Protein Expression Signatures for Inhibition of Epidermal Growth Factor Receptor Mediated Signaling

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Abstract

Analysis of cellular signaling networks typically involves targeted measurements of phosphorylated protein intermediates. However, phosphoproteomic analyses usually require affinity enrichment of phosphopeptides and can be complicated by artifactual changes in phosphorylation caused by uncontrolled preanalytical variables, particularly in the analysis of tissue specimens. We asked whether changes in protein expression, which are more stable and easily analyzed, could reflect network stimulation and inhibition. We employed this approach to analyze stimulation and inhibition of the epidermal growth factor receptor (EGFR) by EGF and selective EGFR inhibitors. Shotgun analysis of proteomes from proliferating A431 cells, EGF-stimulated cells and cells co-treated with the EGFR inhibitors cetuximab or gefitinib identified groups of differentially expressed proteins. Comparisons of these protein groups identified 13 proteins whose EGF-induced expression changes were reversed by both EGFR inhibitors. Targeted multiple-reaction-monitoring (MRM) analysis verified differential expression of 12 of these proteins, which comprise a candidate EGFR inhibition signature. We then tested these 12 proteins by MRM analysis in 3 other models: 1) a comparison of DiFi (EGFR inhibitor-sensitive) and HCT116 (EGFR-insensitive) cell lines, 2) in formalin-fixed, paraffin-embedded (FFPE) mouse xenograft DiFi and HCT116 tumors, and 3) in tissue biopsies from a patient with the gastric hyperproliferative disorder Menetrier's disease, who was treated with cetuximab. Of the proteins in the candidate signature, a core group, including c-Jun, jagged-1, and claudin 4 were decreased by EGFR inhibitors in all three models. Although the goal of these studies was not to validate a clinically-useful EGFR inhibition signature, the results confirm the hypothesis that clinically-used EGFR inhibitors generate characteristic protein expression changes. This work further outlines a prototypical approach to derive and test protein expression signatures for drug action on signaling networks.
Introduction

Signaling networks and pathways regulate essential cellular functions. Activities of pathways are controlled by posttranslational modification of key pathway intermediates, such as signaling receptors and their downstream effectors, which undergo reversible phosphorylation. Immunoblot methods are most commonly used to monitor protein phosphorylation changes, but this approach is limited by the availability and specificity of antibody reagents. Mass spectrometry-based proteomic approaches aimed at the detection of phosphorylation modifications have proven useful in the investigation of cellular signaling events (1-3) and have been shown to identify protein phosphorylation changes in response to drug treatments (4). Phosphoproteome analysis methods typically require affinity enrichment of phosphorylated proteins or peptides to detect low abundance phosphorylated forms (1, 3-5). The transient nature of phosphorylation modifications also presents the challenge of preserving phosphorylation status during sample preparation. Most work in phosphoproteomics has been done in cell culture models, which offer the advantages of controlled experimental conditions, relatively easy sample workup and scalability to enable analysis of low abundance phosphoproteins. Phosphoproteomic analysis of tissue specimens is complicated by sample heterogeneity, limiting amounts of available tissue, and low abundance of modified peptides. In addition, acquisition practices for biopsies and surgical resections do not permit rigorous control of preanalytical variables, such as ischemic time and temperature, which trigger stress responses that may obscure the status of network intermediates in vivo (6-8). Given these considerations, more robust approaches to measure signaling networks are needed to overcome the shortcomings of direct phosphoproteome analyses.

One of the most extensively studied signaling pathways is driven by the epidermal growth factor receptor (EGFR), a receptor tyrosine kinase that influences a broad range of signaling events and biological processes. Upon ligand binding, EGFR dimerizes with itself or with other ErbB proteins and the receptor is autophosphorylated at multiple residues (9). Signal
transduction occurs by recruitment of adaptor proteins and activation of downstream kinases in
the MAPK, PI3K and mTOR pathways (10). EGFR activation plays a critical role in many
human cancers and several anticancer drugs directed at this receptor tyrosine kinase are in
clinical use (11). EGFR mutations can modify responsiveness to EGFR-inhibiting drugs and
are associated with acquired resistance to inhibitors (12, 13).

Despite the broad importance of EGFR as a therapeutic target, prediction and
assessment of therapeutic responses to EGFR inhibitors presents a significant clinical problem
(14). Negative predictors of response include mutations in KRAS, which constitutively activate
MAPK signaling and block cellular response to EGFR inhibiting drugs (12, 15). Similarly,
mutations in PIK3CA also confer resistance to EGFR inhibition with cetuximab (16, 17). Protein
and phosphoprotein analyses in tumor tissues by reverse phase protein array methods have
identified putative signatures for EGFR inhibitor responses (18-21). Studies in cell models using
global phosphoproteomics and targeted analysis of EGF pathway phosphoprotein intermediates
have provided the most comprehensive analyses of EGFR-driven signaling networks (1, 22-24).
Despite the rapid growth of information about EGFR signaling networks, identification of robust
molecular markers linking network status and therapeutic response remains an open challenge.
Indeed, skin rash remains one of the most effective early indicators of clinical response to
EGFR inhibitors (25).

We asked whether changes in global protein expression levels could produce distinct
protein signatures indicative of a cellular response to EGFR modulation. To address this issue,
we employed a model system in A431 cells using epidermal growth factor (EGF) and two
clinically-used EGFR inhibitors, gefitinib and cetuximab, to manipulate the EGFR signaling axis.
We analyzed differentially treated A431 cells with a standardized shotgun proteomics platform
that combines peptide isoelectric focusing and LC-MS/MS (26, 27). Comparison of these
datasets indicated proteins that differed significantly in expression between treatment conditions
and constitute potential stimulation and inhibition signatures. A set of proteins whose
expression was changed by EGF and reversed by both gefitinib and cetuximab comprised a candidate “EGFR inhibition signature”, which we further verified by multiple reaction monitoring (MRM) analyses. We then tested this EGFR inhibition signature in 3 other models: 1) a comparison of DiFi (EGFR inhibitor-sensitive) and HCT116 (EGFR-resistant) cell lines, 2) in formalin-fixed, paraffin-embedded (FFPE) mouse xenograft DiFi and HCT116 tumors, and 3) in frozen tissue biopsies from a patient with the gastric hyperproliferative disorder Menetrier’s disease, who was treated with cetuximab. The results verified a multiprotein EGFR inhibition signature in all three models and illustrate the application of protein expression changes as surrogate measures of signaling network activation and inhibition.
Experimental procedures

Materials and reagents

Iodoacetamide, ammonium bicarbonate, sodium molybdate, β-glycerophosphate, sodium molybdate, sodium orthovanadate, 4-(2-Aminoethyl)benzenesulfonyl fluoride, aprotinin, leupeptin, betastatin, pepstatin A, dimethyl sulfoxide, and sodium dodecyl sulfate (all >99% purity) were purchased from Sigma (St. Louis, MO). Dithiothreitol was from Bio-Rad (Hercules, CA); 2,2,2-trifluoroethanol (TFE) was from Acros (Geel, Belgium). C-terminal isotopically labeled β-actin peptide (GYSFTTTAE*R) containing U-13C6, U-15N4-arginine was obtained from New England Peptide at 99% isotopic purity and 95% chemical purity. Fetal bovine serum (FBS) was from Atlas Biologicals (Fort Collins, CO). Mass spectrometry grade trypsin (Trypsin Gold) was purchased from Promega (Madison, WI). HPLC grade water and acetonitrile (ACN) were from Mallinckrodt Baker (Phillipsburg, NJ). Phosphate buffered saline, Dulbecco’s modified eagle medium (DMEM), improved minimal essential medium (IMEM), McCoy’s 5A medium, NuPAGE® MOPS and MES SDS running buffer, NuPAGE® LDS sample buffer, NuPAGE® 10% Bis-tris gels and polyvinylidene difluoride membrane were from Invitrogen (Carlsbad, CA). Epidermal growth factor (EGF), EGFR, pY1173, pY998, Jagged 1, c-Jun, caspase 3 and PARP antibodies (#8916LF, 4267s, 4407s, 2641, 2620, 9165, 9542 and 9662 respectively) were purchased from Cell Signaling Technologies (Danvers, MA). Claudin 4 and β-actin antibody (Cat. No. ab53156 and Cat. No. ab8224, respectively) were from AbCam (Cambridge, MA). 4G10 antibody (Cat. No. 05-1050X) was from Milipore (Billerica, MA). AlexaFluor® 680-conjugated fluorescent secondary antibodies were obtained from Molecular Probes (Eugene, OR). Trifluoroacetic acid (TFA), formic acid, urea and tris-carboxyethylphosphine were purchased from Thermo Fisher Scientific. Gefitinib and cetuximab were gracious gifts from Dr. Carlos Arteaga and Dr. Robert Coffey respectively, both at Vanderbilt University. Sub-X was from Surgipath (Richmond, IL).
Tissue specimens

Frozen gastric epithelial biopsy specimens were obtained from our previous study (28), which was a single-arm clinical trial to explore the effectiveness of a 4-week course of cetuximab in patients with clinically and histologically confirmed Ménétrier’s disease. This prospective, open-label trial was approved by the Vanderbilt University Medical Center Institutional Review Board (IRB), and all participants provided written informed consent. DiFi and HCT116 derived mouse xenograft tumor samples were from our previous study (29) and tissues were obtained as archival FFPE sections.

Cell culture

A431 human epithelial carcinoma cell lines were purchased from ATCC (Manassas, VA). A431 cells were maintained in IMEM supplemented with 10% FBS. DiFi human colorectal carcinoma cells were a gift from Dr. Bruce Boman at the Creighton University School of Medicine and were maintained in DMEM and supplemented with 10% FBS (30). HCT116 human colorectal carcinoma cells were from ATCC and were maintained in McCoy’s 5A medium also supplemented with 10% FBS. All cell lines were cultured at 37°C in 5% CO₂. Proliferating cells were grown to 70-75% confluency before collection, whereas all treated cells were grown to ~60-65% confluency before incubation overnight in serum-free media. These serum-starved cells were then either treated with 30 nM EGF only for 4 h (EGF Stimulated), or preincubated with 500 nM gefitinib or 10 µg/mL cetuximab (unless otherwise noted) for 30 minutes before treatment with 30 nM EGF for 4 h in the continued presence of the inhibitors. All cells were harvested on ice using cold magnesium- and calcium-free phosphate buffered saline and supplemented with a phosphatase inhibitor cocktail (1.0 mM sodium orthovanadate, 1.0 mM sodium molybdate, 1.0 mM sodium fluoride, and 10 mM of β-glycerophosphate). Cells were pelleted by centrifugation at 500 x g at 4°C and pellets were flash frozen in liquid nitrogen.
Western blot analysis

Cell pellets were resuspended and lysed in a modified RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Igepal, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) supplemented with phosphatase inhibitor cocktail (see above) and protease inhibitor cocktail (0.5 µM AEBSF, 10 mM aprotinin, 1.0 mM leupeptin, 5.0 µM bestatin and 1.0 µM pepstatin). Lysates were chilled for 20 minutes on ice before sonication with five one-second pulses at 30 watts and 20% output. The lysate was centrifuged at 13,000 x g and the total protein concentration of the supernatant was determined using the bicinchoninic acid assay (Pierce) with bovine serum albumin as the protein standard. Sample loads were normalized for total protein concentration before reducing with dithiothreitol and adding NuPAGE® LDS sample buffer and then were boiled for 7 minutes at 90°C.

Serial frozen tissue biopsy specimens from a Ménétrier’s disease patient were provided for this study as lysates generated using the TFE and ammonium bicarbonate lysis protocol described below. Prior to western blot analysis, aliquots of the lysates were lyophilized and resuspended in HPLC water three times to remove residual TFE before resuspending in RIPA buffer. All denatured samples were then resolved using NuPAGE® 10% Bis-Tris gels at 160 V for ~65 minutes in either MOPS or MES SDS running buffer (Invitrogen). Proteins were electrophoretically transferred from the gel to a polyvinylidene difluoride membrane for 3 h at 35 V at 4°C.

Membranes were probed using primary antibodies to EGFR, pY1173, pY998, Jagged 1, c-Jun, Claudin 4, 4G10 (for global phosphotyrosine analysis), or β-actin overnight at 4°C. AlexaFluor® 680-labeled goat anti-rabbit and goat anti-mouse secondary antibodies were used to detect the corresponding primary antibodies. Immunoreactive proteins were visualized using
the Odyssey™ Infrared Imaging System and Odyssey software as described by the manufacturer (Li-Cor, Lincoln, NE).

**Cell and tissue preparation for MS analyses**

A431, DiFi, and HCT116 cell pellets were resuspended and lysed in 100 µL of ammonium bicarbonate (100 mM, pH 8.0) and 100 µL of TFE and then incubated at 60°C for 60 min at 1000 rpm on an Eppendorf Thermomixer before sonicating at 30 watts and 20% output for 10 one-second pulses. Protein concentration was assessed using the bicinchoninic acid assay and equivalent sample aliquots were reduced with 40 mM tris-carboxyethylphosphine and 100 mM dithiothritol at 60°C for 30 min at 1000 rpm on the Eppendorf Thermomixer then incubated at ambient temperature in the dark with 200 mM iodoacetamide. Samples were diluted with 50 mM ammonium bicarbonate, pH 8.0 (to 10% TFE) before adding Trypsin Gold at a 1:50 (w/w) ratio and incubating overnight at 37°C with shaking. Aliquots (200 µg) of digested samples were lyophilized. Samples were resuspended in 1 mL water, vortex-mixed vigorously, and desalted with Sep-Pak® 100 mg, C-18 columns (Waters) on a vacuum manifold. After washing with water, peptides were eluted with 80% ACN and evaporated to dryness in vacuo. Digests from A431 lysate for shotgun analyses were then subjected to isoelectric focusing (IEF) as described below. Digests for MRM analysis were reconstituted in 0.1% (v/v) aqueous formic acid to a final concentration of 0.5 µg/µL, and the isotopically labeled β-actin peptide reference standard was spiked in at a concentration of 20 fmol/µL.

DiFi and HCT116 derived mouse tumor xenograft samples were acquired as FFPE tumor blocks. Three 30 µm slices were cut from each tumor block and placed into a single 1.5 mL Eppendorf tube. These were processed as we described previously (27). Rehydrated mouse xenograft samples and Ménétrier’s disease lysates were prepared for MRM analysis as described above for cultured cells, except that in the initial protein solubilization step, the tissue
samples were sonicated 3 times at 30 watts and 20% output continuously for 20s both before and after 60 min incubation in 50 mM ammonium bicarbonate with 50% TFE.

Isoelectric focusing of peptides

Tryptic peptide mixtures corresponding to 200 µg protein were resuspended in 155 µL of 6 M urea. Samples were loaded into sample loading wells of the ZOOM IPGRunner cassette (Invitrogen). ZOOM strips (10 cm) with immobilized pH gradient gel from 3.5-4.7 were placed into cassette and loaded with samples at room temperature for 1 h. Cassette was placed into a ZOOM IPGRunner cell and run for 15 minutes at 175 V, ramped from 175 V to 2000 V over 45 minutes, and held at 2000 V for 105 minutes. The gel strips were then cut into 15 equal 4 mm segments, which were each placed in separate wells of a 96-well enzyme-linked immunosorbent assay (ELISA) plate. Peptides were eluted from the strips as follows: 200 µL of 0.1% FA in water for 15 min; 200 µL of 50% acetonitrile (ACN)/0.1% FA for 15 min; 200 µL of 100% ACN/0.1% FA for 15 min. Extracted peptides for each fraction were pooled, evaporated in vacuo, resuspended in 1 ml of 0.1% trifluoroacetic acid (TFA) and desalted over a 96-well, C18 Oasis hydrophilic-lipophilic balance 30 µm (µElution) plate (Waters Corp.). After washing with HPLC water and step-wise elution (200 µL of 30% ACN/0.1% TFA, 200 µL 70% ACN/0.1% TFA, and 200 µL of 100% ACN/0.1% TFA), the eluates for each IPG strip fraction were pooled, evaporated in vacuo, resuspended in 100 µL of 0.1% (v/v) FA in water and placed in sample vials for LC-MS/MS analysis.

Reverse phase LC-MS/MS

LC-MS/MS analyses were performed on an LTQ-XL mass spectrometer from Thermo Fisher Scientific (Waltham, MA) equipped with an Eksigent nanoLC 1D plus pump and Eksigent autosampler (Dublin, CA). Peptides were resolved on 100 µm × 11 cm fused-silica capillary column (Polymicro Technologies, LLC Phoenix, AZ) packed with 5 µm, 300 Å Jupiter C-18 resin
(Phenomenex, Torrance, CA) with an in-line solid-phase extraction column (pre-column, 100 μm × 6 cm) packed with the same C-18 resin (using a frit generated with liquid silicate Kasil) similar to that previously described (31). LC was carried out at ambient temperature over 85 min using a gradient mixture of 0.1% (v/v) FA in water (solvent A) and 0.1% (v/v) FA in ACN (solvent B). A 10 minute load period using 100% solvent A at 1 µL/min was followed by an elution gradient (600 nL/min) from 2-25% solvent B in 30 minutes, 25-90% solvent B over 15 minutes, and held at 90% solvent B for 17 minutes before returning to 2% solvent B to equilibrate column. Peptides eluting from the capillary tip were introduced into the LTQ source in micro-electrospray mode with a capillary voltage of ∼2 kV. A full scan was obtained for eluting peptides in the range of 400–2000 amu followed by five data-dependent MS/MS scans of the most intense ions. MS/MS spectra were recorded using dynamic exclusion of previously analyzed precursors for 60 s with a repeat of 1 and a repeat duration of 1. MS/MS spectra were generated by collision-induced dissociation of the peptide ions at normalized collision energy of 35% to generate a series of b- and y-ions as major fragments.

**MRM analyses**

MRM analyses were performed on a TSQ Vantage triple quadrupole mass spectrometer from Thermo Fisher Scientific (Waltham MA) equipped with an Eksigent NanoLC-ultra 1D Plus pump (Dublin CA) and a capillary column and pre-column similar to that described above. The mobile phase consisted of the gradient mixture of solvent A and solvent B used for LC-MS/MS analysis. Sample solution (2 µL) containing 0.5 ng/µL peptide mixture (based on protein concentration) were loaded for 15 minutes onto the column using 100% solvent A at 1 µL/min followed by a gradient elution (400 nL/min) from 3–20% solvent B in 7 min, 20–60% solvent B over 35 min, 60–95% solvent B in 6 min and held at 95% for 11 minutes before returning to 3% solvent B. MRM analyses of target peptides and β-actin peptides (isotope labeled and endogenous) were performed using a 1300 V electrospray voltage, 210 °C capillary
temperature, and −5 V skimmer offset. Both Q1 and Q3 were set at unit resolution FWHM 0.7 Da and collision gas (He) pressure in Q2 was held at 1.5 mTorr. Scan width was 0.004 m/z and scan time was 10 ms for all analyses. Collision energy for each peptide was calculated using the open source software Skyline (32). Instrument quality control assessment was done as described previously (33).

Quantitative analyses were done by the method we described recently (33) using U-\(^{13}\)C\(_6\), U-\(^{15}\)N\(_4\)-arginine β-actin (BA) peptide (GYSFTTAE*\(\text{R}\)) as the reference standard. At least four MRM transitions were monitored for target peptides and the standard. Signature peptides for each protein measured were required to be between 7-25 amino acids long and were selected based on uniqueness and chemical stability. Although priority was given to peptides that were previously identified in the shotgun data set with high MS/MS spectral quality, additional peptides were selected by in silico digestion. Peptides containing cysteine or methionine residues were not excluded and cysteines were present as carboxyamidomethylated derivatives following treatment with iodoacetamide during sample work-up. Peptide uniqueness was confirmed by BLAST searching sequences against the UniProt database. Skyline software was used to extract and integrate transition peak areas for each target peptide. Dot product scores (≥0.85) and predicted retention times computed by Skyline were used as criteria to confirm peak area identifications (32). Summed peak areas for target peptide transitions were divided by the summed peak area for the reference standard peptide transitions to give normalized peak area (NPA) and coefficient of variations (CVs) were calculated across replicates for each treatment. For MRM analyses in cell culture experiments, 3 replicate cell cultures were analyzed for each cell culture and treatment. For DiFi cell xenografts, 4 replicate samples were analyzed; 5 replicate HCT116 xenografts were analyzed; for Menetrier’s disease, 3 separate gastric tissue biopsy samples were analyzed.
Data analysis

The “ScanSifter” algorithm v2.0.4 read MS/MS spectra stored as centroided peak lists from Thermo RAW files and transcoded them to mzData files (34). Only MS/MS scans were written to the mzData files; MS scans were excluded. If 90% of the intensity of a MS/MS spectrum appeared at a lower \( m/z \) than that of the precursor ion, a single precursor charge was assumed; otherwise the spectrum was processed under both double and triple precursor charge assumptions. MS/MS spectra were assigned to peptides from the IPI Human database version 3.56 (May 5, 2009; 76591 entries) by the MyriMatch algorithm, version 1.6.33 (35). The sequence database was doubled to contain each sequence in both normal and reversed orientations, enabling false discovery rate (FDR) estimation. MyriMatch was configured to expect all cysteines to bear carboxamidomethyl modifications and to allow for the possibility of oxidation on methionines. Candidate peptides were required to feature trypsin cleavages or protein termini at both ends, though any number of missed cleavages was permitted. A precursor error of 1.25 \( m/z \) was allowed, but fragment ions were required to match within 0.5 \( m/z \). The IDPicker algorithm v2.6.1 (36) filtered the identifications for each reverse phase liquid chromatography run to include the largest set for which a 5% peptide-level identification FDR could be maintained, as described by Qian et al. (37). Indistinguishable proteins were recognized and grouped, and parsimony rules were applied to generate a minimal list of proteins (Protein groups) that explained all of the peptides that passed our entry criteria (36). This approach uses bipartite graph analysis to derive a minimal list of protein identifications with shared clusters of peptides. These identifications were pooled for each IEF sample set (15 fractions). Proteins were required to have at least two different peptide sequences observed within an IEF sample set. FDR for peptide identifications were computed by the formula (38): \[ \text{FDR} = \frac{2 \times \text{reverse}}{\text{forward} + \text{reverse}}. \] The algorithm reported the number of spectra and number of distinct sequences observed for each protein and protein group in each sample set.
**Statistical analyses**

Comparisons of protein abundance from shotgun datasets were made with spectral count data (39) using QuasiTel, a statistical software modeling package (40). QuasiTel uses a quasi-likelihood method based on Poisson distribution—commonly used for count data (41)—and applies a regression model to compare spectral count data. This statistical model was used to perform pair-wise comparisons between two treatment conditions (6 replicates per condition) which generated a single combined inventory of protein identifications (Comparison dataset); the model also uses F-tests to compute p-values and the FDR method to correct for multiple hypothesis comparisons of identified proteins (42). Thresholds were set for p-values ($\leq 0.20$), total spectral counts (11), and spectral count log$_2$ rate ratios (fold changes, $\geq 2$) generated by this model and were used as criteria to filter comparison datasets. Significance of measured differences for target proteins of all MRM analytes was determined with two-tailed unpaired t test using Prism 5.0 (GraphPad Software, San Diego, CA).
Results

EGFR modulation in A431 cells

To establish the A431 cell model, we first established conditions for activation and inhibition of EGFR signaling. We measured the phosphorylation status of the EGF receptor as well as global tyrosine phosphorylation by western blot analysis (Figure 1). Cells proliferating in the presence of serum served as baseline controls, whereas serum-starved cells treated with EGF served as the benchmark for EGFR activation. After treatment with 30 nM EGF for 4 h, EGFR phosphorylation at residues Y998 and Y1173 increased dramatically, as did global phosphotyrosine levels indicated activation, as measured with the antiphosphotyrosine antibody 4G10. EGFR protein expression inversely varied with activation, which reflects enhanced receptor internalization and down regulation upon activation (43-46). Increasing concentrations of both cetuximab and gefitinib lead to a decrease of EGFR phosphorylation at Y1173 and Y998 compared to EGF only stimulated cells. At the concentrations used, the tyrosine kinase inhibitor gefitinib produced a more profound inhibition of EGFR phosphorylation than did the ligand binding domain inhibitor cetuximab. The highest concentration of cetuximab (20 µg/mL) only reduced EGFR phosphorylation to basal levels (Figure 1A), whereas gefitinib at 250 nM reduced both Y1173 and Y998 phosphorylation well below basal levels (Figure 1B). Total tyrosine phosphorylation detected in EGF stimulated cells showed an increase over signals detected in proliferating cells, and while both inhibitors reduced detected levels of total tyrosine phosphorylation, only gefitinib was capable of reducing tyrosine phosphorylation below basal levels.

With the exception of pY998 in cetuximab treated cells, minimal differences were noted between EGFR and total tyrosine phosphorylation status for the two highest concentrations of each inhibitor (10 and 20 µg/mL for cetuximab or 500 and 1000 nM for gefitinib). Based on these results, a single concentration each for cetuximab (10 µg/mL) and gefitinib (500 nM) was selected for subsequent experiments. At the selected concentrations, both inhibitors significantly
and reproducibly inhibited EGF receptor signaling. While changes in phosphorylation were noted after 4 hours, EGF treatment did not induce apoptosis (Figure S1). A slight rounding of A431 cells in culture was noted for EGF- treated cells, in agreement with previous reports (47, 48). Cells co-treated with inhibitor and EGF retained an unaltered morphology. Neither EGF nor EGFR inhibitors produced significant detachment of cells from the culture dish.

Global protein expression analyses

We performed shotgun proteome analyses of the four A431 treatment conditions described above: proliferating cells, EGF treated, EGF and gefitinib treated or EGF and cetuximab treated. Cells were collected and lysed, tryptic digests were prepared and resolved by IEF, and the IEF fractions were analyzed by data-dependent LC-MS/MS. A summary of the global protein analysis is shown in Table I. Values displayed are averaged data from 3 separate cultures and 2 process replicates of each culture per treatment condition. Similar values for confident spectral IDs (numbers of spectra matched to database sequences at 5% FDR) and protein groups (numbers of indistinguishable protein identifications supported by the identified peptides) and protein group CVs under 5% demonstrate high reproducibility of the analyses across biological replicates. A complete list of protein identifications is provided in Table S1.

Dataset comparisons, data filtering and derivation of a candidate EGFR inhibition signature

Next, we compared protein expression datasets from the four A431 cell conditions to identify proteins whose levels were changed by EGFR activation and for which the changes were reversed by the inhibitors. Protein expression was compared on the basis of spectral counts using QuasiTel, a quasi-likelihood modeling software package (40). QuasiTel performed pairwise comparison between two treatment/biological conditions based on protein spectral counts and variance across replicate analyses and computed p-values and rate ratios (fold changes) for detected proteins.
Pair-wise comparisons were made between 1) proliferating and EGF stimulated cells, 2) EGF stimulated and EGF/gefitinib treated cells and 3) EGF stimulated and EGF/cetuximab treated cells (Fig. 2, colored boxes). To control the protein-level FDR, comparisons were limited to proteins with ≥11 spectral counts per protein across replicates. Accordingly, the three comparisons included 3783, 3881 and 3750 proteins, which corresponded to protein-level FDRs of 3.3, 3.1 and 3.3% respectively. The colored circles in Figure 2 represent proteins differentially expressed (both up and down) between treatments with a fold change of ≥ 2.0 and p-values ≤ 0.20. The blue circle (191 proteins) represents proteins differentially expressed between EGF stimulated cells and non-treated, proliferating cells. The green circle (237 proteins) and yellow (133 proteins) circles signify proteins whose EGF-stimulated changes were reversed by gefitinib and cetuximab inhibition, respectively. Tables S2-S4 provide a complete list of proteins in each of these groups. The group reversed by cetuximab is only about half as large as the group reversed by gefitinib, which is consistent with the lesser degree of EGFR inhibition produced by cetuximab in our model (Figure 2), as well as with possible off-target effects of gefitinib due to its inhibition of multiple kinases (49, 50).

The Venn diagram (Figure 2) compares these three groups of proteins to explore the common protein expression changes associated with EGF stimulation and EGFR inhibition. The three groups comprised 445 proteins, of which 13 proteins were shared between all three groups. Another 90 proteins were shared between two groups and most (342 proteins) belonged to only a single group. The center overlap contained 13 proteins whose expression was significantly changed by EGF treatment and these changes were reversed by both inhibitors. We will refer to this group hereafter as the “EGFR inhibition signature”. The 13 proteins are: coiled-coiled domain containing 50 (CCDC50), cyclin-dependent kinase inhibitor 1A (CDKN1A, p21), claudin 4 (CLDN4), cordon-bleu-like protein 1 (COBL1), jagged 1 (JAG1), proto-oncogenes c-Jun and JunD (JUN and JUND respectively), DNA primase subunit 1 (PRIM1), RNA binding motif protein 15 (RBM15), translocase of outer mitochondrial membrane 20
(TOMM20), trafficking protein particle complex 3 (TRAPPC3), and tripartite motif containing protein 32 (TRIM 32) and glutamine-fructose-6-phosphatetrasaminase 2 (GFPT2).

The Venn diagram also indicates protein subgroups with expression patterns similar to those in the EGFR inhibition signature. For example, EGF-induced expression changes were reversed only by cetuximab for 15 proteins and only by gefitinib for 57 proteins. Another 18 proteins were reversed by both cetuximab and gefitinib, but these proteins were not significantly elevated by EGF treatment at the selected cut-offs. The proteins found outside of any overlap showed significant differences between EGF stimulation and either non-treated (proliferating) or inhibitor treated cells; however, these protein differentials were unique to individual treatments and comparisons. Table S5 lists the proteins found in each section of the Venn diagram.

These protein groupings, including the EGFR inhibition signature, depend on the selected thresholds for fold change and p-values. More lenient thresholds may increase detection of false-positive differences, whereas more stringent criteria may decrease detection of true differences. Manipulation of thresholds for p-value and fold change can shift some proteins from one classification to another in the Venn diagram (Figure S2).

**MRM verification of EGFR inhibition signature in A431 cells**

To verify expression differences for the 13 proteins in the EGFR inhibition signature, we performed MRM analyses using the labeled reference peptide (LRP) method we described recently (33) to quantitate target peptides with reference to a labeled β-actin peptide standard. A431 cells were treated as described for global proteome comparisons, but no IEF fractionation was performed. The data generated by MRM analyses is displayed together with spectral count data from the shotgun analyses (Figure 3). MRM data were from analyses of 3 separate cultures for each treatment and the normalized peak area (NPA) for a single, proteotypic peptide is shown for each protein. The spectral count data (Figure 3, teal bar) displays the total number of spectra identified for the corresponding protein across six replicate shotgun analyses (3 separate
cultures and 2 process replicates of each culture). The peptide sequences, monitored transitions, peptide and normalized peak areas, and CVs for the MRM data in Figure 3 can be found in Table S6. CVs for NPA values calculated measured peptides across the treatment conditions for all but two of the peptides were below 30%. For 39 of these, CVs were less than 18%. MRM analyses for a second, unique peptide for 10 of the 12 EGFR inhibition signature proteins produced similar results (Figure S3).

MRM analyses detected peptide transitions corresponding to 12 of the 13 EGFR inhibition signature proteins. None of the peptides monitored for glutamine-fructose-6-phosphatetransaminase 2 (GFPT2) produced detectable signals. This protein was not considered further. For the remaining 12 proteins, MRM analyses verified almost all of the expression differences detected by shotgun analyses (Figure 3). EGF-induced protein expression compared to proliferating cells was significantly different by MRM in 8 of the 12 cells and the trends in the others were consistent with spectral count differences, except for TOMM20 and RBM15. Significant reversal of EGF-induced expression was verified by MRM for both inhibitors for all 12 proteins, except for the effect of cetuximab on PRIM1, which fell short of statistical significance, although the trend was consistent with the spectral count comparison. All protein expression differentials measured by spectral counts and MRM were consistent in direction, whereas the magnitude of the measured differences varied between the two analysis platforms. This reflects the greater precision of MRM measurements over the dynamic range of protein expression analyzed.

Confirmation of EGFR inhibition signature protein changes in DiFi and HCT116 cell lines

Our next studies analyzed changes in EGFR inhibition signature proteins in cells that differ in response to EGFR inhibitors. We used MRM analyses to measure the 12 EGFR inhibition signature proteins in DiFi and HCT116 colorectal cancer cell lines. DiFi cells demonstrate both gene amplification and protein overexpression of the EGF receptor and also
harbor an adenomatous polyposis coli gene (APC) mutation frequently found in hereditary colorectal cancers (30, 51). DiFi cells thus would be expected to show similar responses to A431 cells. HCT116 cells show increased autocrine production of the EGFR-stimulating ligand transforming growth factor alpha (TGFA) and carry a mutant allele (G13D) of the KRAS protooncogene (52, 53). Because KRAS mutations block clinical and cellular responses to EGFR inhibitors, such as cetuximab, we hypothesized that an EGFR inhibition signature would be minimal or absent in the HCT116 cells.

Both cell lines were treated with EGF and inhibitors as described above and the 12 EGFR inhibition signature proteins were analyzed. Peptides for 11 of the 12 proteins were successfully monitored in DiFi cells, whereas peptides for only 8 of the proteins were detected in HCT116 cells (Figure 4). The COBLL1, PRIM1 and RBM15 peptides monitored in HCT116 cells produced no detectable signals and CDKN1A peptide signals were absent in both cell lines. Most of the peptide signals in DiFi cells monitored showed statistically significant differences between EGF only and inhibitor treated cells (Figure 4, DiFi(c)) consistent with the trends noted in A431 cell experiments (Figure 4, A431). Only JUND and PRIM1 failed to show significant changes. HCT116 cells were largely resistant to changes in EGFR inhibition signature proteins, with significant differences detected only for CCDC50, JUND and TOMM20. CCDC50 peptide signals were significantly upregulated in both inhibitor treated conditions when compared to EGF only samples—in agreement with A431 and DiFi cell MRM data—whereas JUND and TOMM20 peptides showed substantial differences for only gefitinib or cetuximab treatments respectively (Figure 4, HCT116(c)). Plots of MRM data for DiFi and HCT116 cells are presented in Figures S4 and S5 and Tables S8 and S9 list the peptide peak areas, normalized peak areas, averages and CVs for the EGFR inhibition signature peptides monitored in the two cell lines.

To confirm the expression of the EGFR inhibition signature in the DiFi and HCT116 cells, we required at least half of the detectable signature proteins to show expression changes consistent with changes measured in the A431 model. The MRM data for DiFi cells demonstrate
expression changes consistent with those in A431 cell—11 of the 12 signature proteins were detected and 9 showed significant expression differences. None of the proteins showed changes opposite those observed in A431 cells. This result for DiFi cells is consistent with their overexpression of EGFR and responsiveness to EGFR inhibitors. In contrast, the HCT116 cells display expression changes sensitive to both inhibitors in only one of the 8 detectable signature proteins (CCDC50) and responded only to a single inhibitor for 2 others (JUND and TOMM20). By our criteria, the HCT116 cells do not display an EGFR inhibition signature, a result that is consistent with the effect of the heterozygous G13D KRAS mutation, which blocks responses to EGFR inhibitors (53).

*Immunoblot confirmation of EGFR inhibition signature*

Further confirmation of protein changes in response to EGFR perturbation was done by immunoblotting with antibodies to p-EGFR, JAG1, JUN and CLDN4 in the A431, DiFi and HCT116 cell lines across all treatments (Figure S6). In all cell lines, an increase was observed in pY998 and pY1173 signals in EGF treated cells relative to proliferating. In A431 and DiFi cells treated with gefitinib, EGFR pY998 and pY1173 both were decreased to basal (proliferating) levels, while a less profound, yet still detectable decrease was observed in HCT116 cells. Cetuximab treatment (at 10 µg/mL) in all three cell lines partially inhibited EGFR autophosphorylation. For EGFR pY1173 in DiFi cells and pY998 in HCT116 cells, no change in phosphotyrosine immunoblot signal was detected between EGF only and cetuximab treated cells (Figure S6). Protein expression changes in all three cell lines measured by immunoblot were consistent with MRM data. In A431 and DiFi cell lines, JAG1, JUN and CLDN4 were increased in EGF treated compared to proliferating cells. In both A431 and DiFi cells, gefitinib decreased JAG1, JUN and CLDN4 expression, whereas cetuximab produced partial reversal of EGF stimulation for these proteins. In HCT116 cells, EGF elevated JAG1, but the inhibitors did reverse this effect.
**Confirmation of EGFR inhibition signature protein changes in mouse xenograft tumors**

To further confirm protein changes in the EGFR inhibition signature in tissue specimens, we analyzed mouse xenograft tumors derived from DiFi and HCT116 cell lines. These xenograft samples come from our previous study, in which we used novel optical imaging probes to study therapeutic responses to cetuximab *in vivo* (29). DiFi and HCT116 xenografts were grown in athymic nude mice. Tumor bearing mice were then treated with 40 mg/kg cetuximab or saline vehicle intraperitoneally every 3 days for 1 week (three total injections). Image analysis demonstrated a significant decrease in EGF-probe uptake in DiFi xenografts and increased apoptosis in cetuximab treated mice, whereas HCT116 xenografts showed neither effect. Gefitinib was not employed in this study.

We analyzed FFPE sections from the xenograft tumors by MRM to measure the 12 EGFR inhibition signature proteins. Analyses of xenograft tumors from vehicle treated mice were used for reference to assess the effects of cetuximab treatment. MRM analyses detected 8 of the 12 EGFR inhibition signature proteins in each xenograft type (see Figure 4 (DiFi(x) and HCT116(x)). DiFi derived tumors from cetuximab-treated mice showed decreased expression of CLDN4, JAG1, JUN, and TRIM32 and an increase in TRAPPC3 when compared to tumors from vehicle treated mice. These differences are similar to those between EGF-treated and only to cetuximab/EGF treated DiFi cells, although the xenograft analyses yielded no statistically significant differences for CCDC50, COBLL1, or RBM15. MRM data for these experiments are provided in Figure S7 and Table S10.

HCT116 xenografts from cetuximab treated mice showed a significant increase in TRAPPC3 and RBM15 compared to vehicle treated tumors, but the other proteins in the EGFR inhibition signature were either unchanged or were not detected (Figure 4, HCT116(x)). Of note are the differences between the EGFR inhibition signatures for the treated HCT116 cells and the HCT116 mouse tumors. In HCT116 cells, cetuximab increased CCDC50 and TOMM20, but not in the HCT116 xenografts. The lack of consistent inhibitor-induced protein expression
differences in the HCT116 xenograft samples is consistent with the lack of response to cetuximab in vivo (29). MRM data for these experiments are provided in Figure S8 and in Table S11. An important aspect of this study is the analysis of the EGFR inhibition signature in archival FFPE tissue specimens. Although a few of the measured peptides yielded CVs around 40%, most yielded CVs <30%.

Confirmation of the EGFR inhibition signature was assessed by the criteria described above—changes concordant with the A431 model in at least 50% of the measurable signature proteins. The signature was confirmed in DiFi xenografts (5 concordant changes in 8 detectable signature proteins), but not in HCT116 xenografts (2 concordant changes in 8 detectable signature proteins).

Use of the EGFR inhibition signature to assess therapeutic response to cetuximab in Ménétrier’s disease

A potential use of the EGFR inhibition signature is to assess response to drug treatment. To test the applicability the EGFR inhibition signature to assess clinical response to EGFR inhibition, we analyzed serial biopsies from a single Ménétrier’s disease patient treated with cetuximab, as described in our recent study (28). Ménétrier’s disease is a rare hypertrophic gastropathy characterized at the molecular level by high expression of transforming growth factor-α (TGFA) and constitutively activated EGFR signaling (54, 55). Symptoms of Ménétrier’s disease are highly responsive to cetuximab therapy, which rapidly and dramatically reverses most effects of the disease (28, 56).

One patient from our recent study (28) showed improved symptoms and a histologically normal stomach after the initial one month trial. Baseline (no treatment) specimens as well as serial biopsies from 1 day and 1 week post-initial cetuximab treatment were analyzed in triplicate (three separate analyses of pooled biopsies from several sites in the stomach) from this patient. MRM analyses detected peptides from 9 of the 12 EGFR inhibition signature proteins (Figure 5).
(The MRM data are presented in expanded form in Table S12). Because our cell model for EGFR inhibition reflected an acute response to EGF and inhibitors, we focused on protein expression changes after 1 day and 1 week of cetuximab treatment (biopsies were also collected at 1 and 4 months of continued treatment). CLDN4, JAG1, and JUN all were significantly decreased relative to baseline after 1 day. JUND and TRIM32 appeared decreased, although the effect was not significant. These changes all are consistent with the effects of cetuximab treatment in the A431 and DiFi cell models. In contrast, CCD50, COBBL1, TOMM20 all were decreased by 1 day of cetuximab treatment, in contrast to the increases in these proteins produced by cetuximab in the A431 and DiFi models. At 1 week cetuximab treatment, CLDN4, JAG1 and JUN were still decreased relative to pretreatment, consistent with responses to cetuximab treatment in A431 cells, DiFi cells and the DiFi xenograft model. Expression of JAG1, JUN and CLDN4 expression was also measured by immunoblotting (Figure S9). Consistent with MRM data, JAG1 and CLDN4 were significantly decreased from the baseline sample at 1 day and 1 week posttreatment. JUN expression measured by immunoblotting did not change with cetuximab treatment.

By the criteria described above, these results do not confirm the EGFR inhibition signature. Three of the signature proteins (CCD50, COBBL1, TOMM20) showed changes opposite those in the A431 model. Nevertheless, the 3 proteins (CLDN4, JUN and JAG1) showing changes concordant with the A431 model, were most consistently responsive to EGFR inhibition in all of our experiments.
Discussion

Direct analysis of phosphorylated receptor proteins and their downstream effectors is the most commonly employed method to assess signaling networks. This approach is complicated by the transient nature of protein posttranslational modifications, their low abundance relative to unmodified proteins and potential artifacts due to uncontrolled preanalytical variables. We asked whether analysis of changes in protein expression could indicate responses of a signaling network. The rationale for this concept is two-fold. First, protein expression is easier to measure and is less labile than phosphorylation or other posttranslational modifications. Second, signaling pathways ultimately drive gene and protein expression changes, which are most directly linked to phenotypes. A protein expression-based analysis could be routinely applied to clinical specimens, including archival FFPE tissues, where direct measurements of phosphorylated intermediates may be neither practical nor valid. Here we tested the hypothesis that manipulation of the EGFR signaling axis with clinically-used drugs generates characteristic protein expression changes. Our results confirm the hypothesis and demonstrate a prototypical approach to derive and test protein expression signatures for drug action on signaling networks.

The A431 cell model provided a reproducible and extensively studied (57-61) system to study EGFR-driven signaling events. Four treatment conditions were selected for comparisons: normal proliferating cells, cells stimulated with EGF and cells pre-incubated with either gefitinib or cetuximab and then treated with EGF in the presence of the inhibitors. Our initial immunoblot analyses (Figure 1) confirmed EGFR activation and inhibition. All treated A431 cells were serum-starved overnight to synchronize the cells and to limit potential interference by alternate EGFR ligands, which could complicate results. Serum starving the cells nevertheless introduced an inherent experimental difference between the proliferating (non-treated) cells harvested in the presence of 10% fetal bovine serum and the EGF treated A431 cells incubated in serum-free media. Shotgun and MRM analyses (data not shown) verified elevation of 2 proteins (HMGCR and HMCS1) and a decrease of 1 protein (A2MG) reported to be induced by serum starvation.
These proteins were expressed at similar levels in all EGF-treated A431 cells (even in the presence of the inhibitors) compared to proliferating cells. This example illustrates the potential for experimental artifacts and underscores the importance of understanding the underlying biology of the discovery model.

Shotgun proteomic analyses of treated and non-treated A431 cells yielded highly reproducible proteomic inventories—CVs for numbers of protein identifications were less than 5% across 3 separate cultures for each treatment condition (Table I). The combined inventories comprised nearly 4,300 proteins identified at a protein-level FDR of 4.7%. Quasi-likelihood analyses generated three pairwise comparisons (EGF versus proliferating, EGF versus gefitinib, and EGF versus cetuximab) based on shotgun spectral count data. These datasets were then filtered on the basis of spectral counts ($\geq 11$), fold-change ($\geq 2$), and p-value ($\leq 0.20$) to generate the three protein groups representing EGF-simulated proteins, cetuximab-sensitive proteins and gefitinib-sensitive proteins (Figure 2). A Venn diagram comparison of these generated the EGFR inhibition signature proteins.

Three important points should be emphasized about the comparison process. First, the application of a minimum spectral count threshold (here $\geq 11$ across all analysis runs in each dataset comparison) is required to control protein-level FDR. Without the threshold, protein FDR exceeded 30%, whereas with the threshold, protein FDR was approximately 3%. Second, an important consideration in dataset comparisons is the stringency of the cut-off criteria applied. We employed a relatively loose p-value threshold ($\leq 0.2$) to maximize detection of differences in protein group comparisons. We reasoned that subsequent MRM analyses would identify false positive differences and confirm true positives that might not have been detected in the spectral count comparisons at higher stringency. We employed a 2-fold expression cut-off for the same comparisons. We experimented with different p-value and fold-change thresholds (Figure S2) and ultimately settled on criteria that yielded an EGFR inhibition signature compatible in number of proteins with our capacity to perform subsequent MRM studies. Third, the interplay between
p-value and fold-change threshold variation also affected the number and distribution of proteins in the overlap categories in Venn diagram analysis. For example, at thresholds of \( p \leq 0.2 \) and a 2-fold difference, the central overlap (EGFR inhibition signature) in the Venn diagram contained 13 proteins, but at \( p \leq 0.1 \), the central overlap contained only 4 proteins (JUN, JUND, CDKN1A, and RBM15) (Figure S2, bottom row middle Venn diagram).

Despite the relatively loose criteria used to derive the EGFR expression signature, 11 of the 13 proteins were verified by MRM with expression changes similar to those observed from shotgun data (Figure 4). One protein (GFPT2) proved difficult to monitor selectively by MRM and was not further studied and another (PRIM1) showed significant reversal of EGF-induced change by gefitinib, but not by cetuximab. These MRM analyses targeted a single peptide for each of the proteins, but a parallel set of analyses targeting a second unique peptide for 10 of the EGFR inhibition signature proteins also verified the results of the spectral count comparisons (Figure S3).

Although our shotgun analyses represented an empirical approach to discover a candidate signature, the component proteins have interesting and biologically plausible relations to EGFR signaling. A core group of proteins (e.g. JAG1, JUN, and CLDN4) displayed the most robust responses to EGFR inhibition. JAG1 (Jagged1) is a Notch1 receptor ligand with reported links to EGFR expression (62, 63); moreover, up-regulation of JAG1 expression and Notch1 signaling has been noted in human colon adenocarcinomas and intestinal tumors (64-66) and associated with poor prognosis in breast cancer (67). An increase in JUN detection in EGF treated samples is consistent with literature reports that indicate an increase in mRNA levels for the transcription factor upon EGF stimulation (68-70). Overexpression of JUN has also been linked with increased invasiveness and hormone resistance in breast cancer cells (71). Increased expression of the tight junction protein CLDN4 upon EGF treatment (72) is in agreement with our data. JUN and CLDN4 also are linked to EGF via the transcription factor Sp1 (73, 74). EGFR manipulation dramatically affected CDKN1A, a TP53-regulated cyclin
dependent kinase inhibitor (75), but only in A431 cells, which lack a functional TP53 protein. This protein may have utility to assess EGFR inhibition only in TP53 mutant tumors.

Once a candidate EGFR expression signature was established, we asked whether this set of protein expression changes can consistently represent the output of the EGFR signaling network in cells and tissues that differ in responsiveness to inhibitors. The EGFR inhibition signature in the DiFi cells was nearly identical to that in the A431 model, which is consistent with the high EGFR expression and responsiveness to inhibitors for both lines. In contrast, the HCT116 cells displayed only one consistent change (increase in CCDC50 by both inhibitors) observed in the A431 cells. This result is consistent with the impact of the KRAS mutant status of these cells, which blocks response to EGFR inhibition (53).

Analyses of DiFi and HCT116 derived mouse xenograft models extended confirmation of the EGFR inhibition signature to tissue specimens. Although fewer targeted EGFR inhibition signature proteins were detected overall (8 of 12 for each xenograft type), there was again a clear difference in response of the signature proteins. Five of 8 detected signature proteins displayed the expected expression changes in DiFi xenografts, whereas only 2 did in HCT116 xenografts (Figure 5). An important aspect of the xenograft studies is that they demonstrate the potential to analyze protein expression signatures in FFPE specimens. Although we have previously demonstrated that shotgun proteome inventories of frozen and FFPE tissues are equivalent (27), yield of proteins may be decreased. Indeed, comparison of the cell line and xenograft data also suggests a “loss of signal”, in that fewer of the signature proteins were quantifiable in the xenografts.

We evaluated the EGFR inhibition signature in Ménétrier’s disease because of the dramatic response of this syndrome to cetuximab therapy (28). Our results indicate that the three most consistent elements of the signature (JUN, JAG1 and CLDN4) respond to cetuximab treatment in Ménétrier’s disease. However, CCDC50, COBBL1 and TOMM20 expression changes were opposite to those in the A431 model. This discrepancy could reflect that fact that
Ménétrier’s disease is a hyperproliferative disorder, but not a cancer, and thus may differ in other ways from the biology of the A431 discovery model and the DiFi and HCT116 models, which are all cancer cell lines. This point underscores the importance of matching the discovery model as closely as possible to the clinical application of an expression signature.

One potential complication in comparing EGFR-mediated responses in cell models and tissues is that endogenous levels of EGF and exposure cannot be controlled in tissues. Moreover, signaling effects driven in vivo by other cell types, such as stroma may influence the expression of candidate signature proteins. These factors could contribute to a lack of complete agreement between cell and in vivo models. However, the most EGF- and EGFR inhibitor-responsive protein changes observed (JUN, JAG1 and CLDN4) were consistent across all of the model systems studied. Thus, proteins whose expression was significantly elevated by EGF and reversed by both inhibitors proved to be the most consistent indicators of EGFR inhibition in the other models.

Our main objective in this work was to determine whether protein expression signatures can represent the effects of drugs on a signaling network, rather than to develop and refine a clinically useful EGFR inhibition signature. This latter objective would entail a more elaborate process that includes 1) multiple discovery systems to generate a more broadly-based candidate signature, 2) integration of quantitative measurements with a valid statistical model to establish score thresholds and account for variability and 3) systematic validation in a carefully selected, valid patient cohort. Nevertheless, some of the proteins identified in this study would appear likely to contribute to a clinically useful EGFR inhibition protein profile. Our results establish proof of concept for this approach by demonstrating that protein expression signatures detect activation and inhibition of dynamic signaling networks. Clinically useful response signatures developed through this approach could have broad impact in the field of cancer therapeutics.
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Table I. LC-MS/MS data summary.

|                               | Proliferating | EGF Stimulated | Gefitinib + EGF | Cetuximab + EGF |
|-------------------------------|---------------|----------------|-----------------|-----------------|
| Confident spectral IDs        | 32567         | 36156          | 34105           | 35322           |
| Protein groups                | 5203          | 5294           | 5347            | 5250            |
| Protein group CV (%)          | 4.4           | 4.8            | 4.9             | 4.5             |

The table lists confident identifications (# of spectra successfully matched to peptides), protein groups, and %CV of replicate analysis for each of the treatment conditions. Protein group numbers are averages of 6 replicates (3 separate cultures, and 2 process replicates of each) per treatment (30 nM EGF, 10 µg/mL cetuximab, 500 nM gefitinib). A 5% peptide-level FDR and minimum 2 distinct peptides were required for confident protein identification. *A protein group is defined by a set of proteins that cannot be distinguished based on the peptide identifications. A single protein is chosen to represent each protein group.
Figure Legends

Figure 1. Activation and inhibition of EGFR in A431 cells. A431 cells were serum-starved overnight before being treated with either 30 nM EGF (E) for 4 hrs or co-treated first with cetuximab (A) or gefitinib (B) at the indicated concentrations for 30 minutes prior to 4 hr treatment with EGF. Proliferating cells (P) were not serum starved and serve as a reference control. Immunoblots were performed for total EGFR, sites of EGFR phosphorylation at pY1173 and pY998 and total tyrosine phosphorylation was detected with the 4G10 antibody. β-Actin was used as a loading control.

Figure 2. Filtering and comparison of A431 proteome datasets. Colored rectangles show protein identifications from global proteomic analyses of the four treatment groups. Dashed rectangles show number of proteins in comparison datasets that were filtered to have ≥11 spectral counts per protein across replicates. Comparisons of protein spectral counts between treatment groups were performed by quasi-likelihood analysis; and a p-value and spectral count rate ratio (fold change) were generated. The colored circles represent lists of proteins differentially expressed (both up and down) between treatments with a fold change of ≥2.0 and quasi p-values ≤ 0.20. The resulting protein groups represent proteins differentially expressed in response to EGF (blue), EGF-induced protein changes reversed by gefitinib (green) and EGF-induced protein changes reversed by cetuximab (yellow). The Venn diagram comparison indicates proteins whose expression changes are shared by the different experimental conditions. The central overlap indicates the “EGFR inhibition signature”, which refers to EGF-stimulated protein expression changes reversed by both inhibitors.

Figure 3. Normalized MRM data and spectral count correlation of EGFR inhibition signature proteins. Each panel shows both MRM data (red bar) and spectral count data (teal bar) for each protein of interest across four A431 cell treatment conditions: proliferating cells (P),
EGF-treated (E), EGF and gefitinib (G+E) and EGF and cetuximab (C+E). The left Y-axis is normalized peak area (NPA), which is the total MRM transition peak area for the target peptide divided by the peak area for the β-actin labeled reference peptide. MRM data is representative of 1 unique peptide for each target protein across 3 separate cultures. Spectral counts (SC) are the total number of spectra identified for each protein across replicate analyses. (*) Denotes significant difference compared to EGF treated cells as determined by Student’s two-tailed, unpaired t-test.

Figure 4. Summary of expression changes for EGFR inhibition signature proteins in cell lines and mouse xenograft models. Symbol colors indicate decreased expression (green); increased expression (red); detected, but no significant change (black) compared to EGF treatment in cells (c) or compared to vehicle control in xenografts (x); and not detected (white). Only cetuximab treatment was used in xenograft experiments.

Figure 5. MRM analyses of EGFR inhibition signature proteins in tissue biopsies from a Ménétrier’s disease patient treated with cetuximab. Gastric tissue biopsies were collected from 3 separate locations prior to treatment (baseline control) and at 1 day and 1 week after treatment with cetuximab. Normalized peak area (NPA), which is the total MRM transition peak area for the target peptide divided by the peak area for the β-actin labeled reference peptide. Data points are representative of 3 separate biopsies taken from a single patient with mean and standard deviation shown. (*) Denotes significant difference between baseline and time post initial treatment as determined by Student’s two-tailed t-test.
Figure 1

A

EGFR
p1173
p998
4G10 (pTyr)

β-actin

EGF (30nM)  Cetuximab (μg/ml)
P E 1 3 5 10 20

B

EGF (30nM)  Gefitinib (nM)
P E 25 100 250 500 1000
Figure 2

- **EGF** stimulated
- **Proliferating** reversed by gefitinib
- Reversed by cetuximab

- ≥ 11 spectra per protein
- ≥ 2 fold change and quasi p-value ≤ 0.2

**EGFR inhibition signature**

- EGF stimulated: 191
- Reversed by gefitinib: 237
- Reversed by cetuximab: 133

- Merge and compare

- EGF: 5294
- Proliferating: 5203
- G+EGF: 5347
- C+EGF: 5250
Figure 4

| Gene     | A431 | DiFi (c) | DiFi (x) | HCT-116 (c) | HCT-116 (x) |
|----------|------|----------|----------|-------------|-------------|
| CLDN4    |      |          |          |             |             |
| JUN      |      |          |          |             |             |
| JAG1     |      |          |          |             |             |
| TRIM32   |      |          |          |             |             |
| JUND     |      |          |          |             |             |
| CDKN1A   |      |          |          |             |             |
| TRAPPC3  |      |          |          |             |             |
| RBM15    |      |          |          |             |             |
| COBLL1   |      |          |          |             |             |
| TOMM20   |      |          |          |             |             |
| CCDC50   |      |          |          |             |             |
| PRIM1    |      |          |          |             |             |

Cetuximab  
Gefitinib

- Green = down regulated by inhibitor treatment (compared to EGF)
- Red = up regulated by inhibitor treatment (compared to EGF)
- Black = detected but not statistically significant
- White = not detected
Figure 5

- CCDC50
- CLDN4
- COBLL1
- JAG1
- JUN
- JUND
- TOMM20
- TRAPPC3
- TRIM32