Antitumor Activity of the Epidermal Growth Factor Receptor (EGFR) Tyrosine Kinase Inhibitor Gefitinib (ZD1839, Iressa) in Non–Small Cell Lung Cancer Cell Lines Correlates with Gene Copy Number and EGFR Mutations but not EGFR Protein Levels

Barbara A. Helfrich,[1] David Raben,[2] Marileila Varella-Garcia,[1] Dan Gustafson,[3] Daniel C. Chan,[1] Lynne Bemis,[1] Chris Coldren,[1] Anna Barón,[4] Chan Zeng,[4] Wilbur A. Franklin,[5] Fred R. Hirsch,[5] Adi Gazdar,[6] John Minna,[6] and Paul A. Bunn, Jr.[1]

Abstract

Purpose: Recognition that the epidermal growth factor receptor (EGFR) was a therapeutic target in non–small cell lung cancer (NSCLC) and other cancers led to development of the small-molecule receptor tyrosine kinase inhibitors gefitinib and erlotinib. Clinical trials established that EGFR tyrosine kinase inhibitors produced objective responses in a minority of NSCLC patients. We examined the sensitivity of 23 NSCLC lines with wild-type or mutated EGFR to gefitinib to determine genes/proteins related to sensitivity, including EGFR and HER2 cell surface expression, phosphorylated EGFR expression, EGFR gene copy number, and EGFR mutational status. Downstream cell cycle and signaling events were compared with growth-inhibitory effects.

Experimental Design: We determined gefitinib sensitivity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, EGFR expression by fluorescence-activated cell sorting and immunohistochemistry, phosphorylated EGFR by Western blotting, EGFR gene copy number by fluorescence *in situ* hybridization, and EGFR mutation by sequencing. The cellular effects of gefitinib on cell cycle were determined by flow cytometry and the molecular effects of gefitinib EGFR inhibition on downstream signal proteins by Western blotting. Gefitinib *in vivo* effects were evaluated in athymic nude mice bearing sensitive and resistant NSCLC xenografts.

Results: There was a significant correlation between EGFR gene copy number, EGFR gene mutations, and gefitinib sensitivity. EGFR protein was necessary but not sufficient for predicting sensitivity. Gefitinib-sensitive lines showed a G_1 cell cycle arrest and inactivation of downstream signaling proteins; resistant cell lines had no changes. The *in vivo* effects mirrored the *in vitro* effects.

Conclusions: This panel of NSCLC lines characterized for gefitinib response was used to identify predictive molecular markers of response to gefitinib. Several of these have subsequently been shown to identify NSCLC patients likely to benefit from gefitinib therapy.

Chemotherapeutic treatment of non–small cell lung cancer (NSCLC) produces objective responses in <30% of patients with advanced-stage disease. Median survival is 8 to 12.5 months (1–3). Future improvements in lung cancer treatments are likely to come from novel agents targeting molecular pathways that promote tumor cell growth and survival. The epidermal growth factor receptor (EGFR) and its associated signaling pathways have emerged as a leading target for NSCLC therapy. EGFR is overexpressed in ~80% of NSCLCs and, with other EGFR family members, plays a key role in signal transduction pathways regulating cell proliferation through the mitogen-activated protein kinase pathway and survival through the phosphatidylinositol 3-kinase/AKT pathway (4).

Gefitinib (ZD1839, Iressa) and erlotinib (OSI-774, Tarceva) are EGFR tyrosine kinase inhibitors (TKI) that received Food and Drug Administration approval as single-agent therapy for the treatment of NSCLC after chemotherapy. Objective responses were reported in 9% to 26% of advanced-stage NSCLC patients in trials (5–7). The majority of patients with objective response had symptom benefit, and overall, 40% to 43% of gefitinib- or erlotinib-treated patients had major symptom improvement (5–7). A randomized trial comparing erlotinib with placebo showed a significant survival advantage for erlotinib (hazard ratio, 0.73; $P = 0.01$), whereas a similar randomized trial failed to show a significant survival advantage

Authors' Affiliations: Tobacco Related Malignancy Program, University of Colorado Cancer Center and Departments of [1]Medicine, [2]Radiation Oncology, [3]Pharmacology, [4]Preventive Medicine and Biometrics, and [5]Pathology, University of Colorado Health Sciences Center, Denver, Colorado and [6]Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas, Texas

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Requests for reprints: Paul A. Bunn, Jr., Division of Medical Oncology, Department of Medicine, University of Colorado Cancer Center, Mail Stop 8117, P.O. Box 6511, Aurora, CO 80010. Phone: 303-724-3028; E-mail: Paul.Bunn@UCHSC.edu.

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for gefitinib (hazard ratio, 0.89; $P = 0.087$; refs. 8, 9). Survival benefit in the two trials was independent of gender or histology (8, 9). Clinical features associated with response included female gender, never-smoking history, and adenocarcinoma histology (10). Several biological markers have been investigated for selection of subjects most likely to benefit from EGFR-TKIs. EGFR protein expression has provided inconsistent results (11–13). EGFR mutations were found in 8% to 17% of primary NSCLC tumors from U.S./European patients with higher fractions in Asian patients (14–19). EGFR mutations are strongly associated with never-smoking status, adenocarcinoma histology, and Asian race (14–16, 18, 19). Response to gefitinib or erlotinib has consistently been higher in patients with EGFR mutations, but responses have been reported in 21% of NSCLC patients without EGFR mutations (14–16). The predictive nature of EGFR mutations for survival is more controversial with the randomized placebo-controlled trial and several single-arm trials showing no significant relationship but other single-arm trials showing a significant relationship (20, 21).

Acquired clinical resistance to gefitinib or erlotinib was shown in lung cancer patients whose tumors have somatic EGFR mutations in exon 20 (22). This mutation, which occurs at nucleotide position 2,369 resulting in a C→T transversion and the substitution of methionine for threonine at position 790 (T790M) in the kinase domain, is found infrequently in NSCLC patients not exposed to EGFR-TKIs (22–24). Therefore, failure of most tumors to respond is due to other factors. Primary resistance to gefitinib or erlotinib has been associated with mutations in *KRAS*, which are more common in heavy smokers (25, 26), and activating mutations in *EGFR* and *KRAS* seem to be mutually exclusive (19, 25).

Because the presumed target of EGFR-TKIs is known and because they benefit only a minority of patients with NSCLC, it is critically important to identify those patients most and least likely to benefit from EGFR-TKI therapy. In the present study, we examined the sensitivity of a panel of 23 NSCLC lines to the EGFR-TKI gefitinib and correlated the sensitivity to the cell surface expression of the EGFR and HER2 proteins by fluorescence-activated cell sorting (FACS) and/or immunohistochemistry, the EGFR gene copy number per cell by fluorescence *in situ* hybridization (FISH), and the presence of mutations in exons 18, 19, and 21 of the EGFR by gene sequencing. To understand the growth-inhibitory effects of gefitinib, we investigated the effects of gefitinib treatment on cell cycle distribution and on EGFR and downstream signaling proteins. Finally, we examined the antitumor effects of gefitinib treatment on athymic nude mice bearing NSCLC cell line xenografts whose sensitivity or resistance to gefitinib was shown *in vitro*.

Materials and Methods

NSCLC cell lines and culture conditions. Drs. John Minna and Adi Gazdar (University of Texas Southwestern Medical School, Dallas, TX) provided the adenocarcinoma lines H441, H820, H1435, H1648, H1650, H1975, H2122, H2279, HCC827, and HCC4006, the squamous lines H226 and H157, and the large cell line H460. The adenocarcinoma lines A549, Calu3, and H125 and the squamous carcinoma lines H520 and H1703 were obtained from the American Type Culture Collection (Rockville, MD). The squamous carcinoma line NE18 was obtained from Dr. Karen Kelly (University of Colorado Health Sciences Center, Denver, CO), and the adenocarcinoma line COLO699 was obtained from Dr. George Moore (Denver General Hospital, Denver, CO). The bronchioloalveolar carcinoma (BAC) line H322 was provided by Dr. Al Moustafa (National Research Council Canada, Biotechnology Research Institute, Montreal, Quebec, Canada), and the BAC line H358 was provided by Dr. Isaiah J. Fidler (University of Texas M. D. Anderson Cancer Center, Houston, TX). The adenocarcinoma line H3255 was obtained from Dr. Bruce Johnson (Dana-Farber Cancer Institute, Boston, MA). These lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT).

Chemicals. Gefitinib (Iressa) was provided by AstraZeneca, Inc. (Macclesfield, United Kingdom).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide growth assay. Inhibition of cell growth by gefitinib was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (27). Briefly, 1,000 to 2,000 viable cells were plated in 100 μL of growth medium in 96-well plates (Corning, Ithaca, NY) and incubated overnight at 37°C. Gefitinib, 0 to 20 μmol/L, was added and the plates were incubated for 6 days, after which the tetrazolium salt was added to a final concentration of 0.4 mg/mL to each well. The medium was aspirated after 4 hours, and the reduced MTT product was solubilized by adding 100 μL of 0.2 N HCl in 75% isopropanol and 23% Milli-Q water to each well. Thorough mixing was done using a Titertek multichannel pipetman. The absorbency of each well was measured using an automated plate reader (Molecular Devices, Sunnyvale, CA).

Identification of EGFR mutations in genomic DNA from cell lines. Six million cells were collected from cells grown in culture, and genomic DNA was extracted using the DNeasy Tissue kit from Qiagen (Valencia, CA). Methods were as described in the kit protocol with the exception of the initial proteinase K digestion, which was conducted at 55°C overnight. Following DNA purification, 50 ng DNA was amplified for EGFR exons 18, 19, and 21 by heminested PCR. The first round of PCR used a reverse outside primer and a forward primer. The second round of PCR used the same forward primer and added an inside reverse primer. Both rounds of the heminested PCR used 2× master mix (Promega, Madison, WI) and touchdown PCR conditions starting with denaturation at 94°C for 1 minute, annealing starting at 10°C above optimal and reducing the temperature 0.5°C at each cycle until 55°C and then an additional 30 cycles at 55°C for 1 minute, and extension at 72°C for 2 minutes. PCR products were separated on 1.5% agarose gel and visualized by UV transillumination of the gels stained with ethidium bromide. The PCR products were then gel purified using a gel purification kit (Qiagen) and submitted for sequence analysis by the University of Colorado Health Sciences Center sequencing core. Sequencing was conducted in both the forward and reverse direction with two investigators examining sequence data independently. Primer sets included exon 18 reverse outside TATACAGCTTGCAAGGACTCTGG, exon 18 forward GCTTTCCAGCATGGTGAGGGC, exon 18 reverse inside CCAGACCATGAGAGGCCCTG, exon 19 reverse outside AGGGTCTAGAGCAGAGCAGC, exon 19 forward CAGATCACTGGGCAGCATGT, exon 19 reverse inside GCCTGAGGTTCAGAGCCAT, exon 21 reverse outside CTGGTCCCTGGTGTCAGGAA, exon 21 forward CATGATGATCTGTCCCTCACAG, and exon 21 reverse inside GCTGGCTGACCTAAAGCCACC.

Fluorescence* in situ *hybridization. Cell cultures were harvested after treatment with colcemid (5 μg/mL) for 1 to 2 hours according to protocol previously published (4, 20). Briefly, the cells were resuspended in the hypotonic solution 0.075 mol/L KCl for 15 minutes at 37°C, fixed in 3:1 methanol/glacial acetic acid, and applied onto microscope slides. Following dehydration, cells were incubated for 5 minutes in pepsin (0.01% in 0.01 mol/L HCl) at 37°C and fixed in 1% formaldehyde at room temperature for 10 minutes. The EGFR/CEP probe (Vysis, Downers Grove, IL) was applied according to the manufacturer's instructions, and codenaturation of probe and target DNA was achieved by incubation at 80°C for 6 minutes. Hybridization

was allowed to occur at 37°C for 20 hours, and the unbound probe was washed out in three incubations in 50% formamide/2× SSC and one incubation in 2× SCC/0.1% NP40, each for 6 minutes at 46°C. Chromatin was counterstained with 4′,6-diamidino-2-phenylindole in Vectashield antifade (Vector, Burlingame, CA). At least 20 metaphases and 200 interphase cells were analyzed per cell line using an epifluorescence microscope coupled with a triple band pass interference filter (blue/red/green) and single band filters for blue, red, and green (Chroma Technology Corp., Rockingham, VT). Images were acquired using a cooled CCD camera (SenSys, Photometrics, Boston, MA) and merged using the SmartCapture software (Vysis).

Mean numbers per cell of EGFR and CEP 7 probes were estimated as well as the EGFR/CEP 7 ratio. According to the frequency of cells with specific number of copies of the EGFR gene and chromosome 7, the FISH pattern of the cell lines was classified as being disomy, low trisomy, high trisomy, low polysomy, high polysomy, and gene amplification (20).

Immunofluorescence staining and immunohistochemistry. EGFR expression by FACS was determined by incubation of 5×10^5 cells with cetuximab, a humanized monoclonal antibody with high binding affinity for the EGFR (ImClone, New York, NY), or an isotype-matched control (Sigma Chemical Co., St. Louis, MO). The cells were counterstained with goat anti-human IgG-FITC (Southern Biotechnology, Birmingham, AL). All staining was on ice for 45 minutes followed by three washes. Following staining, the samples were fixed with 1% formaldehyde and the cell fluorescence was measured by FACS (Coulter EPICS XL-MCL, Beckman Coulter, Hiahleah, FL). The percentage of EGFR-positive cells and their median fluorescence intensity (MFI) was determined using the Coulter software.

For immunohistochemical analysis, NSCLC cell lines were pelleted and paraffin fixed. Antigen retrieval was done at 95°C in citrate buffer for 40 minutes. The slides were cooled at room temperature for 20 minutes and washed thrice for 3 minutes with Tris buffer. The slides were peroxidase blocked for 5 minutes and washed as above. The slides were incubated for 30 minutes with the primary antibody, anti-EGFR clone 31G7 (Zymed, San Francisco, CA), followed by the secondary antibody (Visualization Reagent), followed by the substrate-chromogen solution (3,3′-diaminobenzidine), and finally counterstained with hematoxylin. Two pathologists (W.A.F. or F.R.H.) independently quantified membrane staining as 0 to 3+.

The methods and levels of HER2 expression in these NSCLC cell lines as assessed by immmunohistochemistry and flow cytometry were previously published (28).

Cell cycle distribution and apoptosis. Cell cycle distribution was determined by FACS analysis as described previously (28). After 24 hours of incubation with gefitinib (0.1, 1, or 10 μmol/L), cells were stained with saponin, propidium iodide, and RNaseA solution and analyzed by FACS using a Coulter EPICS, and ModFit software (Verity Software House, Topsham, MN) was used to calculate the cell cycle distributions. Apoptosis was evaluated by FACS using the Vybrant Apoptosis Assay kit 4 (Molecular Probes, Eugene, OR).

Western blotting. NSCLC cells were seeded to reach 80% confluency and treated with 1 μmol/L gefitinib for 24 hours. Western blot analysis was used to assess phosphorylation and total protein of EGFR, AKT, and extracellular signal-regulated kinase 1/2 (ERK1/2). Actin served as the loading control. Protein concentration was determined using the DC Microplate Protein Assay by Bio-Rad (Hercules, CA), and 50 μg protein was loaded for each sample. Two blots were set up from the same sample: one for phosphorylated proteins and one for total protein. Molecular weight markers (Bio-Rad) were loaded to ensure proteins of interest were at the appropriate size. Cellular lysates were separated on a 7.5% or 10% SDS-PAGE gel and transferred to polyvinylidene difluoride paper. Immunoblots were blocked in 3% albumin bovine fraction V (MP Biomedicals, Inc., Solon, OH) and probed with the following antibodies from Cell Signaling Technology (Beverly, MA): anti-AKT, anti-phosphorylated AKT (Ser^{473}), anti-EGFR antibody, anti-phosphorylated EGFR (Tyr^{1068}), anti-p44/p42 ERK1/2, and anti-phosphorylated p44/42 ERK1/2 (Thr^{202}/Tyr^{204}). The immunoblots were detected by enhanced chemiluminescence Western blotting kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Nude mouse xenograft tumor model. Athymic nude mice (4- to 6-week-old females) obtained from the National Cancer Institute (Bethesda, MD) were maintained in accordance with University of Colorado Health Sciences Center institutional guidelines. The sensitive NCI-H322 cell line and the resistant NCI-H157 cell line were selected because we had prior experience with nude mouse xenografts from these cell lines. Cultured cells were injected into the flanks of the nude mice ($\sim 2 \times 10^6$) at day 0. Animals were divided into two groups, control (vehicle) and gefitinib-treated mice (50 or 60 mg/kg i.p. daily 5/7 days for 3-4 weeks), starting 5 days after tumor heterotransplantation. Tumor volume measurements were evaluated by caliper measurement and calculated by the following formula: $\pi \times$ short diameter$^2 \times$ long diameter / 6.

Statistical methods. Wilcoxon rank sum tests were used to examine associations between continuous variables, such as gefitinib IC_{50}s, EGFR, or HER2 protein expression, and categorical variables, such as gender, smoking status, histology, EGFR mutation status, and FISH patterns. Spearman correlation coefficients were estimated for continuous variables, such as gefitinib IC_{50}, EGFR or HER2 protein expression, and EGFR gene copy number. Fisher's exact tests were used to examine the association between categorical variables, such as gender, smoking status, EGFR mutation status, FISH pattern, and histology. The Kruskal-Wallis test was used to determine changes in the percentage cells arrested in the G_1 phase of the cell cycle following gefitinib treatment.

Results

Gefitinib mediated growth inhibition of human NSCLC cell in vitro. Table 1 summarizes gefitinib growth inhibition in 23 NSCLC lines by MTT assays. There was a wide range of sensitivities with IC_{50}s of 5 nmol/L to >12 μmol/L. Nine cell lines, Calu3, H322, H358, H1648, H1650, H2279, H3255, HCC827, and HCC4006, had IC_{50}s of ≤1 μmol/L. Intermediate sensitivity to gefitinib was observed in seven NSCLC lines with IC_{50}s of 3 to 6 μmol/L. Resistance to gefitinib, represented by IC_{50}s of ≥8 μmol/L, was observed in seven NSCLC lines.

Patient characteristics and response to gefitinib. The gefitinib sensitivity of these 23 lines along with the clinical characteristics of the NSCLC patients from whom the cell lines were derived is summarized in Table 1. There was a significant association between gefitinib IC_{50}s and histology. Adenocarcinomas (including BAC subtypes) were more sensitive (median IC_{50}, 2.0 μmol/L; range, 0.005-12.9) than nonadenocarcinomas (median IC_{50}, 10 μmol/L; range 5-13.6; $P = 0.015$). All the sensitive cell lines (IC_{50}, <1 μmol/L) were of adenocarcinoma (or BAC) histology. There was no significant association between gender and gefitinib IC_{50}s, although male values were somewhat higher (females median IC_{50}, 3.0 μmol/L, range, 0.01-8.0 versus males median IC_{50}, 6.0 μmol/L, range, 0.18-13.6; $P = 0.078$). There was also no significant association between histology and gender, although adenocarcinomas were more frequent in females (7/7 = 100% versus 9/15 = 60% in males; $P = 0.13$). No significant association was found between smoking and gefitinib IC_{50}s (never smokers median IC_{50}, 8 μmol/L, range, 0.015-13.6 versus smokers median IC_{50}, 2.0 μmol/L, range, 0.25-8; $P = 0.29$). Similarly, smoking status was not significantly associated with gender ($P = 0.60$) or histology ($P = 1.0$).

EGFR mutation status. EGFR mutations were present in 7 of the 23 NSCLC cell lines: 5 lines harbored exon 19 deletions,

Table 1. NSCLC lines: gefitinib sensitivity, patient characteristics, EGFR mutation status, EGFR gene copy number, and EGFR and HER2 cell surface expression

Cell line	MTT	Patient characteristics			EGFR gene copy number (dual-color FISH analysis)			EGFR expression (flow cytometry)			HER2 expression (flow cytometry)		
	IC_{50} (μmol/L)	Gender	PY	EGFR status	Mean CEP 7	Mean EGFR	EGFR gene pattern	%EGFR+	MFI	IHC	%HER2+	MFI	IHC
BAC													
H358	0.18	M	0	WT	3	3	HP	97	4	ND	98	5.2	ND
H322	0.25	M	60	WT	6.92	3.64	LP	100	16	3+	96	5.5	2+
Adenocarcinoma													
HCC827	0.005	F	Unknown	exon 19	16.6	>25.3	GA	81	15	ND	29	3.9	ND
H3255	0.015	F	0	L858R	12.96	>44.08	GA	90	7.3	ND	47	2.7	ND
HCC4006	0.02	F	0	exon 19	>9.94	>12.39	GA	94	6.8	ND	ND	ND	ND
H2279	0.03	Unknown	Unknown	exon 19	9.12	15.98	GA	97	9.8	ND	25	2	ND
Calu3	0.3	M	Unknown	WT	4.01	4.61	HP	98	8.9	3+	100	37	3+
H1648	0.38	M	40	WT	3.33	3.33	LP	89	11.7	ND	100	7.8	ND
H1650	1	M	10	exon 19	>3.32	>8.85	HP	85	12.8	ND	89	4.5	ND
H125	3	M	50	WT	3.4	3.36	HT	100	13	3+	91	2.8	1+
H1435	3	F	0	WT	5.2	3.8	HP	98	14	3+	83	3.2	1+
H820	3	M	0	exon 19	5.35	8.37	HP	25	4	ND	32	3.3	ND
COLO699	4.2	F	Unknown	WT	2.94	2.93	HT	0	0	0+	0	0	0+
H2122	5.9	F	30	WT	3	3.07	HT	94	5.1	2+	73	4	1+
H441	6	M	0	WT	4.3	4.3	HP	94	6	ND	84	4	ND
H1975	8	F	0	L858R, T790M	6.20	6.24	HP	96	7.7	ND	92	3.4	ND
A549	9.6	M	Unknown	WT	2.5	2.5	LT	99	14	3+	78	2.4	0+
Squamous													
NE18	5	M	Unknown	WT	3.18	3.19	HT	100	16	3+	94	3.3	0+
H1703	8	M	50	WT	3.06	4.02	HP	99	15	3+	65	2.2	1+
H226	10	M	0	WT	2.01	2.61	HT	100	49	3+	96	2.4	2+
H157	12.8	M	0	WT	3.1	3.1	HT	93	13	3+	62	1.8	1+
H520	13.6	M	0	WT	2.82	2.65	HT	0	0	0+	0	0	0+
Large cell													
H460	12.9	M	0	WT	1.03	1.04	M	96	12	2+	32	1.9	2+

Abbreviations: PY, pack-years; CEP, chromosome 7; HP, high polysomy; LP, low polysomy; GA, gene amplification; HT, high trisomy; LT, low trisomy; M, monosomy; IHC, immunohistochemistry; ND, not done.

1 harbored the exon 21 point mutations L858R, and H1975 harbored both the L858R mutation associated with sensitivity and the gefitinib resistance mutation T790M (Table 1). The median IC_{50} for the seven EGFR mutated lines was lower (median, 0.03 μmol/L; range, 0.005-8) than that for the 16 wild-type (WT) EGFR lines (median, 5.4 μmol/L; range, 0.18-13.6; Fig. 1A) but not significantly different ($P = 0.057$). Five of the 7 (71%) mutated cell lines had an IC_{50} of ≤1 μmol/L, whereas 4 of 16 (25%) cell lines with WT receptor had an IC_{50} of ≤1 μmol/L. The seven cell lines with EGFR mutations were all of adenocarcinoma histology (Table 1). With respect to mutation status and gender, a marginally higher proportion of cell lines from females had mutations compared with males (4/7 = 57% in females versus 2/15 = 13% in males; $P = 0.054$). Four of the seven mutated cell lines came from never smokers, whereas one came from a smoker with only 10 pack-years, and the smoking status of two was unknown.

FISH analysis of NSCLC cell lines for EGFR gene amplification. Results of dual-color FISH assays to assess EGFR genomic imbalances are shown in Table 1 and Figs. 1B and C and 2C and D. There was a significant correlation between EGFR gene copy number and gefitinib sensitivity ($r = -0.64$; $P = 0.001$; Fig. 1B). Gene amplification was detected in four cell lines (all EGFR mutant), and high polysomy was present in eight cell lines, two of which were EGFR mutant. The correlation between gene copy number and gefitinib sensitivity persisted when evaluated in the WT EGFR lines alone ($r = -0.52$; $P = 0.037$). There was also a significant correlation between gefitinib sensitivity (IC_{50}) and FISH positivity defined as gene amplification/high polysomy (FISH-positive cell lines median IC_{50}, 0.65 μmol/L, range, 0.005-8 versus FISH-negative cell lines median IC_{50}, 5.9 μmol/L, range, 0.25-13.6; $P = 0.03$) as shown in Fig. 1C.

High polysomy and gene amplification was more frequent in adenocarcinoma/BAC cell lines (11 of 17), but the association did not reach statistical significance ($P = 0.16$). With respect to FISH and gender, 5 of the 7 cell lines from females and 6 of 15 cell lines from males were FISH positive ($P = 0.36$). About FISH and smoking status, never smokers (7 of 11) were more often FISH positive compared with smokers (2 of 6); however, this was not significant ($P = 0.33$).

EGFR, phosphorylated EGFR, and HER2 cell surface protein expression levels in NSCLC cell lines. Table 1 and Figs. 1D and E and 2A and B show the levels of EGFR and HER2 protein expression detected by flow cytometry and immunohistochemistry in the panel of NSCLC cell lines. For EGFR expression, 21 of the 23 cell lines had >80% of cells expressing EGFR and each of these had MFIs of ≥4.

Immunohistochemical results were similar with 2+ or 3+ EGFR staining in all cell lines expressing ≥80% positive cells and no expression in two of two cell lines with no EGFR-expressing cells by FACS. There was no significant correlation

between EGFR expression and gefitinib IC_{50} (FACS: $r = -0.02$; $P = 0.93$; immunohistochemistry: $r = -0.39$; $P = 0.19$). However, all 11 cell lines with an IC_{50} of ≤3 μmol/L were EGFR positive (>80% of cells EGFR positive) and all 3 lines with <50% positive EGFR cells had an IC_{50} of ≥3 μmol/L. There was an excellent correlation between levels of EGFR expression detected by immunohistochemistry and FACS ($r = 0.78$; $P = 0.0016$).

A comparison of histology with EGFR protein expression showed high expression (immunohistochemical score 3+) in 80% of squamous cell carcinoma lines and 71% of adenocarcinoma plus BAC lines ($P = 1.0$). By FACS analysis, both females and males had high EGFR protein expression (mean MFI, 8 ± 5 in females versus MFI, 13 ± 11 in males; $P = 0.31$). There was also no difference in EGFR protein expression between never smokers and smokers (mean MFI, 11 ± 13 versus MFI, 12 ± 4, respectively; $P = 0.19$). By FACS analysis, there was also no significant difference in the EGFR MFI between cell lines with mutated (mean, 9.1 ± 3.8) versus WT receptor (mean, 12.4 ± 11; $P = 0.44$). Cell lines with FISH high EGFR gene copy number had higher levels of EGFR protein by immunohistochemistry, but the results were not significant ($r = 0.51$; $P = 0.074$). Phosphorylated EGFR expression determined by Western blotting was examined using an anti-Tyr^{1068} antibody. The highest levels were observed in the mutant cell line H3255; high levels were also observed in the sensitive WT cell lines H322 and Calu3, whereas no phosphorylated EGFR was observed in the resistant lines H157 and H520 (Fig. 3).

HER2 positivity in >50% of cells was present in 15 of 23 cell lines by FACS analysis (MFI, 1.8-37). By immunohistochemistry, 2+ or 3+ staining was present in 4 of 13 cell lines tested, 1+ staining was present in 5 cell lines, and 4 lines had no staining. There was a significant correlation between gefitinib sensitivity (IC_{50}) and HER2 staining by FACS ($r = -0.53$; $P = 0.01$) but not by immunohistochemistry ($r = -0.26$; $P = 0.4$). There was also no significant correlation between EGFR staining and HER2 staining by FACS ($r = 0.039$; $P = 0.86$) or by immunohistochemistry ($r = 0.32$; $P = 0.28$). There was no significant correlation between HER2 expression by FACS and immunohistochemical staining ($r = 0.47$; $P = 0.11$), although all HER2-negative cell lines by immunohistochemistry had IC_{50} of ≥4 μmol/L.

By histology, there was a significant difference in the mean HER2 MFI between adenocarcinomas/BAC (MFI, 5.7 ± 8.5) and squamous carcinomas (MFI, 1.9 ± 1.2; $P = 0.037$). A higher number of adenocarcinoma and BAC-derived lines (28%) than squamous cell carcinoma lines (20%) expressed HER2 at the 2 to 3+ level of immunohistochemical staining, but this was not significant ($P = 1.0$).

HER2 protein expression by FACS analysis was not significantly different between females (mean MFI, 2.9 ± 1.4) and males (mean MFI, 5.6 ± 8.6; $P = 0.79$). HER2 protein expression was not significantly higher in never smokers (mean MFI, 2.8 ± 1.4) than in smokers (mean MFI, 4.5 ± 2.0; $P = 0.1$). HER2 protein expression between mutant EGFR lines (mean MFI, 3.3 ± 0.9) and WT EGFR lines (mean MFI, 5.2 ± 8.7) was also not significantly different ($P = 0.77$).

Effects of gefitinib on G_1 cell cycle arrest and apoptosis assessed by flow cytometry. In the sensitive EGFR mutant line H3255, 24 hours of exposure to 0.1 and 1 μmol/L gefitinib resulted in a

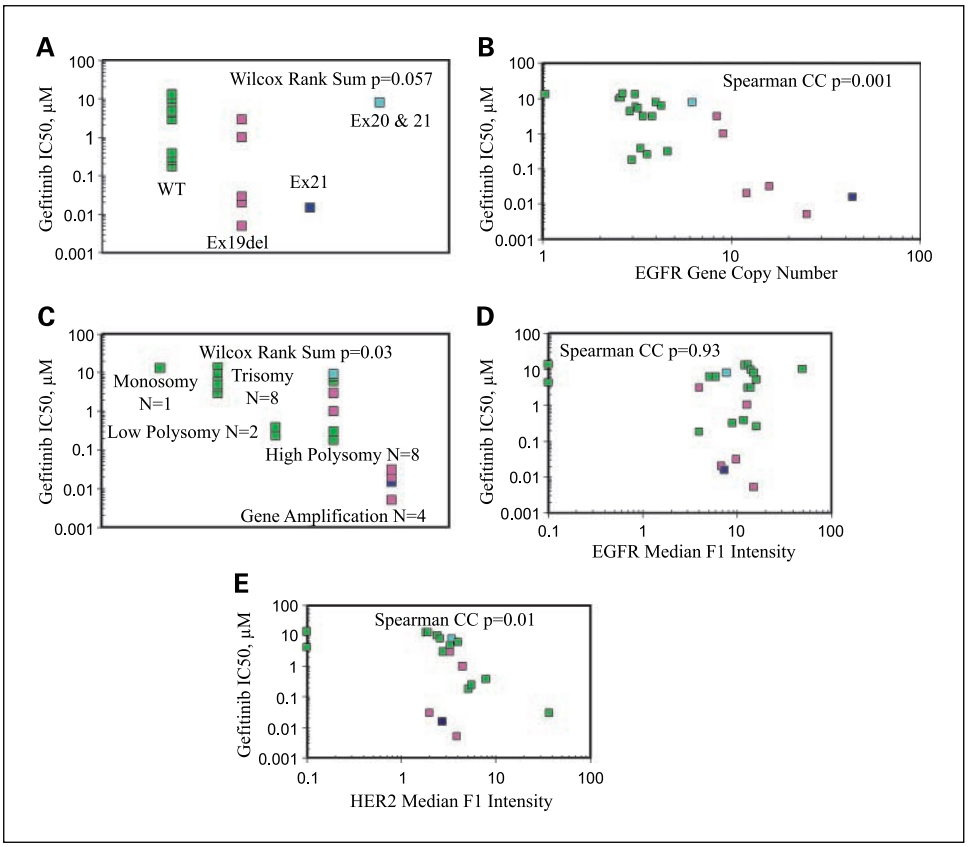

Fig. 1. Comparison of gefitinib IC_{50}s with EGFR mutational status, EGFR gene copy number, and EGFR and HER2 MFI in NSCLC cell lines. Green squares, EGFR WT cell lines; pink squares, cell lines with EGFR exon 19 deletions; dark blue squares, cell lines with EGFR exon 21 point mutations; light blue squares, cell lines with the exon 21 point mutations and exon 20 mutations. *A,* IC_{50}s in the EGFR mutant and WT cell lines were ranked and compared using the Wilcoxon rank sum test ($P = 0.057$). *B, D,* and *E,* Spearman correlation coefficient was used to determine the association between continuous variables i.e., gefitinib IC_{50}s versus EGFR gene copy number, EGFR protein expression, and HER2 protein expression. *B,* there was a significant association between gefitinib IC_{50}s and EGFR gene copy number ($P = 0.001$). *C,* there was a significant association between gene expression pattern and the IC_{50}s using the Wilcoxon rank sum test ($P = 0.03$). *D,* there was no association between gefitinib IC_{50}s and EGFR protein expression ($P = 0.93$). *E,* there was a significant association between gefitinib IC_{50}s and HER2 expression ($P = 0.01$).

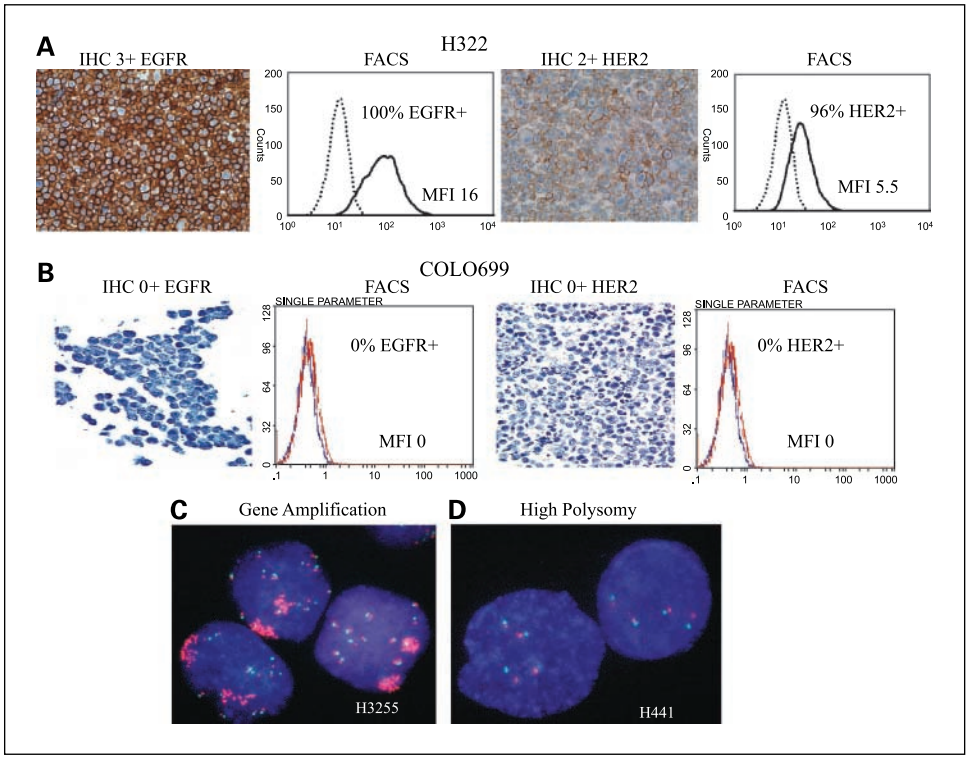

Fig. 2. EGFR protein expression by immunohistochemistry (*IHC*) and FACS and EGFR gene status by dual-color FISH assays. Expression of EGFR and HER2 in two NSCLC cell lines as assessed by immunohistochemistry (*left*) and FACS (*right*). In the FACS staining, the left peak represents staining with the isotype-matched control antibody and the right peak represents the cell surface staining with the anti-EGFR antibody or anti-HER2 antibody. By both immunohistochemistry and FACS, the adenocarcinoma line H322 was positive for both EGFR and HER2 (*A*), whereas the adenocarcinoma line COLO699 was negative for both markers (*B*). EGFR gene status was determined by FISH assay using the EGFR/CEP Vysis DNA probe. *C,* gene amplification was detected in the adenocarcinoma line H3255 as shown in the massive amount of red EGFR signal. The green signal is from the centrosome on chromosome 7 that harbors the *EGFR* gene. *D,* EGFR gene expression pattern in the adenocarcinoma line H441 was high polysomy as the average EGFR gene copy number/cell was 4.3.

31% to 32% cell increase in the G_1 phase if the cell cycle over the percentage observed in untreated control cells (Table 2). The mean percentage G_1 increase in the EGFR WT sensitive lines by 1 μmol/L gefitinib was 14.5 ± 7% and 21.8 ± 12.3% by 10 μmol/L. In the NSCLC cell lines with intermediate gefitinib sensitivity, the mean percentage G_1 increase was 18.5 ± 7.7% after 24-hour 10 μmol/L gefitinib exposure. The gefitinib-resistant NSCLC lines had no significant increases in the percentage G_1 cells (mean, 4.2 ± 3.8%) following a 24-hour exposure to 10 μmol/L gefitinib. Overall, there was a significant difference in the mean percentage G_1 increase between the three gefitinib-treated groups (P = 0.017).

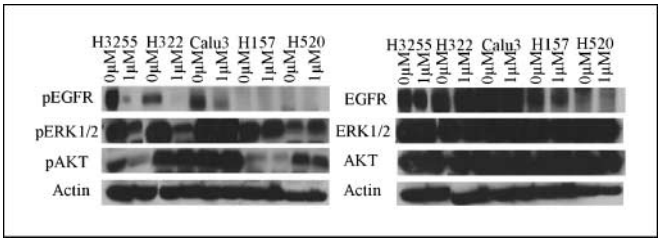

Fig. 3. Gefitinib inhibition of phosphorylated proteins. The inhibition of EGFR phosphorylation (*pEGFR*) and of downstream phosphorylation of AKT (*pAKT*) and ERK1/2 (*pERK1/2*) in gefitinib-sensitive and gefitinib-resistant cell lines was assessed using Western blotting. Cell lines were treated for 24 hours with gefitinib at the physiologic concentration of 1 μmol/L. A single sample was set up for each cell extract with enough protein to load 50 μg of total protein on a gel to detect total protein and a gel to detect phosphorylated proteins. Each gel was then stripped and probed for actin to ensure equal loading. Phosphorylated EGFR was significantly down-regulated in the gefitinib-sensitive lines H3255, H322, and Calu3, which had IC_{50}s of <1 μmol/L, and unchanged in the gefitinib-resistant line (IC_{50}, >8 μmol/L) H157 and in the H520 cell line that does not express EGFR. The phosphorylated ERK1/2 levels were down-regulated in the sensitive lines and unchanged in H157 and H520. Phosphorylated AKT was down-regulated in H3255 and unchanged in H322, Calu3, H157, and H520. Actin served as a loading control.

Furthermore, the mean percentage G_1 increase of the gefitinib sensitive plus intermediate lines was significantly greater than that seen in the gefitinib-resistant lines (P > 0.05). Following a 24-hour treatment with the physiologic concentration of 1 μmol/L gefitinib, we observed no apoptosis in any of the cell lines.

Effect of gefitinib on downstream signaling proteins. Figure 3 shows the results of immunoblotting for phosphorylated and total EGFR, HER2, ERK1/2, and AKT after exposure of gefitinib-sensitive and gefitinib-resistant lines to 1 μmol/L gefitinib for 24 hours. Gefitinib (1 μmol/L) inhibited phosphorylated EGFR in the EGFR mutant line H3255 and in the EGFR WT EGFR lines H322 and Calu3 (Fig. 3). In the gefitinib-resistant line H157, there were no changes in phosphorylated EGFR (Fig. 3). As expected, no phosphorylated EGFR was detected in the EGFR null line H520 (Fig. 3). No changes were seen in total EGFR (Fig. 3).

In the gefitinib-sensitive lines H3255 and H322, phosphorylated ERK1/2 was decreased significantly by 1 μmol/L gefitinib (Fig. 3). Gefitinib (1 μmol/L) did not inhibited phosphorylated ERK1/2 in the sensitive Calu3 cells (Fig. 3). No changes in cellular phosphorylated ERK1/2 were observed in the gefitinib-resistant lines H157 and H520 (Fig. 3). Decreased phosphorylated AKT expression was seen in the gefitinib-sensitive line H3255 by 1 μmol/L (Fig. 3). No changes were seen in phosphorylated AKT in the gefitinib-sensitive lines H322 and Calu3 and in the gefitinib-resistant lines H157 and H520 by 1 μmol/L gefitinib (Fig. 3). No changes were seen in total ERK1/2 and AKT (Fig. 3).

Treatment of human NSCLC xenografts with gefitinib alone. We evaluated the *in vivo* efficacy of gefitinib treatment on an $EGFR^+$-sensitive (NCI-H322) and $EGFR^+$-resistant (NCI-H157) cell lines to determine whether *in vivo* results would

mimic *in vitro* results. Gefitinib treatment produced no retardation in tumor growth of the resistant NCI-H157 cell lines compared with that observed in control tumors treated with vehicle (Fig. 4A). Xenografts established from the *in vitro* – sensitive line H322 were significantly growth inhibited during a 4-week treatment with gefitinib compared with control mice treated with vehicle. However, once gefitinib treatment was halted, tumor growth resumed at a rate similar to that seen in control animals (Fig. 4B).

Discussion

The results of our studies present the largest series of cell lines studied for their responsiveness to EGFR-TKIs. The similarities between these preclinical results and clinical results suggest that the study of a broad panel of cell lines is necessary to account for the heterogeneity observed in human solid tumors. We found a broad range (5 logs) in sensitivity with IC_{50}s ranging from 5 nmol/L to >10 μmol/L, with about one third of cell lines having no response to a 10 μmol/L concentration. These data are consistent with clinical data indicating progressive disease in about one third of patients. We found that adenocarcinoma cell lines were more sensitive than other histologies, although EGFR protein levels were highest in squamous carcinomas. We found that cell lines with EGFR mutations had lower IC_{50}s than those with WT receptors, but many cell lines with WT receptors had IC_{50}s of <1 μmol/L. These data are also consistent with reported clinical data. The data did show a relationship between gender and smoking status of the individuals from whom the cell lines were derived and gefitinib sensitivity, although the numbers were quite small.

With respect to biological markers, we found that EGFR mutations and EGFR gene copy number and expression pattern by FISH were significantly correlated with gefitinib sensitivity. EGFR protein levels were necessary for growth inhibition by gefitinib, but EGFR protein level was not significantly associated with gefitinib sensitivity. There was an apparent relationship between expression of phosphorylated EGFR expression and EGFR-TKI sensitivity in the five cell lines that we tested. However, Tracey et al. (29) found high levels of phosphorylated EGFR in mutant H3255 cells but also in some resistant cell lines. Some cell lines with WT phosphorylated

Table 2. Effects on cell cycle distribution in NSCLC cell lines sensitive (IC_{50}, <1 μmol/L), intermediate (IC_{50}, 3-6 μmol/L), and resistant (IC_{50}, ≥8 μmol/L) after 24-hour exposure to 1 and 10 μmol/L gefitinib

Response to gefitinib	Cell line	IC_{50} (μmol/L)	Gefitinib (μmol/L)	MFI	%G_1	G_0-G_1 (%)	S (%)	G_2-M (%)
Sensitive	H3225	0.015	0	7.3		64	34	2
			0.1		31	95	5	0
			1		32	96	2	2
	H1648	0.38	0	11.7		45	31	24
			1		22	67	33	0
			10		30	75	25	0
	Calu3	0.3	0	8.9		43	35	20
			1		19	62	32	6
			10		4	47	13	40
	H322	0.25	0	16		41	38	21
			1		9	50	32	18
			10		30	71	19	10
	H358	0.18	0	4		35	53	12
			1		8	43	46	11
			10		23	58	30	12
Intermediate	COLO699	4.2	0	0		51	33	16
			10		27	78	15	7
	H2122	5.9	0	5.1		39	39	22
			10		25	64	26	10
	H1435	3	0	14		38	48	14
			10		23	61	29	10
	H441	6	0	6		43	39	18
			10		17	60	27	13
	NE18	5	0	16		52	27	21
			10		10	62	20	18
	H125	3	0	13		43	38	19
			10		9	52	33	15
Resistant	A549	9.6	0	14		43	43	14
			10		10	53	38	9
	H1703	8	0	15		49	42	9
			10		7	56	36	8
	H157	12.8	0	13		43	40	17
			10		4	47	39	14
	H460	12.9	0	12		49	37	14
			10		3	50	34	16
	H520	13.6	0	0		40	43	17
			10		1	39	43	18
	H226	10	0	49		61	39	0
			10		0	61	37	2

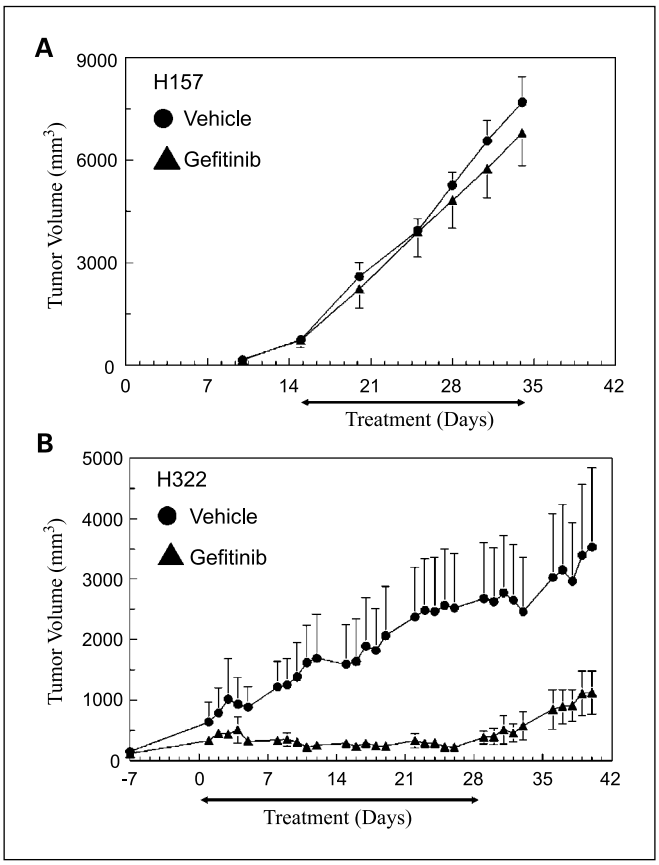

Fig. 4. *In vivo* effects of gefitinib. Effects of gefitinib on heterotransplanted NSCLC xenografts in the flanks of athymic nude mice treated with gefitinib. *A,* no tumor growth delay in athymic nude mice bearing the gefitinib-resistant NSCLC line H157 was observed during gefitinib therapy (50 mg/kg i.p. daily 5/7 days for 3 weeks). *B,* significant tumor growth delay was observed by gefitinib therapy in the athymic nude mice bearing the sensitive NSCLC line H322 (60 mg/kg i.p. daily 5/7 days for 4 weeks). However, H322 tumor growth rate returns to the rate seen in controls once treatment was discontinued.

EGFR receptors that were not inhibited by 1 μmol/L gefitinib were inhibited at concentrations of 10 μmol/L gefitinib for 4 hours (data not shown). These data would suggest that higher doses could produce superior results in humans. These data are also consistent with a better outcome from erlotinib compared with placebo that could relate to potentially high "equivalent dosing" at its maximum tolerated dose compared with lower "equivalent dosing" of gefitinib that is considerably lower than its maximum tolerated dose.

High expression levels of HER2 were found less frequently than high EGFR levels, and expression was significantly higher in adenocarcinomas compared with other histologies. Consistent with clinical findings, there was a significant correlation between HER2 expression by FACS and gefitinib sensitivity (but not for immunohistochemistry; ref. 30).

We found a significant relationship between the growth-inhibitory effects of gefitinib and cell cycle arrest in G_1 irrespective of the mutation status of the receptor, including a large G_1 arrest in H3255 (mutant) cells. Sensitive cell lines (IC_{50}, ≤10 μmol/L) had increases in the G_1 fraction averaging 14%, whereas resistant cells had no significant increase in the G_1 fraction. Similarly, Tracey et al. (29) also reported that gefitinib produced a G_1 cell cycle arrest in the sensitive WT cell line H1666 but no arrest in the WT resistant lines A549 and H441. These data could explain the lack of synergy and possible antagonism when sensitive cell lines are treated concurrently with taxanes that are most active in the G_2-M phase of the cell cycle. Tracey et al. (29) also found an increase in the sub-G_1 apoptotic peak in mutant H3255 cells following a 24-hour exposure to 1 μmol/L gefitinib when the cells were analyzed 72 hours later. We did not find an increase in the apoptotic fraction in either sensitive or resistant cell lines regardless of mutation status; however, we analyzed the cells immediately following the 24-hour treatment of 1 μmol/L gefitinib. This may indicate that the effects of gefitinib on cell growth are due both to cell cycle arrest and apoptosis.

The proper sequence of chemotherapy and EGFR-TKIs could provide synergistic growth inhibition with chemotherapy in sensitive cell lines but is unlikely to provide synergy in EGFR-TKI-resistant cell lines. These cell cycle changes and gefitinib sensitivity patterns were also related to changes in downstream signal proteins. Once again, higher gefitinib concentrations were required to inhibit downstream signal in cell lines with intermediate sensitivity, suggesting that higher doses or other manipulations might produce responses in patients with tumors having these properties.

The similarity between the *in vivo* and *in vitro* effects suggests that the direct signaling effects (as opposed to indirect antiangiogenic effects) are responsible for the antitumor effects and that the *in vivo* models would be useful for determining optimal drug sequencing as reported by Solit et al. (31).

If these *in vitro* effects of gefitinib in human lung cancer cell lines are largely reflective of the effects seen in human lung cancer patients, what is the value of this and similar studies? First, it is reassuring that the effects are similar and the heterogeneity observed indicates that it is important to study a panel of cell lines, not just to study the most sensitive cell lines. Second, the genes and proteins involved in primary resistance to EGFR-TKIs are not well defined. We undertook genomic and proteomic approaches to determine differentially expressed genes and proteins in the sensitive compared with the resistant cell lines from this study (32).

The results of this study and other *in vitro* studies provide insights into our understanding of EGFR-TKI sensitivity. Sensitive cell lines have increased expression of phosphorylated EGFR but not total EGFR. EGFR mutant cell lines had the highest levels of phosphorylated EGFR. It is possible that EGFR mutations and high EGFR gene copy numbers can increase sensitivity of the EGFR to its ligands without affecting total receptor levels. In other reports, we showed that sensitive cell lines have high levels of Rab-25, high levels of E-cadherin, high levels of HER3, and low levels of Zeb-1 gene and protein expression (32, 33). Rab-25 is a cytoplasmic protein involved in EGFR receptor recycling and may contribute to persistent receptor activation. E-cadherin and Zeb-1 are proteins associated with the epithelial to mesenchymal transition that have been reported to associate with EGFR-TKI sensitivity in several reports. Whether they are directly or indirectly related to EGFR mutation status and/or EGFR gene copy number requires further investigation. One study suggests that E-cadherin expression is associated with EGFR-TKI sensitivity in NSCLC patients treated with erlotinib (34).

Because Zeb-1 is a transcriptional repressor of E-cadherin and requires histone acetylation for activity, it was logical to ask

whether inhibition of Zeb-1 by histone deacetylase inhibitors would increase E-cadherin expression in resistant cell lines and increase gefitinib sensitivity. We recently showed that several histone deacetylases do increase E-cadherin expression and gefitinib sensitivity (33). Sordella et al. (35) reported that EGFR mutations increase constitutive levels of phosphorylated AKT and phosphorylated signal transducers and activators of transcription 3. This may indicate one mechanism for gefitinib sensitivity in mutant cell lines. We did not find a consistent relationship between phosphorylated AKT levels and gefitinib sensitivity in the WT cell lines. These data suggest that a broad panel of well-characterized cell lines, such as this, could help determine genes and proteins associated with sensitivity/resistance to other targeted agents. Such studies are in progress.

Acknowledgments

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Barbara A. Helfrich, David Raben, Marileila Varella-Garcia, et al.

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