = .0009$).

Responsiveness of NSCLC to Gefitinib

The response of NSCLC patients with different genotypes to single-agent gefitinib was evaluated in the IDEAL studies. In these studies, all patients received either of two doses of gefitinib, which showed no difference in response and hence, are grouped together in this analysis. Patients whose tumor had an *EGFR* mutation had a better response to gefitinib, with an OR of six of 13 (46%), compared with those lacking such mutations (six of 61 (10%); $P = .005$). Patients with more than four-fold amplification of *EGFR* also had a higher, but not statistically significant, probability of response to gefitinib (two of seven cases [29%]), compared with those with diploid or less than four-fold *EGFR* gene copy numbers (12 of 79 [15%]; $P = .319$). However, of the only two gefitinib-responsive patients with *EGFR* amplification, one had amplification of the mutant allele while the other had multiple copies of the wild-type allele. Given these small numbers of responses, the independent contribution of wild-type *EGFR* amplification to gefitinib-responsiveness cannot be determined. Altogether, in tumors analyzed for both mutations and amplification of *EGFR*, six of 10 patients (60%) with either genetic abnormality had a response to gefitinib, compared with five of 52 patients (10%) with neither amplification nor mutation ($P = .0011$). Median TTP for mutation positive cases was longer (116 days; range, 25 to 171), than that for mutation

Table 2. Comparison of Patients With Assessable Sample to Overall Population-INTACT Trials

Variable	Population With Samples Assessable for Mutation (n = 312)		Population With Samples Assessable for Amplification (n = 453)		Overall Population-INTACT I and II (n = 2,130)	
	No.	%	No.	%	No.	%
Gefitinib dose, mg						
250	95	30.4	134	29.6	710	33.3
500	125	40.1	173	38.2	712	33.4
Placebo	92	29.5	146	32.3	708	33.2
Age group, years						
18-64	204	65.4	289	63.8	1,333	62.5
65-74	92	29.5	137	30.2	638	30.0
≥ 75	16	5.1	27	6.0	159	7.5
Gender						
Female	95	30.4	157	34.7	706	33.1
Male	217	69.6	296	65.3	1,424	66.9
Ethnicity						
Asian	7	2.2	13	2.9	87	4.1
Non-Asian	305	97.8	440	97.1	2,043	95.9
Histology						
Adenocarcinoma	164	52.6	220	48.6	1,115	52.3
Other	148	47.4	233	51.4	1,015	47.7
Objective tumor response						
CR	10	3.2	13	2.9	32	1.7
PR/PRNM	115	36.9	162	35.8	748	39.9
SD	87	27.9	125	27.6	569	30.3
PD	29	9.3	50	11.0	235	12.5
Unknown	71	22.7	103	22.7	293	15.6

Abbreviations: CR, complete response; PR/PRNM, partial response/partial response non-measuraible; SD, stable disease, PD, progressive disease.

negative cases (57 days; range, 28 to 170). However, there was no impact on OS (Fig 2).

Additional Genetic Abnormalities in EGFR-*Mutant NSCLC in IDEAL*

Because only 46% of *EGFR*-mutant tumors responded to gefitinib, we addressed the possibility that the unresponsive tumors might have accrued additional genetic alterations modulating the drug sensitivity effect of the *EGFR* mutations. The T790M secondary *EGFR* mutation, recently correlated with acquired resistance to gefitinib,[32-34] was not detected in any tumors from the IDEAL trial. Previous analyses of unselected cases of NSCLC indicated that *EGFR* and *Kras* mutations are mutually exclusive, leading to the hypothesis that activating mutations within *Kras* may prevent clinical response to EGFR inhibitors.[35] However, we found no mutations at hotspot *Kras* codons 12, 13, or 61 in

Table 3. *EGFR* Mutations Within the IDEAL and INTACT Clinical Trials

Nucleotide Change	Amino Acid Change	Exon	No. of Patients (n = 46)
Deletion of nucleotides 2235-2249	Del E746-A750	19	23
Deletion of nucleotides 2236-2250	Del E746-A750		3
Deletion of nucleotides 2237-2255insT	Del E746-S752		1
Deletion of nucleotides 2239-2248insC	Del L746-A750insP		1
Deletion of nucleotides 2240-2254	Del L747-T751		2
Deletion of nucleotides 2240-2257	Del L747-P753insS		3
Insertion of GGT at nucleotide 2311	Ins G771	20	1
Substitution of A for G at nucleotide 2308	Asp770Asn		1
Substitution of T for C at nucleotide 2348	Thr783Ile		1
Substitution of G for T at nucleotide 2573	Leu858Arg	21	7
Substitution of G for T at nucleotide 2573 & T for G at nucleotide 2574	Leu858Arg		1
Substitution of A for G at nucleotide 2588	Gly863Asp		1
Substitution of A for G at nucleotide 2689	Val897Ile	22	1

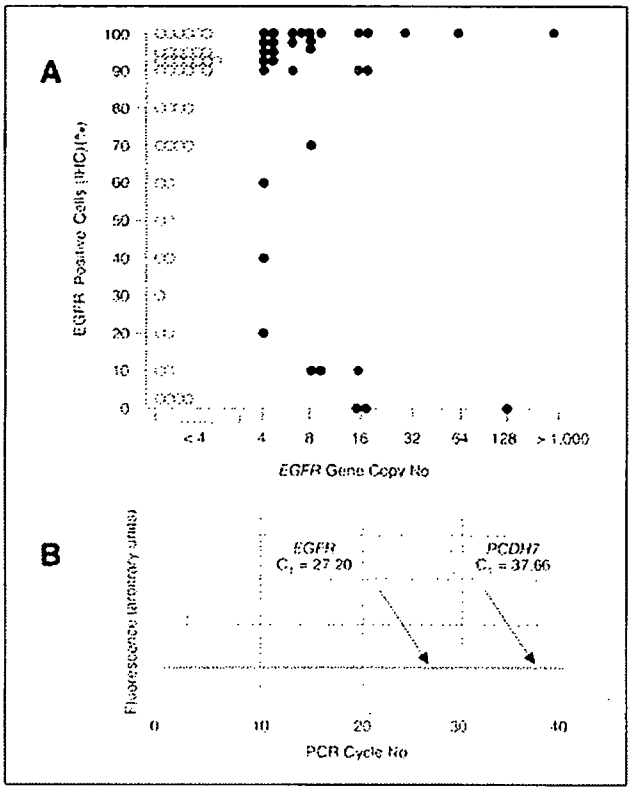

Fig 1. Comparison of EGFR protein expression and gene amplification in tumors from the IDEAL and INTACT trials. (A) Protein expression was determined by immunohistochemistry and *EGFR* copy number was measured by quantitative real-time polymerase chain reaction (qPCR). Open circles represent 10 patients; closed circles denote a single patient. (B) Representative analysis of a sample using qPCR. Cycle numbers (C_T) required for linear amplification of *EGFR* are compared with those needed for equal amplification of the control gene *PCDH7*.

EGFR-mutant tumors that were either responsive (n = 5) or nonresponsive (n = 3) to gefitinib. We also considered the possibility that mutational inactivation of *p53* might suppress apoptotic signals and relieve cells from their dependence on survival signals mediated by mutant EGFR. However, among *EGFR*-mutant tumors, *p53* mutations were found in two out of six patients (33%) who responded to gefitinib and in one out of seven nonresponsive patients (14%; $P = .13$). Finally, given the role of *PTEN* in suppressing AKT activation, which appears to be critical in mediating gefitinib-sensitivity in *EGFR*-mutant tumors, we screened for *PTEN* expression using immunohistochemistry and sequenced mutational hotspots but found no association between loss of expression or mutation of *PTEN* and gefitinib-responsiveness in *EGFR*-mutant tumors. Thus, we did not identify known molecular abnormalities in NSCLC that modulate the response to single-agent gefitinib in *EGFR*-mutant tumors.

Addition of Gefitinib to Chemotherapy

The INTACT studies were randomized, placebo-controlled trials to test the effectiveness of chemotherapy combined with gefitinib in previously untreated NSCLC.[9,10] In contrast to the success of combining trastuzumab (Herceptin; Genentech, South San Francisco, CA) with chemotherapy in breast cancer, the INTACT trials showed no overall benefit to patients treated with both chemotherapy and gefitinib compared with chemotherapy alone, raising concern that use of one therapeutic modality might, in fact, suppress the effectiveness of the other. Analysis of *EGFR*-mutant subgroups from the INTACT studies, therefore, allowed examination of the potential interaction between chemotherapy and gefitinib in patients likely to respond to the EGFR-TKI. Two different chemotherapy regimens were employed in these studies, along with two different doses of gefitinib, which are grouped here for purposes of analysis. Molecular analysis revealed that 13 of 18 *EGFR*-mutation carriers (72%) responded to chemotherapy plus gefitinib, compared with 84 of 152 mutation negative cases (55%), but this difference did not achieve statistical significance ($P = .2$). No difference in OR to combination chemotherapy-gefitinib was seen in cases with *EGFR* amplification (10 of 18; 56%) versus tumors without amplification (114 of 217; 53%). While the increased OR to combined therapy in *EGFR*-mutant tumors, compared with other subgroups, suggests that the addition of gefitinib to chemotherapy provided some benefit in these patients over chemotherapy alone, we could not directly address this question because of the small number of *EGFR* mutant tumors in the placebo arm of the trial: two of five *EGFR*-mutant tumors (40%) responded to chemotherapy alone while 13 of 18 (72%) responded to combination chemotherapy-gefitinib ($P = .3$). Among tumors with wild-type *EGFR*, 26 of 66 (39%) responded to chemotherapy alone versus 84 of 152 (55%) to combination chemotherapy-gefitinib; among tumors with *EGFR* amplification, three of six (50%) responded to chemotherapy versus 10 of 18 (56%) with combination treatment ($P = 1.0$).

OS was not affected by the addition of gefitinib to chemotherapy in patients with *EGFR* mutations (hazard ratio [HR];, 1.77; 95% CI, 0.50 to 6.23; Fig 3; Supplemental Tables 1 and 2). However, *EGFR*-mutant patients treated with chemotherapy alone had a better OS compared with mutation negative patients (median OS, 19.4 months *v* 9.2 months; HR, 0.48; 95% CI, 0.29 to 0.82), raising the possibility that this genetically defined subset of NSCLC may have a more favorable natural history and that *EGFR* mutations may also serve as a prognostic factor. A similar trend toward improved OS irrespective of gefitinib therapy was also seen for chemotherapy-treated patients with amplification of *EGFR* (median OS, > 20 months *v* 10.2 months; HR, 0.46; 95% CI, 0.25 to 0.83; Fig 3; Supplemental Tables 3 and 4). Consistent with this, progression-free survival was also slightly longer for mutation positive (median TTP, 6.7 months *v* 4.5 months; HR, 0.40; 95% CI, 0.23 to 0.71) or amplification positive (median TTP, 7.3 months *v* 4.6 months; HR, 0.37; 95% CI, 0.20 to 0.69) patients treated

Table 4. Demographic Distribution of *EGFR* Mutations and Amplification Within the IDEAL and INTACT Clinical Trials

Demographic Sub-Group	Frequency of *EGFR* Mutations			Frequency of *EGFR* Gene Amplification		
	No.	%	*P*	No.	%	*P*
Gender			.006			.025
Female	23 of 124	18.5		21 of 192	10.9	
Male	23 of 267	8.6		19 of 351	5.4	
Smoking status			.0004			.817
Non-smoker	14 of 55	25.5		5 of 79	6.3	
Smoker	22 of 284	7.7		31 of 381	8.1	
Histology			.0001			.743
Adenocarcinoma/BAC	37 of 213	17.4		19 of 275	6.9	
Other histology	9 of 178	5.1		21 of 267	7.8	
Ethnicity			.346			.758
East Asian	5 of 27	18.5		2 of 39	5.1	
Other ethnicity	41 of 364	11.2		38 of 504	7.5	
Age, years			.031			.0009
< 64	37 of 259	14.3		23 of 351	6.6	
> 65	9 of 132	6.8		17 of 92	18.5	

Abbreviation: BAC, bronchoalveolar.

with chemotherapy irrespective of gefitinib therapy. Clinical characteristics suggest that *EGFR* mutations and amplification identify biologically distinct subsets of lung cancer (Table 4), despite the possibility that both may be associated with a better natural history. However, an improved OS for *EGFR*-mutant NSCLC was not evident in the IDEAL trials, involving pretreated patients rather than first line therapy. Hence, the determination of whether *EGFR*-mutant NSCLC bears a more favorable prognosis irrespective of therapy awaits carefully designed population studies.

The IDEAL and INTACT clinical trials were large, international phase II and phase III studies aimed at defining the clinical role of gefitinib, in NSCLC.[7-10] Despite the virtually universal expression of the gefitinib target, *EGFR*, the trials had the unexpected result of identifying a small subset of NSCLC with dramatic responses to this drug, while many other patients progressed on therapy.[7,8] The identification of *EGFR* mutations in the majority of gefitinib responders[14-16,18-20,23,24,26-28,31] now allows an analysis of these clinical trials to define the contribution of molecular abnormalities in *EGFR*, using an unselected patient population. Although there are a number of biases that are inherent in an analysis such as this one, the patients available for molecular analysis appear to be a representative group.

Our studies confirm the now well-established association between *EGFR* mutations and gefitinib responsiveness. Previous studies have shown that approximately 80% of retroactively identified gefitinib-responsive NSCLC have activating mutations in the EGFR kinase,[14-16,18-20,23,24,26-28,31] while only 50% of responders in the IDEAL trials had such mutations. This difference may reflect a bias in previous retrospective studies, which focused on cases with dramatic responses to gefitinib, whereas, the systematic inclusion of all cases with a PR in the IDEAL trials may have added cases with less remarkable responses attributable to other tumor characteristics. In addition, high throughput sequencing analysis of archival tumor specimens may have resulted in a lower mutation detection rate, compared with the smaller number of selected specimens analyzed previously.

Most significantly, the IDEAL studies allow calculation of the value of *EGFR* mutations in predicting drug response. Approximately 15% of NSCLC patients had a kinase mutation in *EGFR* and 46% of these went on to have an OR, following treatment with gefitinib, compared with 10% of mutation-negative patients ($P = .005$). Although the number of patients was too small to detect a clinically meaningful impact on OS, the presence of *EGFR* mutations was associated with increased TTP, including a small number of long-term responses. Recent studies, largely within Asian populations in which *EGFR*-mutant NSCLC is more prevalent, have reported that these mutations are predictive of both increased TTP and OS.[20,23,27,28] By contrast, molecular analysis of *EGFR* within the recently published BR.21 phase II trial of erlotinib implies that mutations are only weakly predictive of response (16% of mutation positive cases responded *v* 7% of mutation negative cases).[36] A meaningful comparison of data reported here and in the BR.21 study is confounded by differences in criteria used to score mutation-positive cases. All mutations reported here were reproducible in at least two independent PCR amplifications and 41 (89%) consisted of the known recurrent exon

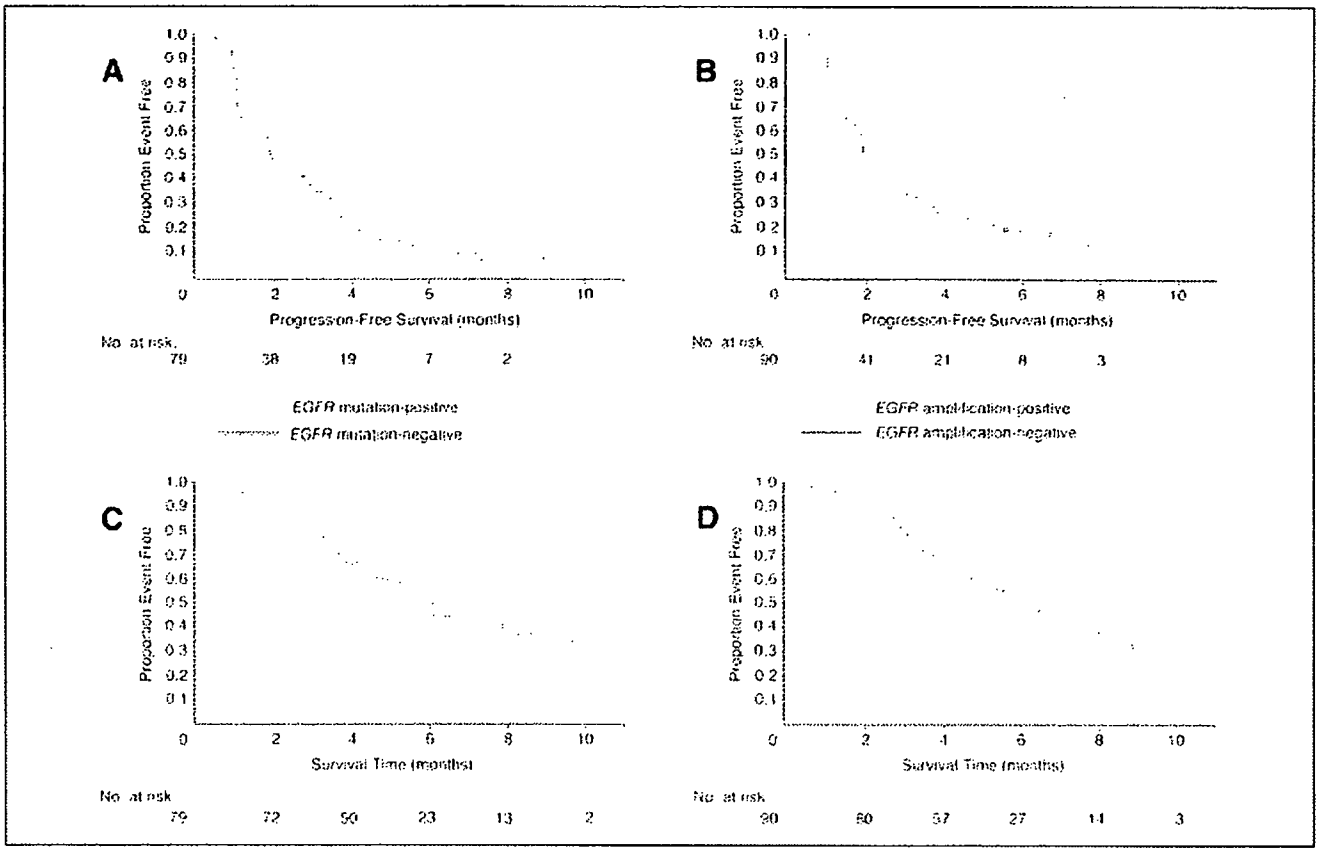

Fig 2. Kaplan-Meier estimates of time to progression (TTP) and overall survival (OS) within the IDEAL trials by (A, C) *EGFR* genotype or (B, D) *EGFR* copy number.

19 deletions and L858R missense mutation. In contrast, 50% of mutations in the BR.21 study, representing 11% of all cases genotyped, were novel and unvalidated sequence variants identified in a single PCR-sequencing analysis.[36] In our experience, the rate of false-positive mutational results using DNA extracted from paraffin-embedded tissue is high, underscoring the importance of duplicate analyses. Among the 77 IDEAL cases analyzed for mutations within exons 18-21, eight irreproducible nucleotide substitutions were identified among six cases (8%). Inclusion of such cases in the analysis of the BR.21 trial may account for the reported lower predictive value of mutations in the response to erlotinib.

Consistent with the oncogene addiction model,[29] it is possible that *EGFR*-mutant cases that failed to respond may have had additional genetic lesions attenuating the dependence of tumor cells on mutant *EGFR* signaling. We did not detect secondary alterations in *EGFR* or mutations in *Kras*, *p53*, or *PTEN* that could explain this apparent resistance to gefitinib in some *EGFR*-mutant tumors. Nonetheless, we note that all mutational analyses were performed on tissue acquired at the time of initial diagnosis, whereas, patients underwent multiple rounds of chemotherapy before entering the IDEAL trials as third-line therapy. Additional mutations in *EGFR* or in other genes may have accrued during these chemotherapy courses. Clinical trials are under way to test whether the predictive value of *EGFR* mutations is enhanced when TKIs are administered at the time of initial diagnosis, tissue acquisition, and *EGFR* genotyping.

Amplification of the *EGFR* gene (> four-fold) was also evident in approximately 7% to 8% of cases, but it was not well-correlated with protein expression data measured by immunohistochemistry. For precise measurement of *EGFR* gene copy number, we used quantitative PCR (qPCR), with one control on the same chromosome as the *EGFR* gene and a second control on an unrelated chromosome with infrequent gene copy number changes in NSCLC. Compared with standard fluorescence in situ hybridization, qPCR allows more reliable distinction between specific amplification of *EGFR* and nonspecific aneuploidy with associated increased *EGFR* gene copies, and it also provides a more consistent measure of the mean gene copy number in a tumor cell population. Most tumors with *EGFR* amplification had multiple copies of the wild-type allele, although some also had amplification of the mutant sequence. The number of cases was too small to allow us to distinguish the effect of wild-type *EGFR* amplification, presumably resulting in increased but normal downstream signaling pathways versus that of mutant-*EGFR* amplification, which is associated with selective activation of

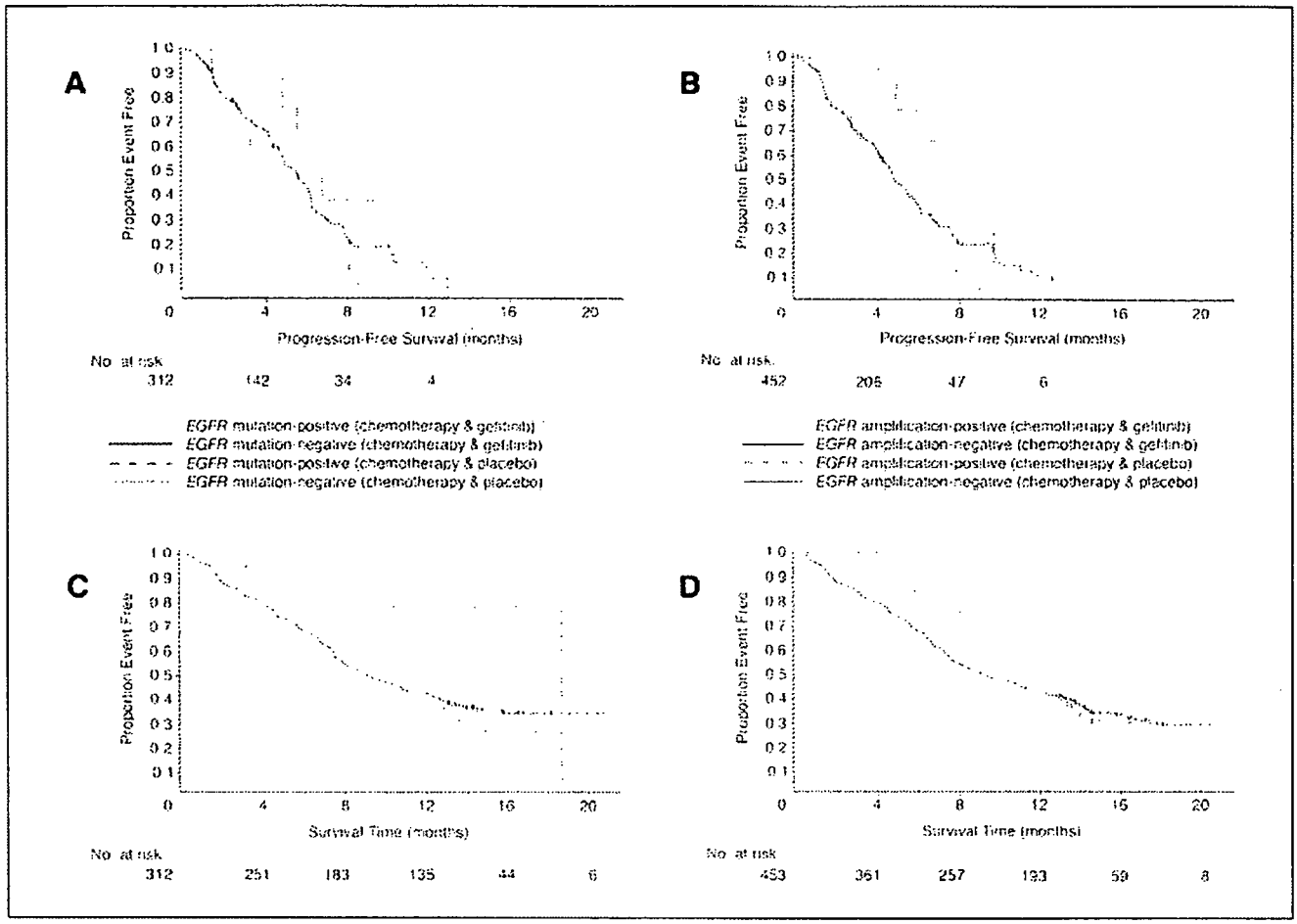

Fig 3. Kaplan-Meier estimates of time to progression (TTP) and overall survival (OS) within the INTACT trials by (A, C) *EGFR* genotype or (B, D) *EGFR* copy number (see supplementary material for calculations of median TTP and OS).

downstream antiapoptotic signals.[29,36] Among gefitinib responders 14% (two of 14) had amplified copies of *EGFR*, and 29% (two of seven) of NSCLC with *EGFR* amplification responded to gefitinib. Further studies will be required to determine whether amplification of wild-type *EGFR* in NSCLC is independently predictive of gefitinib response. Some studies of NSCLC, using fluorescence in situ hybridization, have proposed *EGFR* amplification as an important predictor of response,[28,37] while analysis of glioblastomas, which are not responsive to gefitinib despite frequent *EGFR* amplification, has suggested otherwise.[38] Nonetheless, combining our mutational and amplification data from the IDEAL studies, 60% of cases with either *EGFR* mutations or gene amplification responded to gefitinib, compared with 10% of cases without either genetic abnormality ($P = .0011$), supporting the hypothesis that genetic lesions in *EGFR* are critical in defining drug-susceptible subtypes of NSCLC.

The genotype analysis of the INTACT studies showed that the addition of gefitinib to standard chemotherapy regimens showed a trend toward an increased OR in *EGFR*-mutant patients but not in patients with *EGFR* amplification. The relatively high OR to chemotherapy alone in these previously untreated patients complicated the detection of a further contribution by gefitinib. Given the dramatic effectiveness of gefitinib in patients that do respond, no conclusion can be drawn about the benefit of simultaneous versus sequential therapy with TKIs and chemotherapy. The INTACT studies did show a significant increased survival of *EGFR* mutation-positive patients treated with chemotherapy, irrespective of gefitinib with a similar trend observed in patients with *EGFR* amplification. As noted above, while this difference may point to a relatively favorable natural history of these tumor subtypes, the enhanced survival of *EGFR*-mutant patients was not observed in the IDEAL studies, and hence, awaits confirmation in population-based studies. These findings are in agreement with the molecular analysis of a phase III trial of erlotinib (TRIBUTE) in which *EGFR* mutations appeared to be a positive prognostic indicator irrespective of *EGFR*-TKI treatment.[39]

An unexpected result of these molecular studies is that *EGFR* kinase mutations and gene amplification appear to

identify different genetic subsets of NSCLC with clearly distinct features. As previously reported, kinase mutations are increased in adenocarcinomas, and in tumors arising in women, nonsmokers, and Asians, all features that were initially identified as clinical correlates or strong gefitinib responses in the IDEAL trials.[7,8] We also note a trend toward earlier age of diagnosis, which had not been previously reported. In contrast, *EGFR* amplification is indistinguishable from the majority of NSCLC, with the expected proportion of tumor histologies, smoking history, and without sexual or ethnic predilection. Thus, *EGFR* mutations appear to identify a unique biologic subset of NSCLC, many of which are highly susceptible to therapy with gefitinib. Further studies will be required to determine the contribution of other molecular markers, including *EGFR* amplification, some of which may also provide insight into the more moderate responses to EGFR-TKIs seen in NSCLC patients classified as having stable disease.

The development of targeted therapy for common epithelial cancers has brought hope of effective treatments with relatively modest toxicity, modeled after the success of imatinib in specific hematologic malignancies. However, our understanding of the different genetic lesions driving NSCLC is limited; while *EGFR* mutations appear to be an important predictor of gefitinib responsiveness, some tumors with these mutations do not respond. Even in *EGFR*-mutant tumors that respond to gefitinib, the acquisition of drug resistance in many cases limits the impact on OS. Thus, improved OS in EGFR-TKI trials will require accurate identification of responsive subsets as well as approaches to circumvent the development of drug resistance. By analogy with the initial discovery of gefitinib-responsive NSCLC subsets in the IDEAL studies, further clues as to the determinants of long-term responses in *EGFR*-mutant tumors, may be derived from patients who have remained free from recurrence for 2 to 3 years.

Editor's Note

Two related articles on this subject were published in the September 1, 2005, issue titled, TRIBUTE: A Phase III Trial of Erlotinib Hydrochloride (OSI-774) Combined With Carboplatin and Paclitaxel Chemotherapy in Advanced Non–Small-Cell Lung Cancer; by Roy S. Herbst, Diane Prager, Robert Hermann, Lou Fehrenbacher, Bruce E. Johnson, Alan Sandler, Mark G. Kris, Hai T. Tran, Pam Klein, Xin Li, David Ramies, David H. Johnson, and Vincent A. Miller (J Clin Oncol 23: 5892-5899); and titled, Mutations in the Epidermal Growth Factor Receptor and in KRAS Are Predictive and Prognostic Indicators in Patients With Non–Small-Cell Lung Cancer Treated With Chemotherapy Alone and in Combination With Erlotinib; by David A. Eberhard, Bruce E. Johnson, Lukas C. Amler, Audrey D. Goddard, Sherry L. Heldens, Roy S. Herbst, William L. Ince, Pasi A. Jänne, Thomas Januario, David H. Johnson, Pam Klein, Vincent A. Miler, Michael A. Ostland, David A. Ramies, Dragan Sebisanovic, Jeremy A. Stinson, Yu R. Zhang, Somasekar Seshagiri, and Kenneth J. Hillan (J Clin Oncol 23:5900-5909).

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Supplementary Tables

Supplementary Table 1. Survival Analysis of *EGFR* Mutations in INTACT 1 and 2

	Median Survival (months)		No. of Patients		No. of Events			
	C	C+I	C	C+I	C	C+I	HR*	95% CI
Mutation +	19.4	14.6	9	23	3	12	1.77	0.50 to 6.23
Mutation −	9.2	9.3	83	197	61	130	0.91	0.67 to 1.23

Abbreviations: HR, hazard ratio; C, chemotherapy alone; C+I, chemotherapy + gefitinib.
*HR < 1.0 implies a lower risk on gefitinib.

Supplementary Table 2. Progression-Free Survival Analysis of *EGFR* Mutations in INTACT 1 and 2

	Median Progression-Free Survival (months)		No. of Patients		No. of Events			
	C	C+I	C	C+I	C	C+I	HR*	95% CI
Mutation +	6.7	NR	9	23	6	8	0.55	0.19 to 1.60
Mutation −	4.5	5.5	83	197	56	106	0.73	0.53 to 1.01

Abbreviations: HR, hazard ratio; C, chemotherapy alone; C+I, chemotherapy + gefitinib; NR, median not reached at time of database cutoff.
*HR < 1.0 implies a lower risk on gefitinib.

Supplementary Table 3. Survival Analysis of *EGFR* Amplification in INTACT 1 and 2

	Median Survival (months)		No. of Patients		No. of Events			
	C	C+I	C	C+I	C	C+I	HR*	95% CI
Amplification +	NR	11.5	10	23	4	15	2.03	0.67 to 6.13
Amplification −	10.2	8.8	136	284	96	193	1.01	0.79 to 1.29

Abbreviations: HR, hazard ratio; C, chemotherapy alone; C+I, chemotherapy + gefitinib; NR, median not reached at time of database cutoff.
*HR < 1.0 implies a lower risk on gefitinib.

Supplementary Table 4. Progression-Free Survival Analysis of *EGFR* Amplification in INTACT 1 and 2

	Median Progression-Free Survival (months)		No. of Patients		No. of Events			
	C	C+I	C	C+I	C	C+I	HR*	95% CI
Amplification +	7.3	6.9	10	23	8	9	0.83	0.32 to 2.18
Amplification −	4.6	4.8	136	284	95	163	0.77	0.60 to 1.00

Abbreviations: HR, hazard ratio; C, chemotherapy alone; C+I, chemotherapy + gefitinib.
*HR < 1.0 implies a lower risk on gefitinib.

Authors' Disclosures of Potential Conflicts of Interest

Although all authors completed the disclosure declaration, the following authors or their immediate family members indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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Renee Bailey Iacona	AstraZeneca (N/R)			AstraZeneca (A)				
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CORRESPONDENCE

Erlotinib in Lung Cancer

TO THE EDITOR: Shepherd and colleagues (July 14 issue)[1] report that erlotinib prolongs survival in non–small-cell lung cancer, as compared with placebo, after the failure of first-line or second-line chemotherapy. One disturbing aspect of this trial is that some patients underwent only one prior chemotherapy regimen before randomization. These same authors previously reported that docetaxel is superior to best supportive care after first-line chemotherapy.[2] Subsequent studies have confirmed the efficacy of docetaxel and shown that pemetrexed achieves similar results.[3] Did Shepherd and colleagues think that random assignment to placebo after the failure of first-line chemotherapy was ethically justifiable? The only patients for whom one could justify the assignment to placebo were those with a performance status of 3, who made up only 8.6 percent of all patients. Contrary to the authors' claim that inclusion of a placebo group was ethical, we believe that some patients were denied a therapeutic option known to be effective. Furthermore, the overall survival in the erlotinib group was inferior to that in published results with docetaxel and pemetrexed, suggesting that erlotinib should be used as third-line chemotherapy.

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Dr. Nabhan reports being an investigator in a study that is sponsored by Sanofi-Aventis.

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TO THE EDITOR: Shepherd et al. and Tsao et al.[1] (July 14 issue) report an important study (BR.21) showing a survival benefit of erlotinib, but the results of the molecular analysis confused us. Recent East Asian studies[2-4] have strongly suggested that the mutational status of the epidermal growth factor receptor (EGFR) is the major determinant of tumor response and survival in patients with non–small-cell lung cancer who are treated with gefitinib, another EGFR tyrosine kinase inhibitor. Response rates among patients with an EGFR mutation were consistently higher than 80 percent in those studies. However, in the BR.21 study, the response rate among such patients was only 16 percent, and mutational status had no significant effect on survival, although the EGFR copy number correlated with responsiveness and survival. In our study,[2] the EGFR copy number was associated with gefitinib sensitivity, but we consider it to be a surrogate marker for EGFR mutations, rather than a true determinant.

THIS WEEK'S LETTERS

These discrepancies may be due to differences in the ethnic background of the populations, the drugs, the study design, and, most important, the accuracy of the molecular analyses. To avoid fruitless controversy, standard methods for analyzing EGFR mutations and copy number should be established.

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TO THE EDITOR: Tsao and colleagues suggest that EGFR mutations were not valuable in predicting a benefit of erlotinib in the BR.21 trial. We believe that the mutation data in their report are inconclusive, for several reasons.

First, in Europe and North America,[1,2] the frequency of mutations is approximately 10 percent; Tsao et al. report mutations in more than 20 percent of the tumors. Second, only 47 percent of the mutations reported were drug-sensitive exon 19 deletions and L858R substitutions; these make up approximately 90 percent of the EGFR mutations in aggregate in the published data.[3] Third, the remaining cases showed "novel variant" mutations whose somatic nature was not established and that were not adequately confirmed. Fourth, these novel mutations were predominantly nucleotide transitions (92 percent), suggesting they were artifacts generated in the polymerase chain reaction (PCR).[4]

Finally, of the 427 patients treated with erlotinib, only 19 who had EGFR mutations could be evaluated. Among these 19 patients, only 8 had tumors with the well-established, drug-sensitive EGFR mutations. At our institution, 33 patients who had tumors containing one of these two common mutations have received erlotinib or gefitinib, and of these, 32 patients (97 percent) have had a response according to the Response Evaluation Criteria in Solid Tumors; the aggregate published response rate for both drugs and mutations is nearly 80 percent.

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DRS. SHEPHERD AND SEYMOUR REPLY: Patients entering the BR.21 trial after first-line chemotherapy were considered by their doctors not to be suitable candidates for second-line chemotherapy. Physicians had to attest to this, and reasons were recorded and monitored. Thus, these patients could not be compared with patients who participated in the trials cited by Drs. Nabhan and Bitran. We think, therefore, as did ethics review boards and regulatory authorities, that the inclusion of a placebo-control group was ethical, since further chemotherapy was not an option and alternative systemic treatments were unavailable.

It is inappropriate to compare the results of the BR.21, TAX 317,[1] and JMEI[2] trials, since their patient populations differed considerably. One third of the patients in the BR.21 study had a performance status of between 2 and 3 or 3, as compared with 25 percent of those in the TAX 317 trial and 12 percent of those in the JMEI study. Survival shortens with each successive chemotherapy regimen. In JMEI and TAX 317, 100 percent and 75 percent of patients, respectively, had undergone only one regimen, as compared with 50 percent of the patients in the BR.21 trial. These imbalances in prognostic factors alone could result in shorter survival, independent of treatment.

With regard to patients who were not eligible

for second-line chemotherapy, we think that EGFR inhibitor therapy is ethical on the basis of the BR.21 trial. Whether it should be considered electively for patients who are otherwise suitable candidates for chemotherapy awaits the results of an ongoing study comparing docetaxel with gefitinib.

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1. Shepherd FA, Dancey J, Ramlau R, et al. Prospective randomized trial of docetaxel versus best supportive care in patients with non-small-cell lung cancer previously treated with platinum-based chemotherapy. J Clin Oncol 2000;18:2095-103.
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DR. TSAO AND COLLEAGUES REPLY: Of 177 tumor samples analyzed in the BR.21 trial, 21 samples (from 20 patients) were exon 19 deletions or L858R substitutions. This rate per patient of 11 percent for classic mutations is similar to that in other reports involving non-Asian patients.[1,2] The response rate among patients who could be evaluated who had classic mutations was 25 percent (two of eight). Although the rate is lower than that among Asian patients, it probably falls within the confidence interval of other series involving non-Asian patients who did not have adenocarcinoma.

The patients in the BR.21 trial who had classic mutations did not derive a greater survival benefit from erlotinib (hazard ratio for death, 0.67) than those with novel mutations (hazard ratio, 0.65) or those with wild-type EGFR (hazard ratio, 0.73). In the Tarceva Responses in Conjunction with Taxol and Carboplatin (TRIBUTE) trial,[3] 29 of 274 (11 percent) of the samples contained mutations (86 percent were classic mutations). Patients who had mutations had longer progression-free survival ($P<0.001$) and overall survival ($P<0.001$) than those who did not have mutations, regardless of the type of treatment (chemotherapy with or without erlotinib); the benefit of erlotinib was statistically nonsignificant. Among patients in the placebo group, those with classic mutations had a longer median survival than those with wild-type or novel EGFR variants (9.1, 3.5, and 3.5 months, respectively). This suggests that classic EGFR mutations have a prognostic influence that is independent of treatment and that the superior survival reported for mutation-positive patients in uncontrolled studies may not have been due to heightened sensitivity to the EGFR inhibitor.

Dr. Pao and colleagues suggested that novel variants are PCR artifacts caused by formalin fixation. The probability of the appearance of PCR artifacts correlates inversely with the number of cells used for the PCR.[1] However, we found novel mutations more frequently in large biopsy or resection specimens (61 percent) than in small biopsy specimens (41 percent). Chou et al.[4] also identified several new mutations (V689M, N700D, S720P, V765A, T783A, and G863D) in formalin-fixed tumors from patients who had a response, and the one patient in our series who had a complete response had a transition mutation (V742A[T→C]).

The role of mutations in patients with lung cancer receiving EGFR inhibitors is still evolving. We elected to publish all our mutation results and encourage others to do so as well. Only in this way will sufficient numbers accrue for all mutations to permit clinical correlation. We agree with Takano and Ohe that standard methods for EGFR-mutation analysis and copy number should be established. It is premature to say that EGFR-inhibitor therapy should not be prescribed for patients who do not have EGFR mutations.

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Establishment of a human non-small cell lung cancer cell line resistant to gefitinib

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The epidermal growth factor receptor (EGFR) tyrosine-kinase inhibitor gefitinib (Iressa™, ZD1839) has shown promising activity preclinically and clinically. Because comparative investigations of drug-resistant sublines with their parental cells are useful approaches to identifying the mechanism of gefitinib resistance and select factors that determine sensitivity to gefitinib, we established a human non-small cell lung carcinoma subline (PC-9/ZD) that is resistant to gefitinib. PC-9/ZD cells are ~180-fold more resistant to gefitinib than their parental PC-9 cells and PC-9/ZD cells do not exhibit cross-resistance to conventional anticancer agents or other tyrosine kinase inhibitors, except AG-1478, a specific inhibitor of EGFR. PC-9/ZD cells also display significant resistance to gefitinib in a tumor-bearing animal model. To elucidate the mechanism of resistance, we characterized PC-9/ZD cells. The basal level of EGFR in PC-9 and PC-9/ZD cells was comparable. A deletion mutation was identified within the kinase domain of *EGFR* in both PC-9 and PC-9/ZD, but no difference in the sequence of *EGFR* cDNA was detected in either cell line. Increased EGFR/HER2 (and EGFR/HER3) heterodimer formations were demonstrated in PC-9/ZD cells by chemical cross-linking and immunoprecipitation analysis in cells unexposed to gefitinib. Exposure to gefitinib increased heterodimer formation in PC-9 cells, but not in PC-9/ZD cells. Gefitinib inhibits EGFR autophosphorylation in a dose-dependent manner in PC-9 cells but not in PC-9/ZD cells. A marked difference in inhibition of site-specific phosphorylation of EGFR was observed at Tyr1068 compared to other tyrosine residues (Tyr845, 992 and 1045). To elucidate the downstream signaling in the PC9/ZD cellular machinery, complex formation between EGFR and its adaptor proteins GRB2, SOS, and Shc was examined. A marked reduction in the GRB2-EGFR complex and absence of SOS-EGFR were observed in PC-9/ZD cells, even though the protein levels of GRB2 and SOS in PC-9 and PC-9/ZD cells were comparable. Expression of phosphorylated AKT was increased in PC-9 cells and inhibited by 0.02 μM gefitinib. But the inhibition was not significant in PC-9/ZD cells. These results suggest that alterations of adaptor-protein-mediated signal transduction from EGFR to AKT is a possible mechanism of the resistance to gefitinib in PC-9/ZD cells. These phenotypes including EGFR–SOS complex and heterodimer formation of HER family members are potential biomarkers for predicting resistance to gefitinib.
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Key words: resistance; gefitinib; EGFR; Grb2; SOS; non-small cell lung cancer

Chemotherapy has played a central role in the treatment of patients with inoperable NSCLC for over 30 years, although its efficacy seems to be of very limited value.[1,2] Human solid tumors, including lung cancer, glioblastoma, breast cancer, prostate cancer, gastric cancer, ovarian cancer, cervical cancer and head and neck cancer, express epidermal growth factor receptor (EGFR) frequently, and elevated EGFR levels are related to disease progression, survival, stage and response to therapy.[2–10] The therapies directed at blocking EGFR function are attractive.

Interest in target-based therapy has been growing ever since the clinical efficacy of STI-571 was first demonstrated,[11–13] and small molecules and monoclonal antibodies that block activation of the EGFR and HER2 have been developed over the past few decades. The leading small-molecule EGFR tyrosine-kinase inhibitor, gefitinib (Iressa™, ZD1839), has shown excellent antitumor activity in a series of Phase I and II studies,[14,15] and Phase II international multicenter trials (Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL) 1 and 2) yield an overall RR of 11.8–18.4% and overall disease control rate of 42.2–54.4% (gefitinib 250 mg/day) in patients with advanced non-small cell lung cancer (NSCLC) who had undergo at least 2 previous treatments with chemotherapy. INTACT 1 and 2 ('Iressa' NSCLC Trials Assessing Combination Therapy) have demonstrated that gefitinib does not provide improvement in survival when added to standard first line platinum-based chemotherapy *vs.* chemotherapy alone in advanced NSCLC.[16,17] Two small retrospective studies reported recently that activating mutation of *EGFR* correlate with sensitivity and clinical response to gefitinib and erlotinib.[18–20] Although information of *EGFR* mutation may enable to identify the subgroup of patients with NSCLC who will respond to gefitinib and erlotinib, it would be expected that acquired resistance would develop in such patients after treatment. The problem of acquired resistance to gefitinib might be growing, but there has been no preclinical research about the mechanism of developing resistance to gefitinib. We established resistant subline using PC-9 that is highly sensitive to gefitinib.

Establishment of drug-resistant sublines and comparative investigations with their parental cells to identify their molecular, biological and biochemical properties are useful approaches to elucidating the mechanism of the drug's action. Our study describes the establishment of a gefitinib-resistant cell line and its characterization at the cellular and subcellular levels. The PC-9/ZD cell line is the first human NSCLC cell line resistant to gefitinib ever reported. PC-9 is a lung adenocarcinoma cell line that is highly sensitive to gefitinib at its IC_{50}-value of 0.039 μM, but the PC-9/ZD subline, which has a level of EGFR expression comparable to that of PC-9 cells, is specifically resistant to gefitinib. Thus, PC-9 and PC-9/ZD cells will provide useful information about the mechanism of developing resistance to gefitinib and molecules as surrogate markers for predicting chemosensitivity to gefitinib.

Material and methods

Drugs and cells

Gefitinib(*N*-(3-chloro-4-fluorophenyl)-7-methoxy-6-[3-(morpholin-4-yl)propoxy] quinazolin-4-amine) was supplied by AstraZeneca Pharmaceuticals (Cheshire, UK). AG-1478, AG-825, K252a, staurosporin, genistein, RG-14620 and Lavendustin A were purchased from Funakoshi Co. Ltd (Tokyo, Japan).

NSCLC cell line PC-9 (derived from a patient with adenocarcinoma untreated previously) was provided by Prof. Hayata of Tokyo Medical University (Tokyo, Japan).[21] PC-9 and PC-9/ZD cells were cultured in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% FBS (GIBCO-BRL, Grand Island, NY), penicillin and streptomycin (100 U/ml and 100 μg/ml, respectively; GIBCO-BRL) in a humidified atmosphere of 5%

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CO_2 at 37°C. Gefitinib-resistant PC-9/ZD cells were selected from a subculture that had acquired resistance to gefitinib using the following procedure. Cultured PC-9 cells were exposed to 2.5 μg/ml *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) for 24 hr and then washed and cultured in medium containing 0.2 μM gefitinib for 7 days. After exposure to gefitinib, they were washed and cultured in drug-free medium for 14 days. When variable cells had increased, they were seeded in medium containing 0.3–0.5 μM of gefitinib on 96-well cultured plates for subcloning. After 21–28 days, the colonies were harvested and a single clone was obtained. The subcloned cells exhibited an 182-fold increase in resistance to the growth-inhibitory effect of gefitinib as determined by MTT assay, and the resistant phenotype has been stable for at least 6 months under drug-free conditions.

In vitro *growth-inhibition assay*

The growth-inhibitory effects of cisplatin, carboplatin, adriamycin, irinotecan, gemcitabine, vindesine, paclitaxel, genistein, K252a, staurosporin, AG-825, AG-1478, Tyrophostin 51, RG-14620, Lavendustin A and gefitinib in PC-9 and PC-9/ZD cells were examined by using a 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay.[22] A 180 μl volume of an exponentially growing cell suspension (6×10^3 cells/ml) was seeded into a 96-well microtiter plate, and 20 μl of various concentrations of each drug was added. After incubation for 72 hr at 37°C, 20 μl of MTT solution (5 mg/ml in PBS) was added to each well, and the plates were incubated for an additional 4 hr at 37°C. After centrifuging the plates at 200*g* for 5 min, the medium was aspirated from each well and 180 μl of DMSO was added to each well to dissolve the formazan. Optical density was measured at 562 and 630 nm with a Delta Soft ELISA analysis program interfaced with a Bio-Tek Microplate Reader (EL-340, Bio-Metallics, Princeton, NJ). Each experiment was carried out in 6 replicate wells for each drug concentration and carried out independently 3 or 4 times. The IC_{50}-value was defined as the concentration needed for a 50% reduction in the absorbance calculated based on the survival curves. Percent survival was calculated as: (mean absorbance of 6 replicate wells containing drugs − mean absorbance of six replicate background wells)/(mean absorbance of 6 replicate drug-free wells − mean absorbance of 6 replicate background wells) × 100.

In vivo *growth-inhibition assays*

Experiments were carried out in accordance with the United Kingdom Coordinating Committee on Cancer Research Guidelines for the welfare of animals in experimental neoplasia (2nd ed.). Female BALB/c nude mice, 6-weeks-old, were purchased from Japan Charles River Co. Ltd (Atsugi, Japan). All mice were maintained in our laboratory under specific-pathogen-free conditions. *In vivo* experiments were scheduled to evaluate the effect of oral administration of gefitinib on pre-existing tumors. Ten days before administration, 5×10^6 PC-9 or PC-9/ZD cells were injected subcutaneously (s.c.) into the back of the mice, and gefitinib (12.5, 25 or 50 mg/kg, p.o.) was administered to the mice on Days 1–21. Tumor diameter was measured with calipers on Days 1, 4, 8, 11, 14, 19 and 22 to evaluate the effect of treatment, and tumor volume was determined by using the following equation: tumor volume = $ab^2/2$ (mm^3) (where *a* is the longest diameter of the tumor and *b* is the shortest diameter). Day "x" denotes the day on which the effect of the drugs was estimated, and Day "1" denotes the first day of treatment. All mice were sacrificed on Day 22, after measuring their tumors. We considered absence of a tumor mass on Day 22 to indicate a cure. Differences in tumor sizes between the treatment groups and control group at Day 22 were analyzed by the unpaired *t*-test. A *p*-value of <0.05 was considered statistically significant.

cDNA expression array

The gene expression profile of PC-9/ZD was assessed with an Atlas Nylon cDNA Expression Array (BD Bioscience Clontech, Palo Alto, CA). Total RNA was extracted by a single-step guanidinium thiocyanate procedure (ISOGEN, Nippon Gene, Tokyo, Japan). An Atlas Pure Total RNA Labeling System was used to isolate RNA and label probes. The materials provided with the kit were used, and the manufacturer's instructions were followed for all steps. Briefly, streptavidin-coated magnetic beads and biotinylated oligo(dT) were used to isolate poly A RNA from 50 μg of total RNA and the RNA obtained was converted into ^{32}P-labeled first-strand cDNA with MMLV reverse transcriptase. The ^{32}P-labeled cDNA fraction was purified on NucleoSpin columns and was added to the membrane on which fragments of 777 genes were spotted. Hybridization was allowed to proceed overnight at 68°C. After washing, the radiolabeled spots were visualized and quantified by BAS-2000II and Array Gauge 1.1 (Fuji Film Co., Ltd., Tokyo, Japan). The data were adjusted for the total density level of each membrane.

Quantitative real-time RT-PCR analysis

Total RNAs extracted from PC-9 cells and PC-9/ZD cells (1×10^6 cells each) were incubated with DNase I (Invitrogen, Carlsbad, CA) for 30 min. First-strand cDNA synthesis was carried out on 1 μg of RNA in 10 μl of a reaction mixture with 50 pmol of Random hexamers and 50 U of M-MLV RTase. Oligonucleotide primers for human *EGFR* were obtained from Takara (HA003051, Takara Bio Co., Tokyo, Japan). For PCR calibration, we generated a calibrator dilution series for *EGFR* cDNA in pUSEamp vector (Upstate, Charlottesville, VA) ranging from 10^8–10^2 copies/1 μl. A total of 2 μl of reverse transcriptase products was used for PCR amplification using Smart Cycler system (Takara) according to manufacturer's instructions. Absolute copy numbers were calculated back to the initial cell numbers, which were set into the RNA extraction. As a result we obtained copies/cell:ratio representing the average *EGFR* RNA amount per cell.

Immunoprecipitation and immunoblotting

The cultured cells were washed twice with ice-cold PBS, and lysed in EBC buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 mM Na orthovanadate, and 10 mg/ml each of leupeptin, aprotinin, pepstatin A and phenylmethylsulphonyl fluoride). The lysate was cleared by centrifugation at 15,000 r.p.m. for 10 min, and the protein concentration of the supernatant was measured by BCA protein assay (Pierce, Rockford, IL). The membrane was probed with antibody against EGFR (1005; Santa Cruz, Santa Cruz, CA), HER2/neu (c-18; Santa Cruz), HER3 (c-17; Santa Cruz), HER4 (c-18; Santa Cruz), PI3K (4; BD), Grb2 (81; BD), SOS1/2 (D-21; Santa Cruz), Shc (30; BD, San Jose, CA), PTEN (9552; Cell Signaling, Beverly, MA), AKT (9272; Cell Signaling), phospho-EGFR specific for Tyr 845, Tyr 992, Tyr 1045, and Tyr 1068 (2231, 2235, 2237, 2234; Cell Signaling), phospho-AKT (Ser473) (9271; Cell Signaling), phospho-Erk (9106; Cell Signaling), and phospho-Tyr (PY-20; BD) as the first antibody, and then with by horseradish-peroxidase-conjugated secondary antibody. The bands were visualized by enhanced chemiluminescence (ECL Western Blotting Detection Kit, Amersham, Piscataway, NJ). For Immunoprecipitation, 5×10^6 cells were washed, lysed in EBC buffer, and centrifuged, and the supernatants obtained (1,500 μg) were incubated at 4°C with the anti-EGFR (1005), -HER2 (c-18), and -HER3 (c-17) Ab overnight. The immunocomplexes were absorbed onto protein A/G-Sepharose beads, washed 5 times with lysate buffer, denatured, and subjected to electrophoresis on a 7.5% polyacrylamide gel.

Analysis of the genes of the HER families by direct sequencing

Total RNAs were extracted from PC-9 and PC-9/ZD cells with ISOGEN (Nippon Gene) according to manufacturer's instructions. First-strand cDNA was synthesized from 2 μg of total RNA by using 400 U of SuperScript II (Invitrogen, Carlsbad, CA). After reverse transcription with oligo (dT) primer (Invitrogen) or random primer (Invitrogen), the first-strand cDNA was amplified by PCR by using specific primers for *EGFR*, *HER2* and *HER3*. The

reaction mixture (50 µl) contained 1.25 U AmpliTaq DNA polymerase (Applied Biosystem, Foster City, CA), and amplification was carried out by 30 cycles of denaturation (95°C, 30 sec), annealing (55–59°C, 30 sec), and extension (72°C, 30 sec) with a GeneAmp PCR System 9600 (Applied Biosystem). After amplification, 5 µl of the RT-PCR products was subjected to electrophoretic analysis on a 2% agarose gel with ethidium bromide. DNA sequencing of the PCR products was carried out by the dideoxy chain termination method using the ABI PRISM 310 Genetic Analyzer (Applied Biosystem).

Chemical cross-linking

Chemical cross-linking in intact cells was carried out as described previously.[23] In brief, after 6 hr exposure to 0.2 µM gefitinib, cells were washed with PBS and incubated for 25 min at 4°C in PBS containing 1.5 mM of the nonpermeable cross-linker bis (sulfosuccinimidyl) substrate (Pierce, Rockford, IL). The reaction was terminated by adding 250 mM glycine for 5 min while rocking. Cells were washed in EBC buffer and 20 µg of protein was resolved by 5–10% gradient SDS-PAGE, and then immunoblot analyzed for EGFR, HER2, HER3 and P-Tyr.

Results

Sensitivity of PC-9/ZD cells to cytotoxic agents and tyrosine kinase inhibitors

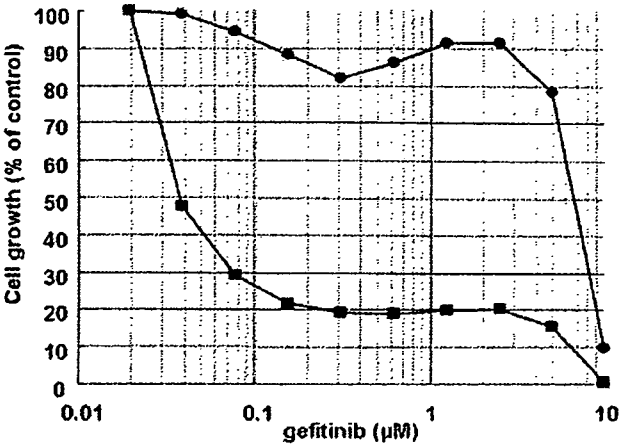
No significant difference between PC-9 and PC-9/ZD cells was observed in *in vitro* cell growth (doubling time of 20.3 hr and 21.4 hr, respectively) and microscopic morphology. Figure 1 shows the growth-inhibitory effect of gefitinib on the parent PC-9 cell line and its resistant subline, PC-9/ZD. The IC_{50}-value of gefitinib in PC-9 cells was 0.039 µM, as compared to 7.1 µM in PC-9/ZD cells (182-fold resistance). PC-9/ZD cells exhibited no cross-resistance to other conventional anticancer agents, including cisplatin, carboplatin, adriamycin, vindesine, paclitaxel and irinotecan. We also examined the growth-inhibitory effect of the EGFR tyrosine kinase inhibitors AG-1478, RG-14620 and Lavendustin A and other tyrosine kinase inhibitors in PC-9 and PC-9/ZD cells. PC-9/ZD cells show cross-resistance to AG1478, but not to all of the tyrosine kinase inhibitors (Tables I, II). It is likely that PC-9/ZD would also be resistant to EGFR-targeted quinazoline derivatives including gefitinib and erlotinib.[20]

PC-9/ZD cells show significant resistance to gefitinib in an in vivo *model*

To ascertain whether the resistance of PC-9/ZD occurs *in vivo*, we investigated the growth-inhibitory effect of gefitinib on PC-9 cells and PC-9/ZD cells in a xenotransplanted model. There was no significant difference in the size of the of PC-9 and PC-9/ZD cell tumor masses in nude mice before the start of gefitinib injection. Figure 2 shows the growth-inhibition curve of PC-9 (Fig. 2*a*) and PC-9/ZD (Fig. 2*b*) cells *in vivo* during the observation period. The PC-9 tumor masses decreased markedly in volume at all doses of gefitinib. In the 50 mg/kg/day p.o. group, the PC-9 masses were eradicated in all mice and did not regrow within the observation period. Growth of the PC-9/ZD masses, on the other hand, was inhibited by gefitinib administration in a dose-dependent manner, but significant tumor reduction was observed only in the 25 and 50 mg/kg/day groups, and the PC-9/ZD masses were not eradicated even in 50 mg/kg/day group. These results clearly demonstrate the significant *in vivo* resistance of PC-9/ZD cells to gefitinib.

Expression of HER family members and related molecules in PC-9 and PC-9/ZD cells

We examined the gene expression and protein levels of HER family members and related molecules by cDNA expression array (followed by confirmation using RT-PCR, data not shown) and immunoblotting. The ratios of the protein expression levels of PC-9 cells to PC-9/ZD cells almost paralleled the expression levels of their genes (Fig. 3*a*). The basal level of EGFR was comparable or slightly higher in PC-9/ZD cells (Fig. 3*a,b*), whereas the HER3 and AKT levels were lower in resistant cells.



	PC-9	PC-9/ZD
IC_{50} value (µM)	0.039 ± 0.002	7.1 ± 0.06
Doubling time (hr)	20.3	21.0

FIGURE 1 – Growth-inhibitory effect of gefitinib on PC-9 and PC-9/ZD cells determined by MTT assay. The cells were exposed to the concentrations of gefitinib indicated for 72 hr. The growth-inhibition curves of PC-9 (■) and PC-9/ZD (●) are shown. Doubling time was determined by MTT assay.

TABLE I – CHEMOSENSITIVITY TO OTHER ANTICANCER DRUGS

Drug	IC_{50} values (µM)[1]		RR[2] 1.6
	PC-9	PC-9/ZD	
Cisplatin	1.9 ± 0.7	3.1 ± 1.5	2.0
Carboplatin	25 ± 21	49 ± 23	1.3
Adriamycin	0.16 ± 0.13	0.20 ± 0.15	2.2
Irinotecan	15 ± 10	32 ± 11	1.5
Etoposide	4.5 ± 1.5	6.6 ± 1.3	1.5
Gemcitabine	18 ± 1.5	27 ± 1.5	0.7
Vindesine	0.0046 ± 0.0004	0.0032 ± 0.0009	1.2
Paclitaxel	0.0041 ± 0.0011	0.0048 ± 0.0004	1.6

[1]As assessed by MTT assay in PC-9 and PC-9/ZD cells. Values are the mean ± SD of >3 independent experiments.–[2]Relative resistance value (IC_{50} of resistant cells/IC_{50} of parental cells).

We carried out quantitative RT-PCR to measure the copy numbers of *EGFR*. Estimated transcript levels of *EGFR* were 786.3 and 712.1 copies/cell for PC-9 cells and PC-9/ZD cells, respectively (Fig. 3*d*). Relative ratio of *EGFR* expression levels in PC-9 cells and PC-9/ZD cells is 1.104. Microarray analysis using CodeLink Bioarray (Amersham Bio, Piscataway, NJ) confirmed equivalent gene expression of *EGFR* with ratio of 1.002 between PC-9 and PC-9/ZD cells (data not shown).

Expression of PI3K, Grb2, SOS, and Shc, the adaptor proteins of EGFR, and PTEN was almost the same in PC-9 and PC-9/ZD cells, and no change in the protein levels was observed after exposure to gefitinib (data not shown). The relative densitometric units of each protein are shown in Figure 3*c*. These results suggest that the difference in protein levels of EGFR, HER2, and related proteins can not explain the high resistance of PC-9/ZD cells to gefitinib.

Sequence of HER family member in PC-9/ZD cells

Several reports suggest that the resistance to receptor tyrosine kinase inhibitor STI-571 is partially due to mutations in the