Laser Capture Microdissection and Protein Microarray Analysis of Human Non-small Cell Lung Cancer

Differential epidermal growth factor receptor (EGFR) phosphorylation events associated with mutated EGFR compared with wild type*

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Little is known about lung carcinoma epidermal growth factor (EGF) kinase pathway signaling within the context of the tissue microenvironment. We quantitatively profiled the phosphorylation and abundance of signal pathway proteins relevant to the EGF receptor within laser capture microdissected untreated, human non-small cell lung cancer (NSCLC) (n = 25) of known epidermal growth factor receptor (EGFR) tyrosine kinase domain mutation status. We measured six phosphorylation sites on EGFR to evaluate whether EGFR mutation status in vivo was associated with the coordinated phosphorylation of specific multiple phosphorylation sites on the EGFR and downstream proteins. Reverse phase protein array quantitation of NSCLC revealed simultaneous increased phosphorylation of EGFR residues Tyr-1148 (p < 0.044) and Tyr-1068 (p < 0.026) and decreased phosphorylation of EGFR Tyr-1045 (p < 0.002), HER2 Tyr-1248 (p < 0.015), IRS-1 Ser-612 (p < 0.001), and SMAD Ser-465/467 (p < 0.011) across all classes of mutated EGFR patient samples compared with wild type. To explore which subset of correlations was influenced by ligand induction versus an intrinsic phenotype of the EGFR mutants, we profiled the time course of 115 cellular signal proteins for EGF ligand-stimulated (three dosages) NSCLC mutant and wild type cultured cell lines. EGFR mutant cell lines (H1975 L858R) displayed a pattern of EGFR Tyr-1045 and HER2 Tyr-1248 phosphorylation similar to that found in tissue. Persistence of phosphorylation for AKT Ser-473 following ligand stimulation was found for the mutant. These data suggest that a higher proportion of the EGFR mutant carcinoma cells may exhibit activation of the phosphatidylinositol 3-kinase/protein kinase B (AKT)/mammalian target of rapamycin (MTOR) pathway through Tyr-1148 and Tyr-1068 and suppression of IRS-1 Ser-612, altered heterodimerization with ERBB2, reduced response to transforming growth factor β suppression, and reduced ubiquitination/degradation of the EGFR through EGFR Tyr-1045, thus providing a survival advantage. This is the first comparison of multiple, site-specific phosphoproteins with the EGFR tyrosine kinase domain mutation status in vivo. Molecular & Cellular Proteomics 7:1902–1924, 2008.

The phenotype of an individual patient’s cancer is a product of the somatic genetic mutations underlying the tumor. One or more of these genetic changes provides a survival advantage for the cancer cells in the context of the tissue microenvironment. Thus, at a functional level, protein-mediated signaling is directly and indirectly influenced by both the genetic underpinnings and the microecology of the tumor. The “oncogene addiction theory” postulates that genetic mutations cause an oncogenic protein to dominate the signaling control of the cancer cell clone, thereby driving it to survive, grow, invade, and metastasize (1, 2). Mutations in the ERBB1 family of

* The abbreviations used are: ERBB, family of epidermal growth factor receptors; AKT, protein kinase B; COX2, cyclooxygenase2; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FKHR, Forkhead homolog1; HE, human endothelial; HER2, receptor tyrosine-protein kinase ERBB2; IGF-1R, insulin-like growth factor receptor; IRS-1, insulin receptor substrate; K-RAS, v-K-ras2 Kirsten rat sarcoma viral oncogene homolog; L858R, leucine to arginine mutation at amino acid 858 of EGFR; LCM, laser capture microdissection; Lys-C, lyszyme C serine protease; MDR, mammalian target of rapamycin; NSCLC, non-small cell lung cancer; RPPA, reverse phase protein microarray; WT, wild type; VEGFR, vascular endothelial growth factor receptor; CREB, cAMP-response element-binding protein; ENOS, endothelial nitric-oxide synthase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; FAK, focal adhesion kinase; T-PER, Tissue Protein Extraction Reagent.
protein receptors are associated with a significant proportion of carcinomas of all types. This class of receptors is particularly poised to play a role in cancer because their normal function involves growth stimulation, prosurvival, motility and migration, and stem cell recruitment (3–7). Current goals of molecular oncology research are to understand how an oncogenic mutation manifests itself at the level of the signal transduction network of the cell, why the altered network provides a survival benefit for the tumor, and how this knowledge can be exploited for individualization of therapy (for a review, see Riedel and Febbo (8)).

Epidermal growth factor receptor (EGFR) is a member of the ERBB family of receptor tyrosine kinases that regulates cellular growth, survival, and proliferation. EGFR has been extensively characterized regarding its kinase activity (9), amino acid sequence (10), receptor abundance (11), autophosphorylation properties (12), substrates (13–15), and mutation sites (16). Overexpression of EGFR in various malignancies (17) as well as the identification of specific EGFR mutations that enhance therapy response to small molecule inhibitors, notably in lung adenocarcinoma patients, and the observation that patients without detectable EGFR kinase domain mutations respond to tyrosine kinase inhibitor therapy qualifies EGFR as a promising molecular end point for individualized therapy (18–21). The dichotomy of objective treatment response to EGFR mutation status in a subset of patients suggests possible alternative mechanisms of receptor or downstream protein activation independent of the EGFR kinase domain, further emphasizing the goals of this study in assessing the phosphoprotein profile of mutant and wild type EGFR lung carcinoma cell populations.

Very little is known about the state of lung carcinoma epidermal growth factor (EGF) kinase pathway signaling within the context of the human lung tissue microenvironment. Within a growing human carcinoma, the tumor cell population is influenced by autocrine and paracrine signaling and cell-cell interactions within the heterogeneous tissue microenvironment. The concentration, source, or temporal fluctuations of cytokines and growth factors influencing the carcinoma cells is unknown. In any individual tumor, ligands that influence the ERBB family of receptors can be generated by the tumor cells themselves or by local host cells. Each individual patient’s carcinoma specimen contains a variable, heterogeneous proportion of stroma, lung parenchyma, bronchial epithelium, inflammatory cells, and endothelial cells. All of these non-carcinoma subpopulations may contain EGF receptors, participate in EGFR signaling, or contribute EGFR-related ligands. Furthermore it is unknown what proportion of the carcinoma population at any point in time is undergoing active signaling for a specific pathway. Consequently the state of the EGFR kinase signaling network within the lung carcinoma cells in a tumor specimen cannot be adequately studied using heterogeneous, ground-up tumor tissue or cultured cell lines (22–24). Laser capture microdissection (LCM) addresses the problem of sample cellular heterogeneity by providing a means to separate tumor cells from the large number of non-tumor cells within the complex microenvironment (22–26). Microdissection also lends itself to studying tissue heterogeneity in respect to 1) spatial orientation of the tissue, i.e. invasive front, necrotic center, and distal portions, or 2) composite diseased/uninvolved cell populations. In this study, we microdissected serial sections of lung adenocarcinoma samples, at various depths of the tissue block, to provide a composite portrait, at the protein level, of the entire tumor cell population.

In the present study we quantitatively profiled the phosphorylation (abundance) of signal pathway proteins relevant to the EGF receptor signal pathway (see Table II) within laser capture microdissected untreated, human non-small cell lung cancer (NSCLC) of known EGFR mutation status. Evaluating the combination of specific receptor protein phosphorylation sites in a tumor sample provides direct functional evidence that the receptor has changed its three-dimensional shape, dimerized, or undergone autophosphorylation on the cytoplasmic region of the receptor. The existence of phosphorylation on the EGFR is transient and may only occur if the receptor is engaged in signaling. Such phosphorylation provides sites of interaction for downstream signaling pathways that drive the growth, survival, differentiation, and motility of cells (3, 6, 27–32). Thus, measurement of the phosphorylation sites provides functional information not obtainable by genomics or transcriptomics measurement of the receptor. For analysis of microdissected carcinoma cells we measured six phosphorylation sites on the EGF receptor and 20 selected downstream signal proteins to evaluate whether EGFR mutation status in vivo was associated with the coordinated phosphorylation or combination of specific multiple phosphorylation sites on the EGFR.

EGFR tyrosine kinase domain gene sequencing was performed for each lung adenocarcinoma case. Thus the EGFR tyrosine kinase domain mutation status of each case in this study set was known and could therefore be compared with the phosphoprotein/signaling protein profile of each case. This is the first comparison of the phosphoprotein profile with the kinase domain mutation status in vivo. 10–20% of non-small cell lung cancer patients in the United States have sensitizing mutations in the EGFR. Specific somatic mutations in the ATP binding pocket of epidermal growth factor receptor have been identified in lung cancer patients who responded favorably to treatment with tyrosine kinase inhibitors directed against EGFR (18, 19). Deletion mutation DelE746A750 in exon 19 and point mutation L858R in exon 21 increase enzyme activity and increase the tyrosine kinase $V_{\text{max}}$ and $K_m$ for ATP (33). Gefitinib (Iressa) and erlotinib (Tarceva) compete for ATP binding of the receptor and have been shown to be more effective in patients harboring EGFR mutations such as DelE746A750 or L858R. These mutations comprise 85% of EGFR mutations (34–37). In addition to L858R mutations,
other important mutations include (a) exon 19 deletions in which 17 different variants have been identified, (b) exon 20 point mutations, (c) exon 18 point mutations, (d) other point mutations in exon 21 in addition to the L858R mutation, and (e) other mutations in the EGFR binding domain (16).

The present analysis of microdissected lung carcinoma cells revealed specific potential correlations and differences between multiple phosphorylation sites on the EGFR and corresponding downstream proteins for the tumors with EGFR mutations compared with tumors without such mutations. Because it is not possible to measure local EGFR ligand levels in human tissue samples and multiple classes of ligand exist, it was not possible to treat whole tissue pieces with ligand. To explore which of these phosphorylation events might be related to the presence or dose of ligand induction we conducted a time course study of EGFR ligand-stimulated cultured NSCLC cell lines of known EGFR tyrosine kinase domain mutation status.

In an initial series of cell culture studies we evaluated 26 end points (see Table II) at one concentration of EGFR ligand stimulation. In a second set of triplicate, independent time course studies we quantified 115 analytes (see Table II) after stimulation by three different EGFR ligand concentrations. The results of the cell line studies showed several important similarities between the cell lines and the human microdissected lung tissue. These include the simultaneous and coordinated expression of multiple phosphorylation sites across the entire class of EGFR tyrosine kinase domain mutations, including the point mutation L858R and deletion mutation DelE746A750. Information gleaned from the quantitative time course cell line data examined in light of the microdissected tissue findings has allowed us to postulate the specific intrinsic effects of the EGFR mutation on the downstream signaling cascade.

**EXPERIMENTAL PROCEDURES**

**Human Adenocarcinoma Tissue**

Tissue Samples and Laser Capture Microdissection—Fresh frozen lung adenocarcinoma specimens, stages I/II/III (n = 25), and relevant clinical data were obtained from the National Institutes of Health, National Cancer Institute, Laboratory of Human Carcinogenesis (38, 39) (see Table I). All patient samples were collected with informed consent as approved by their respective institutional review boards. An independent board-certified pathologist (L. A. L.) verified the presence of adenocarcinoma tissue prior to laser capture microdissection.

Eight-micrometer cryostat sections were sectioned on silanized glass microscope slides (ThermoFisher, Atlanta, GA) or polyethylene naphthalene membrane slides (Molecular Devices, Sunnyvale, CA). The frozen sections were stored at −80 °C prior to staining and microdissection. The frozen section slides were fixed briefly in 70% ethanol, rinsed in water, stained with Mayer’s hematoxylin (Sigma-Aldrich), developed in Scott’s Tap Water (ThermoFisher), and dehydrated in an ethanol gradient (70, 95, and 100%) with a final rinse in xylene (Sigma). The sections were allowed to air dry briefly prior to laser capture microdissection. Pure tumor cell populations were microdissected with a PixCell IIe or Veritas LCM instrument (Molecular Devices). Microdissected cells were stored at −80 °C prior to microarray construction.

Reverse Phase Protein Microarray Construction—The microdissected cells were subjected to lysis with a 2.5% solution of 2-mercaptoethanol (Sigma) in Tissue Protein Extraction Reagent (T-PER™, Pierce)/2× SDS Tris-glycine 2× SDS buffer (Invitrogen). Reverse phase protein microarrays were printed in duplicate with whole cell protein lysates as described by Petrocin et al. (40). Briefly the lysates were printed on glass-backed nitrocellulose array slides (FAST slides, Whatman) using a GMS 417 arrayer (Affymetrix, Santa Clara, CA) equipped with 500-µm pins or an Aushon 2470 arrayer equipped with 350-µm pins (Aushon Biosystems, Billerica, MA). Each lysate was printed in a dilution curve representing undiluted lysate and 1:2, 1:4, 1:8, 1:16, and negative control dilutions. The slides were stored with desiccant (Drierite, W. A. Hammond, Xenia, OH) at −20 °C prior to immunostaining.

Reverse Phase Protein Microarray Immunostaining—Immunostaining was performed on an automated slide stainer according to the manufacturer’s instructions (Autostainer catalyzed signal amplification (CSA) kit, Dako, Carpinteria, CA). Each slide was incubated with a single primary antibody at room temperature for 30 min. Each array was probed with a single polyclonal or monoclonal primary antibody (see Table II). The negative control slide was incubated with antibody diluent. Secondary antibody was goat anti-rabbit IgG heavy + light (1:5000) (Vector Laboratories, Burlingame, CA) or rabbit anti-mouse IgG (1:10) (Dako). Subsequent protein detection was amplified via horseradish peroxidase-mediated biotinyl tyramide with chromogenic detection (diaminobenzidine) according to the manufacturer’s instructions (Dako). Total protein per microarray spot was determined with SYPRO Ruby blot stain (Invitrogen) according to the manufacturer’s directions. Imaging was performed with an Alpha Innotech (San Leandro, CA) FluorChem imager.

Antibody Validation and Phosphoprotein Specificity—Primary antibodies (see Table II) were validated prior to use by immunoblotting with complex cellular lysates such as commercial cell lysates or human tissue lysates (supplemental Fig. 2). Criteria for antibody validation were (a) a single band at the correct molecular weight or (b) if two bands were present 80% of the signal must have been at the correct molecular weight. Specificity of the phosphoprotein-specific antibodies was verified by peptide competition on an immunoblot (supplemental Fig. 2) when the corresponding peptide/antibody pair was available. The specificity criterion was a reduction in signal intensity in the presence of the corresponding peptide compared with the antibody alone. Further specificity for phosphospecific antibodies was verified by peptide/antibody reactivity on a reverse phase protein microarray (RPPA). A series of peptides and peptide mixtures were immobilized on a nitrocellulose-coated slide. The slide was probed with a single antibody. An antibody was considered specific if the spot signal intensity was not greater than 2 S.D. above background for any peptide other than its corresponding peptide or a mixture of peptides containing the cognate peptide (supplemental Fig. 3). The antibody was specific if it bound to its cognate peptide or a mixture containing its corresponding peptide.

Cell Culture and EGF Stimulation

Evaluation of EGF Stimulation on Wild Type and EGFR Mutant Cell Lines—The human NSCLC cell lines A549 (WT), H1650 (DelE746A750), and H1975 (L858R/T790M) were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were routinely cultured at 37 °C in a humidified 5.0% CO2 atmosphere in F-12K (A549) or RPMI 1640 medium (H1650 and H1975) (ATCC) supplemented with 10% heat-inactivated fetal bovine serum (ATCC). Cells were passaged using trypsin/EDTA (ATCC). Approximately 5.0 × 10⁴ A549, 1.5 × 10⁴ H1650, and 10.0 × 10⁴ H1975 cells were subcultured into 24-well plates and cultured until 80% confluent. Cells were grown in serum-free medium for 24 h prior to treatment.
with 100.0 ng/ml recombinant EGF in medium (Cell Signaling Technology, Danvers, MA). Treated and untreated cells were subsequently collected at 0, 1, 2, 5, 10, 15, 30, and 60 min. At each time point the cells were washed once with PBS and then lysed directly in a 2.5% solution of 2-mercaptoethanol (Sigma) in T-PER (Pierce)/2× SDS Tris-glycine 2× SDS buffer (Invitrogen) and protease inhibitor mixture (Sigma). Cell lysates were stored at ~80 °C prior to microarray construction.

**EGF Stimulation Time Course Assays**—Additional cell stocks of NSCLC cell lines A549 and H1975 were obtained from ATCC and were cultured as described above. Approximately 5.0×10^4 A549 (WT) and 6.5×10^4 H1975 (L858R/T790M) cells were subcultured into 6-well plates and cultured for 3 days. On day 4, cells were grown in serum-free medium for 24 h prior to treatment with recombinant EGF in medium (Cell Signaling Technology). On day 5, at 80% confluence, cells were treated with EGF at 5.0, 50.0, or 500.0 ng/ml; 1 mM pervanadate-supplemented medium; or medium alone. Treated and untreated cells were subsequently collected at 0, 1, 3, 5, 7, 15, 30, and 180 min. At each time point the cells were washed twice with PBS and lysed in 100 μl of a 2.5% solution of 2-mercaptoethanol (Sigma) in T-PER (Pierce)/2× SDS Tris-glycine 2× SDS buffer (Invitrogen). Time 0 samples were a mock treatment consisting of immediate removal of medium, washing, and lysis. Cell lysates were stored at ~80 °C prior to microarray construction. The time course was repeated on three individual days.

**Reverse Phase Protein Microarray Printing of Cell Line Samples**—Samples were diluted 1:4 to obtain an approximate 1.0 mg/ml total protein concentration and then denatured by heating at 100 °C for 7 min. Two-fold dilution curves (undiluted, 1:2, 1:4, and 1:8) of A549 and H1975 time course lysates and A431 and A431 + EGF control cell lysates (BD Biosciences) were printed in an array format onto FAST nitrocellulose slides (Whatman) with an Aushon 2470 arrayer (Sigma) in T-PER (Pierce)/2× SDS-Tris-glycine 2× SDS buffer (Invitrogen). Time 0 samples were a mock treatment consisting of immediate removal of medium, washing, and lysis. Cell lysates were stored at ~80 °C prior to microarray construction.

**Immunoblotting Analyses**—Cell lysates were prepared from 50.0 ng/ml EGF-treated A549 and H1975 cells. Proteins were resolved by SDS-PAGE (4–20% gel) and electrophoretically transferred to PVDF (Millipore). Membranes were probed with polyclonal antibodies to EGFR, EGFR L858R, and AKT Ser-473 (Cell Signaling Technology). Immunoreactive bands were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG (Vector Laboratories) and ECL substrate (Pierce). β-Actin (Cell Signaling Technology) staining was performed as a loading control. Imaging analysis was performed with an Eastman Kodak Co. 4000MM imager.

**RESULTS**

**PCR and Genomic DNA Sequencing of EGFR**—Sequencing of EGFR exons 17–22 in H1975 and A549 adenocarcinoma cells confirmed previously published results that A549 cells do not harbor mutations in the tyrosine kinase domain, whereas H1975 cells carry mutation L858R in exon 21 and resistance mutation T790M in exon 20 (16). Additionally K-RAS sequencing confirmed the presence of point mutation G12S in A549 cells. K-RAS mutations were not found in the H1975 cells. NSCLC patient samples were sequenced previously for EGFR tyrosine kinase domain mutations (exons 18–21) (38, 39). In this small study set (n = 25), eight tissue samples had mutations in the EGFR tyrosine kinase domain (Table I). It is noteworthy that a higher percentage of patients harboring the L858R mutation in this study were female non-smokers (60%) with advanced disease stage; this is consistent with data published previously (18, 41).

**EGFR Mutations Are Associated with Site-specific Phosphorylation of EGF Receptor in NSCLC Cells Procured by LCM**—To elucidate cell signaling pathways relevant to human lung adenocarcinomas, we used laser capture microdissection to procure pure tumor cell populations from human lung biopsy specimens (Fig. 1). An advantage of this study was the collection of snap frozen tumor specimens at the time of primary surgical diagnosis. Therefore we were not evaluating the effects of treatment on the cellular signaling pathways but rather the state of the tumor at the time of procurement in treatment-naïve patients. A total of 31 cases were available for this study. We applied a set of quality assessment criteria to the samples to judge their adequacy for microdissection (22). Criteria were: (a) presence of tumor cells, (b) sufficient size and quantity of tissue, and (c) absence of dehydration or freeze-thaw artifacts. One case was judged inadequate because of freezer dehydration artifacts. Four cases consisted of connective tissue with minimal tumor cells. One case was not
analyzed because of insufficient total protein on the microarray. 83% of the cases were judged to be adequate for microdissection and array analysis. 10–15 cryosections were prepared for each case with 1–2 sections/slide. The average number of laser pulses (shots) per slide was 1,149 with an average microdissection efficiency of 95%, resulting in 15,000–30,000 cells/sample collected from multiple cryosections. This was sufficient material for adequate sensitivity and specificity on the RPPA (40, 42–44). Human endothelial (HE) cell lysates treated with pervanadate were used as a model of phosphorylated vascular endothelial growth factor receptor sensitivity and precision (supplementalFig. 1). Human endothelial cells are known to express 100,000 vascular endothelial growth factor receptors/cell. Sensitivity of the arrays probed with anti-VEGFR Tyr-951 was found to be 3,000–4,000 receptor molecules. To determine interslide precision, HE cells treated with pervanadate were printed in duplicate on eight slides and probed with anti-VEGFR Tyr-951. Excellent dose-response curves were observed between arrays (percent coefficient of variation, 5.0–17.8%; n = 8). Within-run variation (n = 12) was found to be within 2.0–18.1% for the HE + pervanadate cell lysate with good linearity (r² = 0.9693) (supplemental data).

Post-translational modifications of EGFR and associated downstream proteins (Table II) were quantitatively measured using RPPA technology. To assess the global activity of the EGF receptor, we measured the activation state of six EGFR phosphorylation sites (Tyr-845, Tyr-992, Tyr-1045, Tyr-1068, Tyr-1148, and Tyr-1173) as well as total EGFR. We used the L858R mutation-specific antibody to validate our mutation sequencing results. This antibody recognizes EGFR with the L858R point mutation, which is one member of the group of mutations found to correlate with sensitivity to gefitinib therapy. This antibody only stained the H1975 mutant cell line and the known L858R tissue samples, providing an independent means of cross-checking the array data and mutation analysis (Figs. 2 and 3 and Table I).

Unsupervised hierarchical clustering analysis revealed the presence of two major groups with six of eight mutants clustered distinctly from the 17 wild type samples (Fig. 3). All the mutation cases were associated with higher levels of EGF receptor phosphorylation on residues Tyr-1148 and Tyr-1068 and lower levels of phosphorylation for SMAD/IRS-1/HER2 end points. This was confirmed by t test and Wilcoxon rank sum analysis of the individual analytes (Table III).

Five of six patient samples possessing either an L858R or deletion exon 19 EGFR mutation had a concomitant unique double phosphorylation of EGFR residues Tyr-1068 and Tyr-1148 as well as a reduction in HER2 Tyr-1248, IRS-1 Ser-612, and SMAD2 Ser-465/467 compared with the wild type. More-

| Sample number | Age  | Sex | Race | Smoking status | Stage | EGFR tyrosine kinase domain mutation |
|---------------|------|-----|------|----------------|-------|-------------------------------------|
| 1186          | 73   | M   | W    | NS             | II    | Point mutation exon 21 L858R        |
| 1498          | 67   | F   | W    | FS             | I     | WT                                  |
| 1604          | 58   | F   | W    | CS             | I     | WT                                  |
| 1712          | 74   | M   | W    | CS             | I     | WT                                  |
| 1747          | 60   | M   | B    | CS             | I     | WT                                  |
| 6             | 56   | W   | W    | NS             | II    | Deletion exon 19 – 15 bp            |
| 10198         | 62   | F   | W    | NS             | III   | Point mutation exon 21 L858R        |
| 10372         | 62   | M   | W    | CS             | I     | WT                                  |
| 10403         | 55   | F   | B    | CS             | I     | WT                                  |
| 10419         | 72   | F   | W    | FS             | I     | WT                                  |
| 10622         | 57   | M   | B    | CS             | I     | WT                                  |
| 11005         | 77   | F   | W    | CS             | I     | WT                                  |
| 11184         | 67   | F   | W    | FS             | I     | WT                                  |
| 11191         | 47   | M   | W    | CS             | I     | WT                                  |
| 11541         | 70   | M   | B    | CS             | I     | WT                                  |
| 11671         | 53   | M   | W    | CS             | I     | WT                                  |
| 11777         | 72   | F   | W    | CS             | I     | WT                                  |
| 11807         | 73   | F   | B    | FS             | I     | WT                                  |
| 11823         | 62   | M   | W    | CS             | I     | WT                                  |
| 12120         | 74   | M   | W    | CS             | I     | WT                                  |
| 15479         | 72   | F   | W    | NS             | II    | Point mutation exon 21 L858R        |
| 146           | 71   | M   | W    | FS             | III   | Point mutation exon 21 L858R        |
| 1064          | 71   | F   | W    | NS             | I     | Deletion exon 19 – 15 bp            |
| 11199         | 47   | F   | W    | NS             | II    | Point mutation exon 21 L858R        |

* NS, non-smoker; FS, former smoker; CS, current smoker.
over the mutant samples exhibited reduced levels of EGFR Tyr-1045, Tyr-845, and Tyr-1173 compared with the wild type (Fig. 2). Thus, the mutant EGFR as a class appeared different from the wild type with regard to elevated phosphorylation of specific EGFR sites, whereas the wild type as a class had elevation in HER2 Tyr-1248, IRS-1 Ser-612, and SMAD2 Ser-465/467 (Fig. 4).

EGFR Mutant Tissue Carcinoma Cell Populations Exhibit Distinct Pairwise Correlations Compared with Wild Type—To further understand these correlations, Spearman’s $\rho$ non-parametric analysis was conducted to examine the strength of the linear relationship between pairs of end points among all the end points analyzed. Spearman’s $\rho$ correlation coefficient is computed on the ranks of the data using the formula for Pearson’s correlation. The Pearson product-moment correlation coefficient measures the strength of the linear relationship between two variables. The results are shown in Table IV, which displays those correlations that have a significant $p$ value ($p < 0.01$) and correlation coefficient $>0.80$. As a class, the mutant carcinomas were found to have a correlation between pairs of phosphorylation sites on EGFR and AKT (Table IV). In contrast the wild type samples lacked correlations of these same two proteins and phosphorylation sites. The wild type samples were scored as having strong correlations between MTOR Ser-2481 and IRS-1 Ser-612 (Fig. 5), COX2 and IRS-1 Ser-612, and EGFR Tyr-992 and 14-3-3 $\gamma$. These correlations were not observed in the mutant. Instead the mutant samples showed a significant correlation between 11 protein pairs including SRC Tyr-527 and SHC Tyr-317 as well as EGFR Tyr-1045 and 14-3-3 $\gamma$ (Fig. 5). The point mutation-specific antibody, anti-EGFR L858R, did not show any statistically significant differences in our study set of eight EGFR mutated samples and 17 wild type samples, although there was clustering of L858R mutant samples by unsupervised hierarchical clustering (Fig. 2). This is most likely because of the inclusion of multiple classes of mutations in the mutated group and the specificity of the antibody for the L858R mutation. Although we do not know the extent to which other gene mutations such as $p53$, K-RAS, or $RB$ (retinoblastoma) may influence AKT signaling (45), the observed signaling profiles in this study set may provide clues indicating the organization of the EGFR-related protein network linkages regarding differences between the wild type and mutant adenocarcinoma samples.

EGF Stimulation of Wild Type and EGFR-mutated Cell Lines Confirms Cell Signaling Differences—To explore which of these phosphoprotein end points might potentially be influenced by ligand presence or dosage, we conducted a 60-min time course study of EGF ligand stimulation (100.0 ng/ml) with cell lines representing wild type EGFR (A549) and known
Validated primary antibodies used for reverse phase protein microarray analysis

MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; rec., receptor; PDGF, platelet-derived growth factor; IκB, inhibitor of NF-κB; MARCKS, myristoylated alanine-rich C kinase substrate; AMPK, AMP-activated protein kinase; PI, phosphatidylinositol; PKA, protein kinase A, PKC, protein kinase C; PKR, double-stranded RNA-dependent protein kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SAPK, stress-activated protein kinase; JNK, c-Jun NH2-terminal kinase; p70S6, ribosomal protein S6 kinase; PARP, poly(ADP-ribose) polymerase; PLC, phospholipase C; pT, phosphothreonine; pY, phosphotyrosine.

| Antibody/subcellular location<sup>a</sup> | Source<sup>b</sup> | LCM non-small cell lung cancer | A549, H1975, and H1650 cell line time course | Cell line screening time course<sup>c</sup> | A549 and H1975 cell line time course (triplicate) |
|----------------------------------------|-----------------|---------------------------------|----------------------------------------------|-----------------------------------------------|------------------------------------------------|
| Nucleus                                |                 |                                 |                                              |                                               |                                               |
| ATF-2 (Thr-71)                         | 1               | X                              | X                                           |                                               |                                               |
| BUB3                                   | 2               | X                              |                                              |                                               |                                               |
| CHK1 (Ser-345)                         | 1               | X                              |                                              |                                               |                                               |
| CHK2 (Ser-33/35)                       | 1               | X                              |                                              |                                               |                                               |
| Cleaved PARP (Asp-214)                 | 1               | X                              | X                                           |                                               |                                               |
| CREB (Ser-133)                         | 1               | X                              |                                              |                                               |                                               |
| Cyclin D1                              | 2               | X                              |                                              |                                               |                                               |
| Cyclin E                               | 2               | X                              |                                              |                                               |                                               |
| ELK-1 (Ser-383)                        | 1               | X                              |                                              |                                               |                                               |
| FOXO1 (FKHR) (Ser-256)                 | 1               | X                              |                                              |                                               |                                               |
| FOXO1 (FKHR) (Thr-24)                  | 1               | X                              | X                                           | X                                             |                                               |
| FOXO3 (FKHR1) (Ser-253)                | 1               | X                              |                                              |                                               |                                               |
| Histone H3 (Ser-10) mitosis marker     | 3               | X                              |                                              |                                               |                                               |
| MSK1 (Ser-360)                         | 1               | X                              |                                              |                                               |                                               |
| β-Catenin (Ser-33/37/Thr-41)           | 1               | X                              |                                              |                                               |                                               |
| β-Catenin (Thr-41/Ser-45)              | 1               | X                              |                                              |                                               |                                               |
| STAT1 (Tyr-701)                        | 1               | X                              | X                                           | X                                             | X                                             |
| STAT3 (Ser-727)                        | 1               | X                              | X                                           |                                               |                                               |
| STAT5 (Tyr-694)                        | 1               | X                              |                                              |                                               |                                               |
| STAT6 (Tyr-641)                        | 1               | X                              |                                              |                                               |                                               |
| Mitochondria                           |                 |                                 |                                              |                                               |                                               |
| BAK                                    | 1               | X                              | X                                           | X                                             | X                                             |
| BCL-2 (Ser-70)                         | 1               | X                              |                                              |                                               |                                               |
| BCL-2 (Thr-56)                         | 1               | X                              |                                              |                                               |                                               |
| BAD (Ser-112)                          | 1               | X                              | X                                           |                                               |                                               |
| BAD (Ser-136)                          | 1               | X                              |                                              |                                               |                                               |
| BAD (Ser-155)                          | 1               | X                              | X                                           | X                                             |                                               |
| Membrane                               |                 |                                 |                                              |                                               |                                               |
| Adducin (Ser-662)                      | 3               | X                              | X                                           | X                                             | X                                             |
| β-Actin<sup>d</sup>                    | 1               | X                              | X                                           | X                                             | X                                             |
| c-KIT (Tyr-703)                        | 4               | X                              | X                                           | X                                             | X                                             |
| c-KIT (Tyr-719)                        | 1               | X                              |                                              |                                               |                                               |
| c-KIT (Tyr-721)                        | 4               | X                              |                                              |                                               |                                               |
| EGFR                                   | 1               | X                              | X                                           | X                                             | X                                             |
| EGFR L858R                              | 1               | X                              | X                                           | X                                             | X                                             |
| EGFR (Tyr-1045)                        | 1               | X                              | X                                           | X                                             | X                                             |
| EGFR (Tyr-1068)                        | 1               | X                              | X                                           | X                                             | X                                             |
| EGFR (Tyr-1148)                        | 5               | X                              | X                                           | X                                             | X                                             |
| EGFR (Tyr-1173)                        | 5               | X                              | X                                           | X                                             | X                                             |
| EGFR (Tyr-845)                         | 1               | X                              | X                                           | X                                             | X                                             |
| EGFR (Tyr-992)                         | 1               | X                              | X                                           | X                                             | X                                             |
| Estrogen receptor α (Ser-118)          | 1               | X                              |                                              |                                               |                                               |
| HER2 (Tyr-1248)                        | 1               | X                              | X                                           | X                                             | X                                             |
| HER3 (Tyr-1289)                        | 1               | X                              | X                                           | X                                             | X                                             |
| IGF-1 rec. (Tyr-1131)/insulin rec. (Tyr-1146) | 1 | X | X | X | X |
| IRS-1 (Ser-612)                        | 1               | X                              | X                                           | X                                             | X                                             |
| MET (Tyr-1234/1235)                    | 1               | X                              |                                              |                                               |                                               |
| PDGF receptor β (Tyr-716)              | 3               | X                              | X                                           |                                               |                                               |
| PDGF receptor β (Tyr-751)              | 1               | X                              |                                              |                                               |                                               |
| PLC-γ-1                                | 1               | X                              |                                              |                                               |                                               |
| PLC-γ-1 (Tyr-783)                      | 1               | X                              |                                              |                                               |                                               |
| Antibody/subcellular location<sup>a</sup> | Source<sup>b</sup> | LCM non-small cell lung cancer | A549, H1975, and H1650 cell line time course | Cell line screening time course<sup>c</sup> | A549 and H1975 cell line time course (triplicate) |
|------------------------------------------|----------------|-------------------------------|-------------------------------------------|---------------------------------|---------------------------------|
| VEGFR 2 (Tyr-1175)                      | 1             |                               |                                           |                                 |                                 |
| VEGFR 2 (Tyr-951)                       | 1             |                               |                                           |                                 |                                 |
| VEGFR 2 (Tyr-996)                       | 1             |                               |                                           |                                 |                                 |
| 14-3-3 / / / η                          | 3             | X                             |                                           |                                 |                                 |
| 4EBP1 (Ser-65)                          | 1             |                               |                                           |                                 |                                 |
| 4EBP1 (Thr-37/46)                       | 1             | X                             |                                           |                                 | X                               |
| AKT (Ser-473)                           | 1             | X                             |                                           |                                 |                                 |
| AKT (Thr-308)                           | 1             |                               |                                           | X                               |                                 |
| AMPKα1 (Ser-485)                        | 1             |                               |                                           |                                 |                                 |
| AMPKβ1 (Ser-108)                        | 1             |                               |                                           |                                 |                                 |
| APC2                                    | 6             | X                             | X                                         |                                 |                                 |
| A-Raf (Ser-299)                         | 1             |                               |                                           |                                 |                                 |
| ASK1 (Ser-83)                           | 1             |                               |                                           |                                 |                                 |
| B-Raf (Ser-445)                         | 1             |                               |                                           |                                 |                                 |
| c-ABL (Thr-735)                         | 1             |                               |                                           |                                 | X                               |
| Cleaved caspase 3 (Asp-175)             | 1             |                               |                                           |                                 |                                 |
| Cleaved caspase 6 (Asp-162)             | 1             |                               |                                           |                                 |                                 |
| Cleaved caspase 7 (Asp-198)             | 1             |                               |                                           |                                 |                                 |
| Cleaved caspase 9 (Asp-330)             | 1             |                               |                                           |                                 |                                 |
| COX2                                    | 3             | X                             | X                                         | X                               |                                 |
| C-RAF (Ser-338)                         | 1             |                               |                                           |                                 |                                 |
| EIF4E (Ser-209)                         | 1             |                               |                                           |                                 |                                 |
| EIF4G (Ser-1108)                        | 1             | X                             |                                           | X                               | X                               |
| ENOS (Ser-1177)                         | 1             |                               |                                           | X                               |                                 |
| ENOS/NOS III (Ser-116)                  | 3             |                               |                                           |                                 | X                               |
| ERK1/2 (Thr-202/Thr-204)                | 1             | X                             |                                           | X                               | X                               |
| ETK (Tyr-40)                            | 1             |                               |                                           |                                 |                                 |
| FAK (Tyr-576/577)                       | 1             |                               |                                           |                                 | X                               |
| GAB1 (Tyr-627)                          | 1             |                               |                                           |                                 | X                               |
| GSK3αβ (Ser-21/9)                      | 1             | X                             |                                           |                                 |                                 |
| GSK3αβ (Ser-21)                        | 1             |                               |                                           | X                               |                                 |
| IκBα (Ser-32)                          | 1             |                               |                                           |                                 |                                 |
| JAK1 (Tyr-1022/1023)                    | 1             |                               |                                           |                                 |                                 |
| LCK (Tyr-505)                           | 5             |                               |                                           | X                               | X                               |
| LKB1 (Ser-334)                          | 1             |                               |                                           |                                 |                                 |
| LKB1 (Ser-428)                          | 1             |                               |                                           |                                 |                                 |
| MAPK (pTεpY)                           | 7             |                               |                                           |                                 |                                 |
| MARCKS (Ser-152/156)                    | 1             |                               |                                           |                                 | X                               |
| MEK1 (Ser-298)                         | 1             |                               |                                           |                                 |                                 |
| MEK1/2 (Ser-217/221)                    | 1             |                               |                                           |                                 | X                               |
| Mtor (Ser-2448)                        | 1             |                               |                                           |                                 |                                 |
| Mtor (Ser-2481)                        | 1             |                               |                                           | X                               | X                               |
| NF-κκβ p65 (Ser-536)                    | 1             |                               |                                           | X                               | X                               |
| p38 MAP kinase (Thr-180/Tyr-182)        | 1             |                               |                                           |                                 |                                 |
| p70S6 kinase (Ser-371)                  | 1             |                               |                                           |                                 | X                               |
| p70S6 kinase (Thr-389)                  | 1             |                               |                                           |                                 |                                 |
| p70S6 kinase (Thr-412)                  | 3             |                               |                                           |                                 | X                               |
| p90RSK (Ser-380)                        | 1             |                               |                                           | X                               | X                               |
| PAK1 (Ser-199/204)/PAK2 (Ser-192/197)   | 1             |                               |                                           |                                 |                                 |
| PDK1 (Ser-241)                          | 1             |                               |                                           |                                 |                                 |
| PI 3-kinase                             | 1             |                               |                                           |                                 |                                 |
| PKA C (Thr-197)                         | 1             |                               |                                           |                                 |                                 |
| PKC (pan) (gill Ser-660)                | 1             |                               |                                           |                                 | X                               |
| PKC α (Ser-657)                        | 3             |                               |                                           |                                 | X                               |
| PKC α/β (Thr-638)                      | 1             |                               |                                           | X                               | X                               |
| PKC / (Thr-410/403)                     | 1             |                               |                                           |                                 |                                 |
| PKR (Thr-446)                           | 1             |                               |                                           |                                 |                                 |
common mutations in exons 19 and 21 of the EGFR gene (DelE746A750 (H1650) and L858R (H1975)). We measured the same end points as in the tissue analysis with the addition of protein analytes in prosurvival and stress pathways (Table II). For many of the end points examined, the amplitude of phosphorylation change over time was similar for the mutant and wild type, for example EGFR Tyr-1173 (Fig. 6). Nevertheless specific differences were noted between the mutants and wild type. Sustained phosphorylation over time was observed for AKT Ser-473, SHC Tyr-317, and FOX01 (FKHR) Thr-24 for both mutated cell lines H1975 and H1650 compared with wild type (Fig. 6). For these end points, phosphorylation returned to base line within 30 min in the A549 wild type cells, whereas phosphorylation remained elevated within the 60-min time course for the H1975 L858R mutant cells. The behavior of the H1975 L858R cell lines was more dissimilar to the wild type compared with the H1650 DelE746A750 cell line. Of note, the H1650 mutant cell line did not show the enhanced phosphorylation of EGFR Tyr-1068 and Tyr-1148 found in the H1975 L858R mutant cell line (Fig. 6). This initial study showed differences in EGFR phosphorylation and associated downstream proteins for H1975 L858R mutant cell lines compared with wild type and H1650 over a 60-min time course for a single concentration of EGF (100.0 ng/ml). Therefore in the subsequent experiments with three doses of EGF ligand stimulation over a 180-min time course we did not evaluate the H1650 cell line.

Experimental EGF Ligand Stimulation of Mutant and Wild Type EGFR Cell Lines—Because the concentration of ligand in tissue is unknown and the initial time course study was relatively short, we extended these studies to a series of EGF doses and a longer time course in A549 and H1975 cell lines. The H1975 cell line contains the same point mutation (L858R) found in the majority of the mutated patient samples. These data also provided us with the opportunity to (a) observe pathway protein fluctuations that occur in response to EGF stimulation in vitro and (b) examine the distinct dose-response differences between WT and mutant (L858R) EGFR cell lines. We determined the effects in vitro when A549 (WT) and H1975 (L858R) cells were stimulated with 5.0, 50.0, or 500.0 ng/ml EGF at time points 0, 1, 3, 5, 7, 9, 15, 30, 60, and 180 min. We evaluated 115 different end points to screen for changes in intrinsic EGFR-related signaling between WT (A549) and L858R (H1975) cell lines. 47 protein end points of 115 tested (Table II) changed more than 20% compared with base line (untreated cells) for either the wild type or mutant cell lines (data not shown).

Dose-dependent EGF Stimulation in WT and Mutant EGFR Cell Lines—To understand the impact of experimental variability we performed three independent experiments with the 47 end points described above that appeared to change more

| Antibody/subcellular location | Source | LCM non-small cell lung cancer | A549, H1975, and H1650 cell line time course | Cell line screening time course | A549 and H1975 cell line time course (triplicate) |
|------------------------------|--------|-------------------------------|---------------------------------------------|---------------------------------|-----------------------------------------------|
| pRAS 40 (Thr-246)            | 5      | X                            | X                                           | X                               | X                                             |
| PTEN (Ser-380)               | 1      | X                            | X                                           | X                               | X                                             |
| RAS-GRF1 (Ser-916)           | 1      | X                            | X                                           | X                               | X                                             |
| RSK3 (Thr-356/Ser-360)       | 1      | X                            | X                                           | X                               | X                                             |
| S6 ribosomal protein (Ser-235/236) | 1   | X                            | X                                           | X                               | X                                             |
| S6 ribosomal protein (Ser-240/244) | 1   | X                            | X                                           | X                               | X                                             |
| SAPK/JNK (Thr-183/Tyr-185)   | 3      | X                            | X                                           | X                               | X                                             |
| SHC (Tyr-317)                | 1      | X                            | X                                           | X                               | X                                             |
| SHIP1 (Tyr-1020)             | 1      | X                            | X                                           | X                               | X                                             |
| SMAD2 (Ser-465/467)          | 1      | X                            | X                                           | X                               | X                                             |
| SRC (Tyr-527)                | 1      | X                            | X                                           | X                               | X                                             |
| SRC family (Tyr-416)         | 1      | X                            | X                                           | X                               | X                                             |
| Tuberin/TSC2 (Tyr-1571)      | 1      | X                            | X                                           | X                               | X                                             |
| TYK2 (Tyr-1054/1055)         | 1      | X                            | X                                           | X                               | X                                             |

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* Many proteins are known to translocate within the cell depending on post-translational modification status. The subcellular location listed is one cellular compartment that the protein may occupy at some point in time in a cell.

1. Cell Signaling Technology; 2. BD Biosciences; 3. Upstate/Millipore, Billerica, MA; 4. Zymed Laboratories Inc., South San Francisco, CA; 5. Biosource/Invitrogen; 6. Lab Vision/ThermoFisher, Fremont, CA; 7. Promega Biosciences, San Luis Obispo, CA.

* Cell line screening time course was a single replicate experiment for global evaluation of cell signaling differences among multiple protein end points. The goal was to determine which end points changed ±20% after EGF ligand stimulation and use this subset of proteins for further in-depth analysis.

* β-Actin was included as a control protein and was analyzed in microdissected cells and cell lines. There was not a significant difference for β-actin in the mutant cells compared with wild type.
than 20% over base line for the three doses of EGF stimulation. β-Actin was analyzed as a control protein in this time course. As expected, the values for β-actin were not significantly different between the mutant and wild type cell lines (data not shown). These studies were conducted on three different days with three independent cell cultures. Table V shows 32 end points that changed significantly over the entire time period (by t test or Wilcoxon rank sum depending on the normality of the data distribution), of 47 end points tested, above unstimulated basal levels for each cell line and ligand dose ($p < 0.01$). A549 cells exhibited 26 different protein end points that responded significantly to EGF stimulation, compared with untreated, basal cells ($p < 0.01$), to at least one of the concentrations of EGF. Levels of 27 protein end points were significantly ($p < 0.01$) different from controls in H1975 cells following EGF ligand stimulation (Table V).

A major finding of the initial time series was recapitulated in these triplicate series. AKT Ser-473 at 50 and 500 ng/ml dosages of EGF was higher over time in mutant compared with WT (Fig. 7). This finding was confirmed by Western blotting and appeared unrelated to any change in the levels of total EGFR (Fig. 8). Similar to what was found for the microdissected tissue, phosphorylation of HER2 Tyr-1248 was significantly up-regulated in the wild type compared with the mutant for all concentrations of ligand ($p < 0.0001$) (Fig. 9). A parallel control arm of the experiment consisted of cell treat-
ment with 1 mM pervanadate to judge the maximum potential phosphorylation level of the analytes being measured. End points that were stimulated following 500.0 ng/ml EGF exposure were within 80% of the maximum value obtained with pervanadate treatment (Fig. 7).

The analysis provided in Table V provides a survey of major differences in the downstream or associated signal proteins that may interact with the EGFR. For this analysis we make the assumption that any protein that exhibits changes in its phosphorylated state in response to EGF ligand stimulation, compared with the unstimulated cells, over 180 min is directly or indirectly interacting with the EGFR. As shown in Table V, there was a time- and concentration-dependent change in 27 (of the original 115 analytes) analyzed for either the mutant or the wild type. Although most of the same proteins were found to be affected by EGF ligand stimulation in the wild type versus the EGF mutant cell line, FOXO1 (FKHR) Thr-24 and CREB Ser-133 were reactive in the wild type but not the

**Fig. 3. Supervised hierarchical clustering of analyte subset determined by Wilcoxon rank sum analysis.** Each row (horizontal axis) represents a patient sample listed by known EGFR mutation status; each column (vertical axis) represents a protein end point found to be significantly different (p < 0.04; Table III) between the wild type EGFR and mutated groups.
mutant at 50 and 500 ng/ml EGF, whereas LKB1 Ser-428 and ENOS Ser-116 were reactive in the mutant but not the wild type for at least two concentrations of EGF. Of note, phosphorylation of EGFR Tyr-1148 in the wild type cells was not significantly reactive until a concentration of 50.0 ng/ml EGF was reached, and for this same residue with the mutant H1975 cells, there was no significant change for any dose of EGF tested (Fig. 9).

Phosphorylation of the following proteins found to be correlated with mutation status in the tissue by Spearman’s \( p \) analysis did not appear to be EGF ligand-stimulated in a time-dependent manner (not greater than 20%) for either the wild type or mutant cell lines: IRS-1 Ser-612 (Fig. 9) and SMAD2 Ser-465/467. SMAD2 Ser-465/467 did not exhibit any changes greater than 20% in the initial screening experiment with multiple doses of EGF for the cell lines.

**DISCUSSION**

EGFR signaling involves five distinct steps: 1) ligand binding, 2) conformational change in the receptor, 3) homodimerization and/or heterodimerization with other ERBB family receptors, 4) autophosphorylation of tyrosine residues, and 5) transphosphorylation of downstream kinases (16, 46). Although the underlying molecular mechanisms are continually being elucidated in greater detail and breadth, a “cancer biomarker problem” still exists for predicting response to EGFR-targeted therapies (47). Somatic mutations within the kinase domain may affect each of these steps independently or result in a cascade of altered downstream signaling events dependent on the specific receptor mutation (30, 36, 48–50).

Genomics and transcriptomics assays do not provide an effective recapitulation of the post-translational, fluctuating signaling events occurring at the proteomic level (33, 47, 51). We utilized reverse phase protein microarrays as a means of quantitatively monitoring the in vivo state of selected phosphoprotein kinase pathways using laser microdissected human NSCLC. We used the same assay technology to monitor in vitro effects of ligand stimulation on NSCLC cell lines with mutated EGFR. The goal of this comparison was to obtain clues about the differential influence of the ligand presence or dose on the mutant EGFR carcinoma phosphoprotein phenotype.

**Value of Laser Capture Microdissection—Jaramillo et al. (6)** recently reported the limited ability of monolayer growth as-
says with A549 cells to effectively recapitulate or predict the in vivo effect of EGFR inhibitors on cell behavior. Although anchorage-dependent growth assays may underestimate the in vivo effects of motility and invasion on cell behavior compared with anchorage-independent assays, information regarding time-dependent EGFR phosphorylation events cannot be assessed in vivo, particularly for human tumors. As shown in the time course of EGF ligand activation (Fig. 9 and Table V) phosphorylation/dephosphorylation events occur in a matter of minutes, and intrinsic differences between the mutants and wild type are found in both the cell lines and human tissue. These differences may reflect the unique ligand-rich tissue microenvironment that affects cross-talk between other receptor systems and the EGFR receptor pathway. Ligands such as EGF cannot be delivered to tissue ex vivo by the bloodstream or in a manner that recapitulates their in vivo delivery state. For these reasons, proteomics analysis of microdissected tissue cells, with complimentary data from in vitro cell culture models, appears to be a critical technology to understand the lung carcinoma cells in the context of the tissue microenvironment (22–25, 42).

Multiple Phosphorylation Sites on the EGFR Are Altered in the EGFR Mutant Carcinomas—More than 13 distinct phosphorylation sites exist on the EGFR (16). The specific type of phosphorylation, the simultaneous phosphorylation of multiple sites, and the time course of the phosphorylation can all influence the proteins that dock with the cytoplasmic tail of the receptor (52–54). In this way the timing and pattern of phosphorylation events can regulate the downstream pathway interconnections emanating from the hetero- or homodimerization of the EGFR with other cell surface receptors. The autophosphorylation sites inside the intracellular tail often serve as docking sites for a range of proteins and initiate cascades of separate and functionally distinct downstream signal pathways (55, 56). Sequence mutations in the cytoplasmic tail are known to affect phosphorylation and the resultant intracellular protein binding (18, 19, 57). Previous studies of post-translational modifications of the epidermal growth factor receptor have indicated that phosphorylation events are transient and rapidly fluctuating over time (32, 56, 58). Indeed the temporal fluctuation of EGFR is thought to be the major mechanism by which a small family of receptors can influence a wide diversity of cellular functions such as regulation, differentiation, and even apoptosis (59, 60). These spatial and temporal fluctuations of EGFR phosphorylation could be causal determinants in diseases such as cancer (56, 61).

In these in vitro studies, the EGF ligand-stimulated fluctuations in the EGFR phosphorylation sites agree generally with those found using other approaches (52–54). The rapid rise followed by a dramatic non-linear decline supports the complex nature of cell signaling in which kinase-driven increases in phosphorylation and phosphatase-induced decreases in phosphorylation orchestrate the output signal of the network.

![Fig. 4. Examples of individual analyte differences shown for microdissected patient samples. LCM-procured non-small cell lung carcinoma cells were analyzed by reverse phase protein microarray. Samples harboring EGFR tyrosine kinase domain mutations (exons 18–21) were compared with wild type EGFR. A, EGFR Tyr-1068 (mean mutant/WT = 0.299 (n = 8), mean WT = 0.093 (n = 17), p < 0.024). B, IRS-1 Ser-612 (mean mutant/WT = 0.170 (n = 8), mean WT = 0.546 (n = 17), p < 0.0004). C, SMAD2 Ser-465/467 (mean mutant/WT = 0.409 (n = 8), mean WT = 0.634 (n = 17), p < 0.0122); 25th and 75th quantile, ∆upper/lower quantile ± 1.5(interquartile range).]
Figs. 6 and 9). Prior studies (Okabe et al. (37)) of EGF ligand stimulation in the same cell lines was confined to a single dose of 100 ng/ml for 15 min, and the readout was Western blotting. In the present study we quantitatively examined a large number of endpoints (Table II) over three dosages of EGF (greater and less than 100 ng/ml) over a series of 10 time points. The results provide an in-depth survey of the potential network remodeling of pathway signaling in mutant versus wild type (Table V).

Unsupervised hierarchical clustering revealed simultaneous up-regulation of EGFR residues Tyr-1148 and Tyr-1068 and down-regulation of HER2 Tyr-1248 and EGFR Tyr-1045 in the mutated EGFR patient cell populations compared with wild type (Figs. 2 and 3). Based on what is known from cell line studies of the wild type receptor, these phosphorylation sites play a role in the docking of the receptor with downstream signal pathways in the phosphatidylinositol 3-kinase/AKT/MTOR, SHC, and MEK/ERK networks (46). In theory, stimulated EGFR could activate a wide variety of downstream signaling pathways (62). The limited number of downstream hyperphosphorylated, activated proteins is in keeping with the expected docking partners for the EGFR phosphorylation sites that are simultaneously up-regulated in the mutant. Thus an elevated level of phosphorylation at AKT Ser-473, ERK Thr-202/Tyr-204, SHC Tyr-317, FOXO1 (FKHR) Thr-24, 4EBP1 Ser-65, HER2 Tyr-1248, ENOS Ser-1177, SRC Y527, SRC Tyr-416, BCL-2 Ser-70, MTOR Ser-2481, platelet-derived growth factor receptor β Tyr-716, EIF4G Ser-1108, and/or Fig. 5. Scatter plots representative of pairwise Spearman’s ρ correlations for the designated analytes from LCM-procured non-small cell lung carcinoma. A and C, wild type EGFR microdissected patient carcinoma cells. B and D, mutated (all classes) EGFR. Correlations judged to have a significant non-parametric correlation coefficient are found in A (IRS-1 Ser-612 correlated with MTOR Ser-2481 in WT, ρ < 0.0001) and D (SHC Tyr-317 correlated with SRC Tyr-527 in EGFR-mutated patient cells, ρ < 0.004). Correlations were not found to be significant for B (IRS-1 Ser-612 and MTOR Ser-2481 in mutant EGFR patient cells) and C (SHC Tyr-317 and SRC Tyr-527 in WT EGFR patient cells).

TABLE IV
Spearman’s ρ non-parametric correlations for microdissected human NSCLC samples

| Protein end point | Protein end point | Spearman ρ | Prob > ρ (p value) |
|-------------------|-------------------|------------|-------------------|
| AKT Thr-308       | AKT Ser-473       | 0.9762     | 0.000003          |
| COX2              | EGFR Tyr-1148     | 0.9762     | 0.000003          |
| COX2              | EGFR Tyr-845      | 0.9322     | 0.0007            |
| EGFR Tyr-992      | EGFR Tyr-1045     | 0.8838     | 0.004             |
| EGFR              | EGFR Tyr-1148     | 0.8809     | 0.004             |
| MTOR Ser-2481     | APC2              | 0.8809     | 0.004             |
| SRC Tyr-527       | SHC Tyr-317       | 0.8809     | 0.004             |
| EGFR Tyr-845      | EGFR Tyr-1148     | 0.8684     | 0.005             |
| 14-3-3 γ γ        | EGFR Tyr-1045     | 0.8625     | 0.006             |
| MTOR Ser-2481     | EGFR              | 0.8571     | 0.006             |
| EGFR              | COX2              | 0.8095     | 0.015             |
| MTOR Ser-2481     | IRS-1 Ser-612     | 0.8767     | 0.000006          |
| COX2              | IRS-1 Ser-612     | 0.8878     | 0.00001           |
| 14-3-3 γ γ        | EGFR Tyr-992      | 0.8612     | 0.00006           |

Molecular & Cellular Proteomics 7.10 1915
FAK Tyr-576 (Table V) suggests that the signaling pathways associated with these EGFR docking proteins are activated in both the wild type and mutant cell lines. This would drive oncogenic cell proliferation/survival, cell migration/invasion, and cell differentiation (46).

Two pieces of evidence point to multiple EGFR phosphorylation events existing at the same point in time for the mutated receptor population. The first is the augmented amplitude of phosphorylation sites on multiple residues for the mutant microdissected carcinomas. The second is a very strong Spearman's correlation ($p < 0.0001$) for pairs of phosphorylation sites on the EGFR within the mutant but not the wild type receptor. The existence of multiple simultaneous phosphorylation sites on the receptor may be a relatively unusual event and could potentially point to an oncogenic state for the mutated receptor. In a previous study (54) we analyzed phosphorylation over six Lys-C fragments of EGFR following EGF ligand stimulation. The long fragments generated by our digestion scheme (e.g. one fragment was over 10 kDa) allow potential estimates of the extent of multiple phosphorylations. We found that diphosphorylation was estimated to be at least 10-fold lower than monophosphorylation. In the previous study most peptide fragments appeared to be monophosphorylated. This observation is a reflection of the average over the population of EGFR molecules where it is unknown what proportion is engaged with EGF. Within the population of tumor cells in the lung tissue microenvironment, the spatial and temporal fluctuations, cell of origin, and molecular class of EGFR ligand is unknown. Consequently the finding of multiple simultaneous and correlated (Figs. 2 and 3) phosphorylation sites on the mutated receptor cases may provide physiologically significant insights.

EGF Receptor Ubiquitination-associated Phosphorylation— Reduced phosphorylation of EGFR Tyr-1045 in the mutant, found to be discriminative in the tissue, also appeared to be altered in a time- and dose-dependent manner in vitro for the EGF ligand-stimulated wild type cell line, reaching a higher amplitude compared with the mutant (Fig. 10). Reduced phosphorylation of EGFR Tyr-1045 noted in EGFR mutant tissue cases has previously been associated in mutant EGFR breast cancer cell lines with modified receptor internalization and degradation (63). Huang et al. (64) reported mutagenesis studies in which EGF receptors that displayed minimal ubiquitination were nonetheless internalized at a rate similar to that of wild type EGF but not degraded. Previous work studying EGFR Tyr-1045, ubiquitination, and degradation has been performed on cell lines or transfected cells (55, 65). Oksvold et al. (55, 66) demonstrated via mutagenesis studies that EGFR serine residues 1046/1047 and tyrosine residue 1045 are essential for receptor ubiquitination in wild type EGFR cell lines.

An interesting observation from our tissue analysis was the strong correlation of 14-3-3 with EGFR Tyr-1045 in the Spearman's $\rho$ analysis of microdissected NSCLC samples (Table IV). We also noted a concomitant hypophosphorylation at Tyr-1045 and reduced levels of 14-3-3 in the EGFR mutant NSCLC samples compared with wild type (Fig. 2). 14-3-3 regulates its binding partners via various mechanisms including formation of protein complexes (66–68) by binding phosphoserine motifs (69). Serine residues in the vicinity of EGFR Tyr-1045 are thought to be critical for receptor degradation as well as attenuation of receptor activity (70, 71).

Site-directed mutagenesis studies of wild type EGFR in NIH3T3 cells revealed that the cytoplasmic tail (amino acids 1029–1186), but not specific serine sites 1046/1047, were necessary for EGF-stimulated binding of 14-3-3 to EGFR.
Because the same finding was present for the wild type versus wild type, for the class of EGFR mutations studied. We speculate that EGFR Tyr-1045 is hypophosphorylated in mutant EGF-treated cultured cells this may reflect one possible intrinsic phenotype of the mutant receptor independent of ligand induction.

As shown in Table III, SMAD phosphorylation was augmented in association with the microdissected lung carcinoma cells containing the wild type EGFR but was reduced in the cases of the EGFR mutation. If this difference reflects an oncogenic difference at the molecular level, then reduced SMAD phosphorylation may reflect a reduced signaling through the transforming growth factor β pathway (72–74).

COX2, an enzyme inducible by cytokines, growth factors, and other stimuli, has been reported to be constitutively elevated in NSCLC (75–77). In the present study we found COX2 levels were correlated with total EGFR, EGFR Tyr-1148, and EGFR Tyr-845 for mutant EGFR, but not the wild type, in the tissue samples. On the other hand, in the wild type tissue samples, COX2 was correlated with IRS-1 Ser-612 but not EGFR (Table IV). In previous studies we have noted an EGF ligand augmented HO-1 production in H23 cell lines (K-RAS 12 mutation) (78).

**TABLE V**

Survey of cell signaling proteins that show a reaction to EGF ligand stimulation over a 180-min time course

| Cell signaling protein | H1975 EGF-treated vs. basal | A549 EGF-treated vs. basal |
|------------------------|-------------------------------|----------------------------|
| n = 32 | | |
| Membrane | | |
| EGFR Tyr-1045 | <0.0001 | <0.0001 | <0.0001 |
| EGFR Tyr-1068 | <0.0001 | <0.0001 | <0.0001 |
| EGFR Tyr-1148 | 0.0005 | <0.0001 | <0.0001 |
| EGFR Tyr-1173 | <0.0001 | <0.0001 | <0.0001 |
| EGFR Tyr-992 | 0.0006 | <0.0001 | <0.0001 |
| HER2 Tyr-1248 | 0.0001 | <0.0001 | <0.0001 |
| FGFR2 Tyr-716 | 0.001 | <0.0001 | <0.0001 |
| EGFR | 0.001 | <0.0001 | <0.0001 |
| c-KIT Tyr-703 | 0.0085 | <0.0001 | <0.0001 |
| Cytoplasm | | |
| AKT Ser-473 | <0.0001 | <0.0001 | <0.0001 |
| AKT Thr-308 | 0.0007 | 0.0056 | 0.0042 |
| B-Raf Ser-445 | 0.0311 | 0.0009 | 0.0017 |
| EIF4G Ser-1108 | 0.0007 | 0.0074 | 0.0001 |
| ERK Thr-202/Tyr-204 | 0.0036 | 0.0001 | 0.0001 |
| FAK Tyr-376 | <0.0001 | <0.0001 | <0.0001 |
| FOXO1 (FKHR) Thr-24 | 0.0029 | <0.0001 | <0.0001 |
| GAB1 Tyr-627 | 0.0001 | <0.0001 | <0.0001 |
| GSK3β Ser-21/9 | <0.0001 | <0.0001 | <0.0001 |
| LCK Tyr-505 | <0.0001 | <0.0001 | <0.0001 |
| LKB1 Ser-428 | <0.0001 | <0.0001 | <0.0001 |
| MAPK pTEpY | <0.0001 | <0.0001 | <0.0001 |
| p70S6 Thr-389 | 0.0005 | 0.003 | 0.0002 |
| p70S6 Thr-412 | 0.0001 | <0.0001 | <0.0001 |
| PTEN Ser-380 | 0.0001 | <0.0001 | <0.0001 |
| SHC Tyr-317 | <0.0001 | <0.0001 | <0.0001 |
| SRC Tyr-416 | 0.0001 | <0.0001 | <0.0001 |
| TYK2 Tyr-1054 | <0.0001 | <0.0001 | <0.0001 |
| Nucleus | | |
| CREB Ser-133 | <0.0001 | <0.0001 | <0.0001 |
| ENOS III Ser-116 | 0.0139 | 0.0042 | 0.0001 |
| p90RSK Ser-380 | 0.0225 | <0.0001 | <0.0001 |
| RSK3 Thr-356 | 0.0139 | 0.0042 | 0.0001 |

(66). Although the authors did not draw any definitive conclusions regarding the significance of this interaction, we may speculate that a mutation in the tyrosine kinase inhibitor domain site, such as L858R, also affects the binding of 14-3-3 proteins. Dysregulation of 14-3-3 binding may possibly interfere with phosphorylation of Tyr-1045 and/or interfere with c-CBL binding, thus further reducing the rate of receptor ubiquitination.

The present study with tissue samples supports the concept that EGFR Tyr-1045 is hypophosphorylated in vivo, compared with wild type, for the class of EGFR mutations studied. Because the same finding was present for the wild type versus mutant EGF-treated cultured cells this may reflect one possible intrinsic phenotype of the mutant receptor independent of ligand induction.

Implications of Phosphorylation Patterns Influenced by the EGFR Network—The mutant and wild type samples could be influenced by unknown mutations in non-EGFR genes that could directly or indirectly affect the EGR pathway. Indeed through these alternative routes the EGF pathway may be active and involved in the pathogenesis of the tumor without...
FIG. 7. Experimental treatment with EGF: H1975 cells exhibit greater levels of phosphorylation on AKT Ser-473 post-EGF stimulation as compared with wild type A549 cells. Shown are triplicate time course series of NSCLC cell lines treated with 5, 50, and 500 ng/ml EGF or 1 mM pervanadate at time 0. Cells were harvested at 0, 1, 3, 5, 7, 9, 15, 30, 60, and 180 min poststimulation. Each plot compares the response over time for AKT Ser-473 of A549 wild type cells with the H1975 L858R mutant cells. For A–C, the full 180-min time course is shown in the left panel; an expanded view of the first 15 min is displayed in the right panel. A, 5.0 ng/ml EGF; B, 50.0 ng/ml EGF; C, 500.0 ng/ml EGF; D, 1.0 mM pervanadate; (mean, n = 3; ± S.D.).
the requirement for a mutation in the EGF receptor itself. In the microdissected tissue, there were six of 17 patients without known EGFR mutations who exhibited phosphorylation of one or more individual sites on the EGFR and associated increased phosphorylation of IRS-1 Ser-612, AKT Ser-473, AKT Thr-308, and ERK Thr-202/Tyr-204. These patients may benefit from tyrosine kinase inhibitor therapy even though they do not harbor a known mutation in the EGF receptor. One challenge facing rational drug design of ERBB family receptors is receptor homodimerization and heterodimerization with other ERBB family members including VEGFR, insulin-like growth factor receptor (IGF-1R), and ERBB2 (HER2) (28). Therefore, we included an analysis of ERBB2 (HER2)- and IGF-1R-related signaling. Phosphorylation of HER2 Tyr-1248 was up-regulated in the wild type EGFR carcinoma cells for both tissue samples (Figs. 2 and 3) and EGF-stimulated cell lines compared with the mutant (Fig. 9). This difference may reflect a higher level of heterodimerization between EGFR and HER2 in the wild type cells and may be an intrinsic phenotype of the mutant versus the wild type.

In both sets of time course EGF ligand-treated cell line experiments AKT was persistently elevated for the EGFR mutant cell line compared with the wild type for all dosages of EGF stimulation (5, 50, 100, or 500 ng/ml). The defect produced a longer lasting up-regulation of phosphorylated AKT in the EGFGR mutation carcinoma compared with the wild type receptor following EGF ligand stimulation. Translating this hypothesis to the in vivo carcinoma cell population, we can postulate that, for the case of the known mutated EGFR carcinoma population, at any point in time a higher proportion of the cells will exhibit activation of the AKT pathway thus suppressing apoptosis and providing a survival advantage.

The differential phosphorylation events for the mutant EGFR (regardless of the class of the receptor mutation in vivo Table I) could be a consequence of the altered three-dimensional conformation of the receptor itself or a difference in its propensity for heterotypic dimerization with other ERBB members. This is supported by the altered and less robust phospho-EGFR dose response for the mutant compared with the wild type receptor and the correlation with HER2 phos-
phosphorylation. A specific mechanism that could extend the phosphorylation state of AKT noted for the mutant EGFR is the activity of the negative feedback loop down-regulating AKT from MTOR through IRS-1 (79). IGF-1R induces antiapoptotic signaling via IRS-1, SHC, and 14-3-3 in A549 cells. The IGF-1R tyrosine kinase inhibitor AG1024 has been demonstrated to decrease both IRS-1 and SHC phosphorylation in A549 cells (80). It has previously been shown that the MTOR pathway (MTOR is a substrate of AKT) is connected in a negative feedback loop through the IRS-1 Ser-612 phosphorylation site (40, 81–84). In the wild type receptor this residue was one of the few residues correlated with MTOR Ser-2481 and COX2 (Table IV). In contrast, a correlation with IRS-1 Ser-612 was not noted for the mutant. This finding is similar to

**Fig. 9.** Triplicate time course of NSCLC cell lines treated with 5, 50, or 500 ng/ml EGF. The first 30 min of the 180-min time course is shown to illustrate the early fluctuations noted in response to EGF ligand stimulation compared with untreated cells. Cells were harvested at 0, 1, 3, 5, 7, 9, 15, 30, 60, or 180 min poststimulation. Each plot compares the mean response over time for three different EGF dosages shown for one cell line (data shown are triplicate mean treated spot intensity values/triplicate mean untreated control spot intensity values ± S.D.).

A, HER2 Tyr-1248. A significant difference was noted for all EGF doses over time in both the A549 WT and H1975 L858R cell lines (t test, p < 0.001). Note the scale differences between A549 WT cells and H1975 L858R mutated cells. B, IRS-1 Ser-612. No significant variance was noted between EGF-treated WT or mutated cell lines over time. C, EGFR Tyr-1148. Significant variance over time was noted for A549 WT cells only at 500 ng/ml EGF (t test, p < 0.0001); (mean, n = 3; ± S.D.).
that observed for microdissected rhabdomyosarcoma for which the aggressive tumors were associated with an increase in AKT Ser-473 and a lack of correlation of MTOR with IRS-1 Ser-612 (40).

As shown in Fig. 2, total EGFR levels were variable between both the mutant and wild type patient samples. As noted previously, neither histoclinicopathologic features nor the number of receptors can accurately predict an individual’s therapeutic response (85, 86). Thus, a combination of genomics and proteomics molecular analyses may be the best theranostic indicator for individualized therapy (86, 87).

Despite the fact that our tissue and cell line results led us to conclusions about signaling differences in the mutant relevant to the oncogenic phenotype, we cannot exclude that the differences in the tissue samples between the mutant and the wild type are indirectly influenced by known or unknown epidemiologic or genetic differences in the carcinoma cells or the non-tumor tissue of the patient. In this small study of lung carcinoma tissue, any conclusions we make about molecular differences have to be considered in this context. For this reason, the comparison of the in vitro EGF ligand-treated cell line studies with the findings based on microdissected cells provides some degree of assurance that the conclusions based on the LCM-procured carcinoma cells reflect a true difference between the mutant and wild type cells.

These findings, based on microdissected NSCLC, have relevance to the individualized therapy of lung cancer with drugs that inhibit the EGFR pathway. An elevated level of phosphorylation of one or more of these residues in proteins of associated downstream pathways, compared with a baseline value, may indicate that the patient is likely to be responsive to (a) EGFR therapy (e.g. to treatment with an agent that inhibits the kinase activity of EGFR), (b) a kinase inhibitor directed toward an associated downstream pathway, or (c) a combination of an EGFR inhibitor and an inhibitor of a member of one of the identified downstream pathways. This is because activity of the downstream proteins indicates that the entire EGF-associated pathway is active and in use in the cancer cells. If the entire pathway is active, then the cancer cell is more likely to be driven by this pathway and thus would be effectively treated by blocking multiple nodes along this pathway.

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LCM and Protein Microarray Analysis of Human NSCLC

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REFERENCES

1. Jain, M., Arvanitis, C., Chu, K., Dewey, W., Leonhardt, E., Trinh, M., Sundberg, C. D., Bishop, J. M., and Felsher, D. W. (2002) Sustained loss of a neoplastic phenotype by brief inactivation of MYC. Science 297, 102–104

2. Weinstein, I. B. (2000) Disorders in cell circuitry during multistage carcinogenesis: the role of homeostasis. Carcinogenesis 21, 857–864

3. Haigler, H. T., Schlaepfer, D. D., and Burgess, W. H. (1987) Characterization of the Src-related tyrosine kinase activity of the epidermal growth factor receptor expressed on A-431 cells. J. Biol. Chem. 262, 9418–9425

4. Ayuso-Sacido, A., Graham, C., Greenfield, J. P., and Boockvar, J. A. (2006) The duality of epidermal growth factor receptor (EGFR) signaling and neurexin cell phenotype: cell enhancer or cell transformer? Curr. Stem Cell Res. Ther. 1, 387–394

5. Haugh, J. M., Huang, A. C., Wiley, H. S., Wells, A., and Lauffenburger, D. A. (2004) The dimerization potential of mutant EGFRs. J. Biol. Chem. 279, 45299–45307

6. Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Siegfried, J. M. (2007) Gastrin-releasing peptide activates Akt through Akt-3/4/5 downstream effector of mutant epidermal growth factor receptor signaling. Cancer Res. 67, 11389–11398

7. Citri, A., and Yarden, Y. (2006) EGF-ERBB signalling: towards the systems level. Nat. Rev. Mol. Cell Biol. 7, 505–516

8. Burgess, A. W., Cho, H. S., Eigenbrot, C., Ferguson, K. M., Garrett, T. P., Leahy, D. J., Lemmon, M. A., Slwikowski, M. X., Ward, C. W., and Yokoyama, S. (2003) An open-and-shut case? Recent insights into the activation of EGFR/ErbB receptors. Mol. Cell 12, 541–552

9. Sharma, S. V., Bell, D. W., Settleman, J., and Haber, D. A. (2007) Epidermal growth factor receptor mutations in lung cancer. Nat. Rev. Cancer 7, 169–181

10. Hwang, D. L., Tey, Y. C., Lin, S. S., and Lev-Ran, A. (1986) Expression of epidermal growth factor receptors in human lung tumors. Cancer 58, 2260–2263

11. Lynch, T. J., Bell, D. W., Sordella, R., Gurubhagavatula, S., O’Brien, R. A., Brannigan, B. W., Harris, P. L., Haserlat, S. M., Supko, J. G., Haluska, F. G., Louis, D. N., Christiani, D. C., Settleman, J., and Haber, D. A. (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N. Engl. J. Med. 350, 2129–2139

12. Paez, J. G., Janne, P. A., Lee, J. C., Tracy, S., Greulich, H., Gabriel, S., Herman, P., Kaye, F. J., Lindeman, N., Boggon, T. J., Naoki, K., Sasaki, H., Fuji, Y., Eck, M. J., Sellers, W. R., Johnson, B. E., and Meyerson, M. (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 304, 1497–1500

13. Sordella, R., Bell, D. W., Haber, D. A., and Settleman, J. (2004) Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science 305, 1163–1167

14. Takano, T., Ohe, Y., Sakamoto, H., Tsuta, K., Matsuou, Y., Tateishi, U., Yamamoto, S., Nokihara, H., Yamamoto, N., Seiken, I., Kunitoh, H., Shibata, T., Sakiyama, T., Yoshiida, T., and Tamura, T. (2005) Epidermal growth factor receptor gene mutations and increased copy numbers predict gefitinib sensitivity in patients with recurrent non-small-cell lung cancer. J. Clin. Oncol. 23, 8629–8637

15. Espina, V., Wulfkuhle, J. D., Calvert, V. S., VanMeter, A., Zhou, W., Coukos, G., Gehe, D. H., Petricoin, E. F., III, and Liotta, L. A. (2006) Laser-capture microdissection. Nat. Protoc. 1, 586–603

16. Espina, V., Helby, M., Pierobon, M., and Liotta, L. A. (2007) Laser capture microdissection technology. Expert Rev. Mol. Diagn. 7, 647–657

17. Wulfkuhle, J. D., Speer, R., Pierobon, M., Laird, J., Espina, V., Deng, J., Mammano, E., Yang, S. X., Swain, S. M., Nitti, D., Esserman, L. J., Belluco, C., Liotta, L. A., and Petricoin, E. F. (2008) Multiplexed cell signaling analysis of human breast cancer applications for personalized therapy. J. Proteome Res. 7, 1508–1517

18. Emmert-Buck, M. R., Bonner, R. F., Smith, P. D., Chuaqui, R. F., Zhuang, Z., Goldstein, S. R., Weiss, R. A., and Liotta, L. A. (1996) Laser capture microdissection. Science 274, 988–1001

19. Bonner, R. F., Emmert-Buck, M., Cole, K., Pohida, T., Chuaqui, R., Goldstein, S., and Liotta, L. A. (1997) Laser capture microdissection: molecular analysis of tissue. Science 278, 1481–1483

20. Morgillo, F., Woo, J. K., Kim, E. S., Hong, W. K., and Lee, H. Y. (2006) Heterodimerization of insulin-like growth factor receptor/epidermal growth factor receptor and induction of survivin expression counteracts the antitumor action of erlotinib. Cancer Res. 66, 10100–10111

21. Giardiello, F., Troiani, T., Bianco, R., Orditura, M., Morgillo, F., Martinelli, E., Morelli, M. P., Cascone, T., and Tortora, G. (2006) Interaction between the epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor (VEGF) pathways: a rational approach for multi-target anticancer therapy. Expert Rev. Mol. Diagn. 6, Suppl. 7, vii109–vii114

22. Kobayashi, S., Shimamura, T., Monti, S., Steidl, U., Hetherington, C. J., Lowell, A. M., Golub, T., Meyerson, M., Tenen, D. G., Shapiro, G. I., and Haimos, B. (2006) Transcriptional profiling identifies cyclin D1 as a critical downstream effector of the epidermal growth factor receptor signaling. Cancer Res. 66, 11389–11398

23. Liu, X., Carlisle, D. L., Swick, M. C., Gaither-Davis, A., Grandis, J. R., and Siegfried, J. M. (2007) Gastrin-releasing peptide activates Akt through the epidermal growth factor receptor pathway and abrogates the effect of gefitinib. Exp. Cell Res. 313, 1361–1372

24. Pawson, T., Gish, G. D., and Naah, P. (2001) SH2 domains, interaction modules and cellular wiring. Trends Cell Biol. 11, 504–511

25. Schlessinger, J. (2004) Common and distinct elements in cellular signaling via EGFR and EGFR receptors. Science 306, 1506–1507

26. Gilmer, T. M., Cable, L., Alligood, K., Rusnak, D., Spehar, G., Gallagher, K. T., Woldu, E., Carter, H. L., Truesdale, A. T., Shewchuk, L., and Wood, E. R. (2008) Impact of common epidermal growth factor receptor and HER2 variants on receptor activity and inhibition by lapatinib. Cancer
34. Janne, P. A., Borras, A. M., Kuang, Y., Rogers, A. M., Joshi, V. A., Liyanage, H., Lindeman, N., Lee, J. C., Halmos, B., Maher, E. A., Distel, R. J., Meyerson, M., and Johnson, B. E. (2006) A rapid and sensitive enzymatic method for epidermal growth factor receptor mutation screening. Clin. Cancer Res. 12, 751–758

35. Jackman, M. D., Yeang, B. Y., Sequist, L. V., Lindeman, N., Holmes, A. J., Joshi, V. A., Bell, D. W., Huberman, M. S., Halmos, B., Rabini, M. S., Haber, D. A., Lynch, T. J., Meyerson, M., Johnson, B. E., and Janne, P. A. (2006) Exon 19 deletion mutations of epidermal growth factor receptor are associated with prolonged survival in non-small cell lung cancer patients treated with gefitinib or erlotinib. Clin. Cancer Res. 12, 3908–3914

36. Ueda, A., Hirano, S., Kitaoh, H., Ogino, A., Rai, K., Toyooka, S., Takigawa, N., Tabata, M., Takada, M., Kiura, K., and Tanimoto, M. (2007) Activation of downstream epidermal growth factor receptor (EGFR) signaling provides gefitinib-resistance in cells carrying EGFR mutation. Cancer Sci. 98, 357–363

37. Okabe, T., Okamoto, I., Tamura, K., Terashima, M., Yoshida, T., Satoh, T., Takada, M., Fukuoka, M., and Nakagawa, K. (2007) Differential constitutive activation of the epidermal growth factor receptor in non-small cell lung cancer cells carrying EGFR gene mutation and amplification. Cancer Res. 67, 2046–2053

38. Wang, S. H., Mechanic, L. E., Yang, P., Landi, M. T., Bowman, E. D., Wampfler, J., Meerza, D., Hong, K. M., Mann, F., Dracheva, T., Fukuoka, J., Travis, W., Caparoso, N. E., Harris, C. C., and Jen, J. (2005) Mutations in the tyrosine kinase domain of the epidermal growth factor receptor in non-small cell lung cancer. Cancer Res. 65, 2106–2110

39. Yang, S. H., Mechanic, L. E., Yang, P., Landi, M. T., Bowman, E. D., Wampfler, J., Meerza, D., Hong, K. M., Mann, F., Dracheva, T., Fukuoka, J., Travis, W., Caparoso, N. E., Harris, C. C., and Jen, J. (2005) Mutations in the tyrosine kinase domain of the epidermal growth factor receptor in non-small cell lung cancer. Cancer Res. 65, 7960–7961

40. Petricoin, E. F., III, Espina, V., Araujo, R. P., Midura, B., Yeung, C., Wan, X., Eichler, G. S., Johann, D. J., Jr., Qualman, S., Tsokos, M., Krishnan, K., Helman, L. J., and Liotta, L. A. (2000) The ErbB family of growth factor receptors: identification of circulating tumor markers in lung cancer patients treated with gefitinib or erlotinib. Science 287, 864–867

41. Pao, W., Miller, V., Zakowski, M., Doherty, J., Politi, K., Sarkaria, I., Singh, B., Heelan, R., Rabin, M. S., Rape, B., Heelan, R., Fukuoka, M., and Nakagawa, K. (2006) The role of EGF receptor mutations in lung cancers from “never smokers” and are associated with sensitivity to tumors to gefitinib and erlotinib. Proc. Natl. Acad. Sci. U. S. A. 103, 13618–13623

42. Petricoin, E. F., III, Espina, V., Araujo, R. P., Midura, B., Yeung, C., Wan, X., Eichler, G. S., Johann, D. J., Jr., Qualman, S., Tsokos, M., Krishnan, K., Helman, L. J., and Liotta, L. A. (2000) The ErbB family of growth factor receptors: identification of circulating tumor markers in lung cancer patients treated with gefitinib or erlotinib. Science 287, 864–867

43. Olayioye, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2000) The ErbB signaling network: receptor heterodimerization in development and cancer. Embo J. 19, 3159–3167

44. Rikova, K., Guo, A., Zeng, Q., Possemato, A., Yu, J., Maack, H., Nardone, J., Li, C., Mitchell, J., Wetzel, R., Macneil, J., Ren, J. M., Yuan, J., Bakalets, C. E., Villen, J., Komnaheer, J., Smith, B., Li, D., Zhou, X., Gygi, S. P., Gu, T. L., Polakiewicz, R. D., Rush, J., and Comb, M. J. (2007) Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell 131, 1190–1203

45. Huang, F., Goh, L. K., and Sorkin, A. (2007) EGFR receptor ubiquitination is not necessary for its internalization. Proc. Natl. Acad. Sci. U. S. A. 104, 16904–16909

46. Padron, D., Sato, M., Shah, J. W., Gazdar, A. F., Minna, J. D., and Roth, M. G. (2007) Epidermal growth factor receptors with tyrosine kinase domain mutations exhibit reduced Cbl association, poor ubiquitination, and down-regulation but are efficiently internalized. Cancer Res. 67, 7695–7702

47. Oksvold, M. P., Thien, C. B., Widerberg, J., Chantry, A., Hultfelt, H. S., and Lundqvist, W. Y. (2015) Global proteome analysis for identification of functional protein-protein interactions in human NSCLC. Cancer Res. 75, 3161–3168

48. Fukuoka, J., Travis, W., Caporaso, N. E., Harris, C. C., and Jen, J. (2005) First-line gefitinib or erlotinib treatment in patients with advanced non-small-cell lung cancer: a phase III randomised trial. Lancet 366, 1261–1268

49. Arai, J. Y., Arai, K., Morii, K., Yamada, T., Suehiro, S., and Nakamura, Y. (2005) Identification of novel proteins that show increased binding of the ligand-induced ubiquitination and internalization of the EGFR receptor do not affect c-Cbl association with the receptor. Oncogene 24, 8509–8518

50. Shiogama, N., and Lane, H. A. (2005) EGFR and cancer: the complexity of targeted inhibitors. Nat. Rev. Cancer 5, 341–354

51. Uramoto, H., and Mitsudomi, T. (2007) Which biomarker predicts benefit from EGFR-TKI treatment for patients with lung cancer? Br. J. Cancer 96, 857–883

52. Agar, J. F., Toethtcher, J. E., Endy, D., White, F. M., and Tidor, B. (2008) Stimulus design for model selection and validation in cell signaling. PLoS Comput. Biol. 4, 603

53. Evangelista, H., and Mitsudomi, T. (2007) Which biomarker predicts benefit from EGFR-TKI treatment for patients with lung cancer? Br. J. Cancer 96, 857–883

54. Zhang, J., Kalyankrishna, S., Wislez, M., Thilaganathan, N., Saigal, B., Wei, H., Xia, K., Pallas, D. C., Cui, C., Conroy, K., Narsimhan, R. P., Mamon, H., Collier, R. J., and Roberts, T. M. (1994) Interaction of the protein kinase Raf-1 with 14-3-3 proteins. Science 266, 126–129

55. Engelman, J. A., Zeijnallullah, K., Mitsudomi, T., Song, Y., Hyland, C., Park, J. O., Lindeman, N., Gale, C. M., Zhao, X., Christensen, J., Kozaka, T., Holmes, A. J., Rogers, A. M., Cappuzzo, F., Mok, T., Lee, C., Johnson, B. E., Cantyey, L. C., and Janne, P. A. (2007) MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 316, 1039–1043

56. Huang, F., Goh, L. K., and Sorkin, A. (2007) EGFR receptor ubiquitination is not necessary for its internalization. Proc. Natl. Acad. Sci. U. S. A. 104, 16904–16909

57. Padron, D., Sato, M., Shah, J. W., Gazdar, A. F., Minna, J. D., and Roth, M. G. (2007) Epidermal growth factor receptors with tyrosine kinase domain mutations exhibit reduced Cbl association, poor ubiquitination, and down-regulation but are efficiently internalized. Cancer Res. 67, 7695–7702

58. Oksvold, M. P., Thien, C. B., Widerberg, J., Chantry, A., Hultfelt, H. S., and Lundqvist, W. Y. (2015) Global proteome analysis for identification of functional protein-protein interactions in human NSCLC. Cancer Res. 75, 3161–3168
70. Theroux, S. J., Taglienti-Sian, C., Nair, N., Countaway, J. L., Robinson, H. L., and Davis, R. J. (1992) Increased oncogenic potential of ErbB is associated with the loss of a COOH-terminal domain serine phosphorylation site. J. Biol. Chem. 267, 7967–7970

71. Countaway, J. L., McQuilkin, P., Girones, N., and Davis, R. J. (1990) Multisite phosphorylation of the epidermal growth factor receptor. Use of site-directed mutagenesis to examine the role of serine/threonine phosphorylation. J. Biol. Chem. 265, 3407–3416

72. Akhurst, R. J. (2002) TGF-β antagonists: why suppress a tumor suppressor? J. Clin. Investig. 109, 1533–1536

73. Derynck, R., Akhurst, R. J., and Balmain, A. (2001) TGF-β signaling in tumor suppression and cancer progression. Nat. Genet. 29, 117–129

74. Wakefield, L. M., and Roberts, A. B. (2002) TGF-β signaling: positive and negative effects on tumorigenesis. Curr. Opin. Genet. Dev. 12, 22–29

75. Hida, T., Yatabe, Y., Achiwa, H., Muramatsu, H., Kozaki, K., Nakamura, S., Ogawa, M., Mitsudomi, T., Sugiyama, T., and Takahashi, T. (1998) Increased expression of cyclooxygenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas. Cancer Res. 58, 3761–3764

76. Hida, T., Kozaki, K., Muramatsu, H., Masuda, A., Shimizu, S., Mitsudomi, T., Sugiyama, T., Ogawa, M., and Takahashi, T. (2000) Cyclooxygenase-2 inhibitor induces apoptosis and enhances cytotoxicity of various anti-cancer agents in non-small cell lung cancer cell lines. Clin. Cancer Res. 6, 2006–2011

77. Huang, M., Stolina, M., Sharma, S., Mao, J. T., Zhu, L., Miller, P. W., Wollman, J., Herschman, H., and Dubinett, S. M. (1998) Non-small cell lung cancer cyclooxygenase-2-dependent regulation of cytokine balance in lymphocytes and macrophages: up-regulation of interleukin 10 and down-regulation of interleukin 12 production. Cancer Res. 58, 1208–1216

78. Winters, M. E., Mehta, A. I., Petricoin, E. F., III, Kohn, E. C., and Liotta, L. A. (2005) Supra-additive growth inhibition by a celecoxib analogue and carboxamido-triazole is primarily mediated through apoptosis. Cancer Res. 65, 3853–3860

79. Gual, P., Le Marchand-Brustel, Y., and Tanti, J. F. (2005) Positive and negative regulation of insulin signaling through IRS-1 phosphorylation.

80. Biochimie (Paris) 87, 99–109

81. Cosaceanu, D., Carapancea, M., Alexandru, O., Budiu, R., Martinsson, H. S., Starborg, M., Vrabete, M., Karst, L., Lewensohn, R., and Dricu, A. (2007) Comparison of three approaches for inhibiting insulin-like growth factor I receptor and their effects on NSCLC cell lines in vitro. Growth Factors 25, 1–8

82. Han, C. H., Cho, J. Y., Moon, J. T., Kim, H. J., Kim, S. K., Shin, D. H., Chang, J., Ahn, C. M., and Chang, Y. S. (2006) Clinical significance of insulin receptor substrate-I down-regulation in non-small cell lung cancer. Oncol. Rep. 16, 1205–1210

83. Tremblay, F., and Marette, A. (2001) Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway. A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. J. Biol. Chem. 276, 38522–38526

84. Oliveira, J. C., Souza, K. K., Dias, M. M., Faria, M. C., Ropelle, E. R., Flores, M. B., Ueno, M., Veloso, L. A., Saad, S. T., Saad, M. J., and Carvalheira, J. B. (2003) Antineoplastic effect of rapamycin is potentiated by inhibition of IRS-1 signaling in prostate cancer cells xenografts. J. Cancer Res. Clin. Oncol. 134, 833–839

85. Perez-Torres, M., Guix, M., Gonzalez, A., and Arteaga, C. L. (2006) Epidermal growth factor receptor (EGFR) antibody down-regulates mutant receptors and inhibits tumors expressing EGFR mutations. J. Biol. Chem. 281, 40183–40192

86. Blons, H., Cote, J. F., Le Corre, D., Riquet, M., Fabre-Guilevin, E., Laurent-Puig, P., and Daniel, C. (2006) Epidermal growth factor receptor mutation in lung cancer are linked to bronchioalveolar differentiation. Am. J. Surg. Pathol. 30, 1309–1315

87. Pugh, T. J., Bebb, G., Barclay, L., Sutcliffe, M., Fee, J., Salski, C., O’Connor, R., Ho, C., Murray, N., Melosky, B., English, J., Veliakind, J., Horsman, D., Laskin, J. J., and Marra, M. A. (2007) Correlations of EGFR mutations and increases in EGFR and HER2 copy number to gefitinib response in a retrospective analysis of lung cancer patients. BMC Cancer. 7, 128