Quantitative profiling of protein tyrosine kinases in human cancer cell lines by multiplexed parallel reaction monitoring assays

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Key Words: protein tyrosine kinase, parallel reaction monitoring, mutations, drug resistance, colon cancer, lung cancer
ABBREVIATIONS

bRPLC, basic reverse phase liquid chromatography
CRC, colorectal cancer
CV, coefficient of variation
EGF, epidermal growth factor
FDR, false discovery rate
LC, liquid chromatography
LRP, labeled reference peptide
MRM, multiple reaction monitoring
MS, mass spectrometry
MS/MS, tandem mass spectrometry
SCX, strong cation exchange chromatography
TEAB, triethylamine bicarbonate

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SUMMARY

Protein tyrosine kinases (PTKs) play key roles in cellular signal transduction, cell cycle regulation, cell division, and cell differentiation. Dysregulation of PTK-activated pathways, often by receptor overexpression, gene amplification, or genetic mutation, is a causal factor underlying numerous cancers. In this study, we have developed a parallel reaction monitoring (PRM)-based assay for quantitative profiling of 83 PTKs. The assay detects 308 proteotypic peptides from 54 receptor tyrosine kinases and 29 nonreceptor tyrosine kinases in a single run. Quantitative comparisons were based on the labeled reference peptide method. We implemented the assay in four cell models: 1) a comparison of proliferating versus epidermal growth factor (EGF)-stimulated A431 cells, 2) a comparison of SW480Null (mutant APC) and SW480APC (APC restored) colon tumor cell lines, and 3) a comparison of 10 colorectal cancer cell lines with different genomic abnormalities, and 4) lung cancer cell lines with either susceptibility (11-18) or acquired resistance (11-18R) to the epidermal growth factor receptor tyrosine kinase inhibitor erlotinib. We observed distinct PTK expression changes that were induced by stimuli, genomic features or drug resistance, which were consistent with previous reports. However, most of the measured expression differences were novel observations. For example, acquired resistance to erlotinib in the 11-18 cell model was associated not only with previously reported upregulation of MET, but also with upregulation of FLK2 and downregulation of LYN and PTK7. Immunoblot analyses and shotgun proteomics data were highly consistent with PRM data. Multiplexed PRM assays provide a targeted, systems-level profiling approach to evaluate cancer-related proteotypes and adaptations. Data are available through Proteome eXchange Accession PXD002706.

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Protein tyrosine kinases (PTKs) are critical effectors of cell fate and are expressed ubiquitously during development and throughout the adult body. Ninety PTKs are encoded in the human genome and among them 58 are receptor type and 32 are nonreceptor tyrosine kinases (1, 2). PTKs initiate intracellular signaling events that elicit diverse cellular responses such as survival, proliferation, differentiation, and motility (3). PTK are some of the most frequently altered genes in cancer, either via mutation, overexpression, or amplification. The resultant deregulated cellular signaling contributes to disease progression and drug resistance. Regulation of PTKs is controlled both by extensive post-translational modifications, particularly protein phosphorylation and by changes in PTK abundance (4-6). Thus, there is potential utility in quantifying the expression of PTKs to identify drug response signatures and reveal new biological characteristics.

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Typically, expression of PTKs is measured by enzyme-linked immunosorbent assay, fluorescence activated cell sorting and immunoblotting, which provide information for a limited number of proteins in a single assay. Multiplexed targeted proteomic assays, on the other hand, could reveal simultaneous alterations of protein expression in entire PTK pathways. A widely used targeted proteomics approach for quantification is multiple reaction monitoring (MRM, also termed selected reaction monitoring), which is done on a triple quadrupole or quadrupole-ion trap mass spectrometer (7). In conjunction with standardization by stable isotope dilution, MRM enables precise, accurate measurements of protein concentrations over 4 to 5 orders of magnitude in biological specimens (8-10). With sample prefractionation, MRM can measure proteins at single digit copy numbers per cell (11). Despite the high specificity, sensitivity, and reproducibility of MRM measurements, the two-stage mass filtering using a low-resolution MS instrument does not
completely avoid interfering ions, which can hamper precise and specific protein quantification (12). In addition, MRM relies on a predefined and experimentally validated set of peptides and peptide fragmentations that requires considerable effort to develop (13).

High resolution and accurate mass peptide analysis now can be achieved with new generation mass spectrometers, such as the Q Exactive (ThermoFisher Scientific). These instruments combine the quadrupole precursor ion selection with the high resolution and high accuracy of an Orbitrap mass analyzer. Recent reports describe several modes of operation for targeted peptide analysis, the most powerful of which is termed parallel reaction monitoring (PRM), which generates both high resolution precursor measurements and high resolution full scan MS/MS data, from which transitions can be extracted post-acquisition (14, 15). A key feature of this approach is the highly specific extraction of signals for target peptides of interest, thus reducing interference from nominally isobaric contaminants.

A particularly useful approach to targeted proteome analysis is to configure multiplexed assay panels for proteins and their modified forms involved in specific pathways or networks. Koomen and colleagues first described this approach with their MRM analyses of components of the Wnt signaling pathway (16) and later expanded to multiple signaling pathways (17). Multiplexed MRM assay panels have been used to quantify phosphotyrosine sites in tyrosine kinase signaling networks (18) and to monitor the protein expression status of cellular metabolic pathways (19).

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In this study, we describe a multiplexed PRM-based assay for quantitation of 83 PTKs, which detects 308 proteotypic peptides from 54 receptor tyrosine kinases and 29 nonreceptor tyrosine kinases in a single scheduled run. We demonstrate analysis of PTK expression changes driven by stimulation, genomic abnormalities or drug resistance in tumor cell lines. PTK expression changes are selective and distinct for the models studied. The profiling of PTK expression changes in cell models provides proof of concept for application of the approach to systems-level analysis of PTK signaling and adaptation in cancer.

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MATERIALS AND METHODS

Cell lines and cell culture

Colorectal cancer (CRC) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). SW480APC (APC restored) and SW480Null (APC mutant) cells were a gift from Antony Burgess (Ludwig Institute, Melbourne, Australia) and 11-18 and 11-18R lung tumor cell lines (20) were provided by William Pao (Vanderbilt University School of Medicine). All cell lines were grown in 10% fetal bovine serum and penicillin/streptomycin supplemented medium at 37 °C under 5% CO₂. The 11-18, 11-18R, COLO205, DLD1, HC-15, SW480 and SW480APC cells were grown in RPMI 1640 medium. The HCT116, HT29 and RKO cells were grown in McCoy’s 5A medium, whereas CACO2 (with 20% fetal bovine serum) and LS174T cells were grown in Minimum Essential Medium and LOVO cells were grown in F-12 K medium (21). Proliferating cells were grown to 70–75% confluency before collection, whereas treated cells were grown to 60–65% confluency before incubation overnight in serum-free medium. For studies of EGF stimulation in A431 cells, serum-starved cells were treated with 30 nM EGF for 4 h. All of the cells were harvested on ice using cold magnesium- and calcium-free phosphate-buffered saline and supplemented with a phosphatase inhibitor mixture (1.0 mM sodium orthovanadate, 1.0mM sodium molybdate, 1.0mM sodium fluoride, and 10mM of beta-glycerophosphate) (Sigma-Aldrich). The cells were pelleted by centrifugation at 500 × g at 4°C and pellets were flash frozen in liquid nitrogen.

Cell lysis and protein digestion

Cell pellets were stored at −80 °C until cell lysis was performed. Lysis of cell pellets was done at ambient temperature. Biological replicates (one cell pellet from one cell line) were

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processed in parallel to minimize the effects of systematic errors. Pellets were resuspended in 100 μL 100 mM ammonium bicarbonate (AmBic) and 100 μL trifluoroethanol were added, followed by sonication (3 × 20 s). Samples were incubated at 60°C for 60 min at 1000 rpm on an Eppendorf Thermomixer and sonicated again (3 × 20 s). Protein concentration was estimated with the bicinchoninic acid assay (Pierce, Rockford, IL). Proteins (100 μg for PRM assays; 200 μg for shotgun analyses) were reduced with 40 mM tris(2-carboxyethyl)phosphine (and 100mM dithiothreitol and alkylated 50mM iodoacetamide. Samples were diluted in 50 mM AmBic, pH 8.0 and trypsinized overnight at 37 °C at a trypsin/protein ratio of 1:50, w/w). The resulting peptide mixture was lyophilized overnight and peptides were desalted as described (22).

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The peptides were separated using a linear gradient of 2% - 35% solvent B (0.1% formic acid in acetonitrile) at a flow rate of 300 nL min⁻¹ over 40 min, followed by an increase to 90% B over 4 min and held at 90% B for 6 min before returning to initial conditions of 2% B. For peptide ionization, 1800 V was applied and a 250 °C capillary temperature was used. All samples were analyzed using a multiplexed PRM method based on a scheduled
inclusion list containing the 314 target precursor ions representing PTK and LRP standard peptides. The full scan event was collected using a $m/z$ 380 - 1500 mass selection, an Orbitrap resolution of 17,500 (at $m/z$ 200), target automatic gain control (AGC) value of 3 X $10^6$ and a maximum injection time of 30 ms. The PRM scan events used an Orbitrap resolution of 17,500, an AGC value of 1 X $10^6$ and maximum fill time of 80 ms with an isolation width of 2 $m/z$. Fragmentation was performed with a normalized collision energy of 27 and MS/MS scans were acquired with a starting mass of $m/z$ 150. Scan windows were set to 4 min for each peptide in the final PRM method to ensure the measurement of 6-10 points per LC peak per transition.

All PRM data analysis and data integration was performed with Skyline software (23). This article has been withdrawn by the authors.

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The standard mixture (25 fmol of each standard peptide per sample) was added immediately following tryptic digestion. Five transitions for each peptide were extracted from the PRM data. The intensity rank order and chromatographic elution of the transitions were required to match those of a synthetic standard for each peptide measured. Summed peak areas from the five target peptide transitions were divided by the summed peak area for the five reference standard peptide transitions to give a peak area ratio and coefficients of variation (CVs) were calculated across replicates for each treatment.
Peptide peak areas were calculated using Skyline. Peptide peak area CV was calculated as:

\[
CV = \frac{\text{Peptide peak area standard deviation}}{\text{Average peptide peak area}}
\]

The CV of the three labeled reference peptides were calculated from the three separate PRM analyses per sample. Peptide peak area ratios were calculated by:

\[
\text{Peak area ratio} = \frac{\text{Peptide peak area}}{\text{Labeled reference peptide peak area}}
\]

**Immunoblotting analyses**

Cell pellets were resuspended and lysed in a modified RIPA assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) (Sigma-Aldrich) supplemented with phosphatase inhibitor mixture (see above) and protease inhibitor mixture (0.5 \( \mu \)M 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 10 mM aprotinin, 1.0 mM leupeptin, 5.0 \( \mu \)M bestatin, and 1.0 \( \mu \)M pepstatin)(HALT™, ThermoFisher Scientific). The lysates were chilled for 20 min on ice before sonication with five 1-s pulses at 30 watts and 20% output. The lysate was centrifuged at 13,000 \( \times \) 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. Lysates from each cell line 10 \( \mu \)g protein) were combined with 4\( \times \) SDS loading buffer (Invitrogen), incubated at 90°C for 5 minutes, and proteins were separated on 10% SDS-PAGE mini-gels (Invitrogen). Proteins were transferred to polyvinylidene difluoride membranes, which were probed with primary antibodies for EGFR (#2232), Phospho-EGFR (Tyr998) (#2641), EPHA2 (#3974), SYK (#2712), ZAP70 (#2705), LYN (#2732), and FYN (#4023) (Cell Signaling Technology).

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PTK7 (ab55633) and ACTB (ab199622) (Abcam). Membranes were probed with fluorophore-conjugated secondary antibodies (Invitrogen) and proteins were visualized on a fluorescence scanner (LI-COR Odyssey; LIC-COR). Sample loads were normalized for total protein concentration before reducing with dithiothreitol and adding NuPAGE® lithium dodecyl sulfate sample buffer and then were boiled for 7 min at 90°C.

*iTRAQ labeling and phosphotyrosine enrichment for analysis of 11-18 and 11-18R cells*

Peptide labeling with iTRAQ 4plex (AB Sciex) was performed as previously described (25, 26). Briefly, for each analysis, ~1 X 10^7 cells (equivalent to 400 μg peptide before desalting and labeling) for each cell line was labeled with one tube of iTRAQ 4plex reagent. The 11-18 cells were labeled with the iTRAQ 4-plex as follows: 114- and 116-channels, 11-18 cells; 115- and 117-channels 11-18R cells. Lyophilized samples were dissolved in 60 μL of 500 mM triethylammonium bicarbonate, pH 8.5, and the iTRAQ reagent was dissolved in 70 μL of isopropanol. The solution containing peptides and iTRAQ reagent was vortex mixed and then incubated at room temperature for 1h and concentrated to 40 μL under vacuum. Samples labeled with four different isotopomeric iTRAQ reagents were combined and evaporated to dryness. Peptides then were dissolved in 400 μL of immunoprecipitation buffer (100 mM Tris, 100 mM NaCl, and 1% Nonidet P-40, pH 7.4) and the pH was adjusted to 7.4 before phosphotyrosine immunoprecipitation.

For phosphotyrosine peptide immunoprecipitation, Protein G agarose (80 μL, EMD Millipore) was incubated with three phosphotyrosine antibodies; 12 μg PT66 (Sigma-Aldrich), 12 μg pY100 (Cell Signaling Technology), and 12 μg 4G10 (EMD Millipore) and 200 μL of immunoprecipitation buffer (100 mm Tris, 100mM NaCl, 1% Nonidet P-40, pH 7.4) was

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added and the mixture was incubated for 8 h at 4°C with gentle mixing by rotation. Antibody conjugated Protein G then was rinsed and iTRAQ 4plex labeled peptides were resuspended in the immunoprecipitation buffer, added to the conjugated Protein G and incubated overnight at 4°C with rotation. Conjugated Protein G agarose was rinsed with 400 μL of immunoprecipitation buffer and 4 × 400 μL of rinse buffer (100 mm Tris, pH 7.4), and peptides were eluted into 70 μL of 100 mm glycine pH 2. Peptides were desalted with Stage Tips (ThermoFisher Scientific).

Phosphotyrosine peptide analysis by LC-MS/MS

Phosphotyrosine peptide separations were performed using an Easy nLC-1000 pump and autosampler system (ThermoFisher Scientific). Injections were done with a 10 μL loop and operated in data-dependent mode with a full scan MS spectrum followed by MS/MS for the top 10 precursor ions in each cycle. Maximum injection time for MS was set to 50 ms with a resolution of 70,000 across m/z 350–2000. For MS/MS, maximum injection time was set to 300 ms with resolution of 35,000. All resulting MS/MS spectra were assigned to peptides from the RefSeq human database version 54 (Sep 25, 2012; 69178 Entries) by the MyriMatch 2.1.138 algorithm. Mass tolerance for precursor ions was set to 7 ppm and fragment ion mass tolerance was 10 ppm. MS/MS spectra searches incorporated fixed modifications of carbamidomethylation of cysteine and iTRAQ 4plex modification of lysines and peptide N termini. Variable modifications were oxidized methionine, and
phosphorylation of serine, threonine, and tyrosine residues. MS/MS spectra of tyrosine phosphorylated peptides were manually validated to confirm peptide identification and phosphorylation site localization. Annotated MS/MS spectra for all phosphotyrosine assignments are provided in Supplemental Dataset 1. Phosphotyrosine peptide iTRAQ ratios were normalized based on the mean relative protein quantification ratios obtained from the total protein (i.e. protein expression analysis).

RNA-seq analysis

The RNA samples were sequenced following the protocols recommended by the manufacturer (Illumina). Briefly, poly-A was purified and then fragmented into small pieces. Using reverse transcriptase and random primers, RNA fragments were used to synthesize the first and second strand cDNAs. Following end repair, addition of an "A" base, adapter ligation, size selection and amplification of cDNA templates, samples were sequenced in 5 lanes on the Illumina HiSeq 2000, generating about 70~110 million of 100 pair-end reads per sample. Reads were mapped to human genome hg19 using TopHat version 2.0.9 with the reference annotation file. The GTF file based on Refseq gene annotation downloads from the UCSC table browser on Dec 9th, 2013. The aligned reads were assembled and transcript expression was quantified using FPKM (Fragments Per Kilobase of transcript per Million fragments mapped) by Cufflinks version 2.0.2, which uses a linear statistical model to compute the likelihood that the number of fragments would be observed given the proposed abundances of the transcripts (28).

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Experimental design and statistical rationale

For PRM analyses in cell culture experiments, three replicate cell cultures were analyzed for each cell culture and treatment. For 10 colorectal cancer cell lines, two replicate cell cultures were analyzed. Student’s t-test was performed with the pair-wise comparisons to determine statistical significance of differences.

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RESULTS AND DISCUSSION

Development of PTK PRM assay panel

We developed a PRM assay for quantitation of 83 PTKs, which measures proteotypic peptides from 54 receptor tyrosine kinases and 29 nonreceptor tyrosine kinases in a single run. We experimentally optimized PRM transition parameters with chemically synthesized peptides. Target peptides were required to be between 7 and 25 amino acids long and were selected based on uniqueness and anticipated chemical stability. Peptides containing cysteine or methionine residues were not excluded. Although priority was given to peptides that were previously identified in the shotgun data set with high MS/MS spectral quality (21, 30, 31), additional predicted peptides were selected in silico. Each PTK protein was monitored by 3-4 proteotypic peptides. The complete panel contained 308 proteotypic peptides.

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Although the PRM method monitored 308 target peptides, additional criteria were applied post-analysis to ensure correct identities of the signals attributed to each target peptide. Accordingly, using Skyline, we verified that the 5 most intense transitions attributed to the target peptide co-eluted and displayed identical chromatographic retention with the synthetic peptide standards and that the order of y-ion fragment intensities matched the order for the synthetic peptide standard. An example of application these criteria to evaluate peptide PRM data are provided in Figure S1.

We applied this PRM assay panel to profile 83 PTK proteins in four cell model studies: 1) a comparison of proliferating versus epidermal growth factor (EGF)-stimulated A431 cells, 2) a comparison of SW480Null (mutant APC) and SW480APC (APC restored) colon tumor cell lines, 3) a comparison of 10 colorectal cancer cell lines with different genomic abnormalities, and 4) a comparison of lung cancer cell lines with either susceptibility or acquired resistance to the epidermal growth factor receptor tyrosine kinase inhibitor erlotinib. We compared PRM-based measurements to spectral count-based estimates and immunoblotting analyses. For the A431 cell model, the SW480 cell model and the 10 CRC cell lines, we used the previously published shotgun datasets (21, 30, 31). For the lung cancer cell lines study, we performed shotgun analyses for this work, as described in Supplemental Methods. Three replicate cultures of each cell line were collected for analysis. All lysates in each study were prepared together for analysis. Each sample was spiked with all three LRP reference peptide standards and the CV for each was calculated across all analyses in each of the four studies (Table 1). In each study, the alkaline phosphatase peptide yielded the lowest CV (mean 6.76% across all four studies) and was used for normalization.

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**Study 1: EGF stimulation in A431 cells**

Recently, we reported protein expression changes produced by EGF stimulation and inhibition in A431 cells, as measured by LC-MS/MS label free shotgun proteomics (31). We employed the same model to study EGF-induced PTK expression changes. Twenty seven PTKs were detected in this study, eight of which showed significant differences ($p < 0.05$) ([Figure 1A](#)). Although a decrease in EGFR protein upon ligand stimulation is well-documented (32), other changes we observed appear to be selective and novel, including downregulation of IGF1R, PTK7, AXL and, most notably EPHA1 (0.40 fold, $p < 0.0004$) and upregulation of SRC, FAK2 and RET.

Table S2 summarizes shotgun LC-MS/MS and PRM data for PTKs in the A431 model. PRM measurements agree with LC-MS/MS shotgun proteomics and identical trends in protein expression were observed between the two platforms. However PRM analyses enabled detection of 11 PTKs not detected by shotgun LC-MS/MS, most notably EPHA1, which was most significantly downregulated by EGF treatment. Thus, the data demonstrate that for a subset of differentially expressed proteins, label free shotgun proteomics data and PRM data are broadly concordant.

We also compared PRM with immunoblotting for selected PTK proteins ([Figure 1B](#)). After treatment with EGF, EGFR phosphorylation at Tyr-998 dramatically increased, which reflects EGFR signaling activation. EGFR, EPHA2, PTK7, and LYN proteins, for which antibodies were commercially available, were chosen for confirmation. The immunoblot results were consistent with shotgun and PRM data.

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**Study 2: Effect of APC mutation in a colorectal cancer model**

APC mutations are a hallmark of most colon and rectal cancers, with mutations or allelic losses in 70-80% of adenocarcinomas and adenomas (33). We previously described proteomic consequences of APC loss in SW480 colon carcinoma cells (30). Shotgun LC-MS/MS identified over 5000 proteins, of which 155 were significantly different between SW480Null (mutant APC) and SW480APC (APC restored) (30). As we had previously reported, EGFR was elevated in SW480null cells (2.22 fold, \( p < 0.0001 \)). However PTK analysis with the PRM panel detected 28 PTKs, 9 of which showed significant differences (\( p < 0.05 \)), including EGFR, EPHA2, EPHA4, IGF1R, JAK1, RON, SYK, ZAP70, and TNK1 (Figure 2A). Of six EPH proteins detected, EPHA2 and EPHA4 (2.94 fold, \( p < 0.0001 \)) were selectively over-expressed in SW480Null. These results were consistent with LC-MS/MS shotgun data (Table S3).

A subset of the PTKs found to be differentially expressed by PRM assay also were assessed by immunoblotting (Figure 2B). The five proteins, EGFR, EPHA2, SYK, ZAP70, and PTK7, were analyzed in three SW480Null and three SW480APC cultures and the immunoblot results were consistent with shotgun and PRM data.

**Study 3: PTK expression related to different genomic abnormalities in 10 CRC cell lines**

We recently described shotgun proteomic and integrated proteogenomic analyses of 10 CRC cell lines (21). These cell lines display mutations frequently associated with CRC, including KRAS, APC, TP53, PLK3CA, BRAF, and CTNNB1 (Table S4). Six of the cell lines display microsatellite instability (MSI) and epigenetic silencing or mutation of the DNA
mismatch repair genes MLH1, MSH2 and MSH6. We analyzed PTKs in three replicate cultures from each cell line and then compared PTK status as a function of genomic characteristics. The results of the PTK measurements are presented in Table S5. We then compared PTK abundance in CRC cell lines based on differences in KRAS, TP53, PIK3CA, BRAF and CTNNB2 mutations and in MSI status.

KRAS mutations impact EGFR signaling (34) and clinical responses to EGFR inhibitors (35, 36). Five of the CRC lines (DLD1, HCT116, HCT15, LOVO, and LS174T) contain both a wild type KRAS allele and a codon 12/13 mutant, whereas SW480 contains two mutant (G12V) alleles. EGFR abundance displayed substantial heterogeneity between KRAS mutant CRC cells. As shown in Figure 3A and Figure S2, EGFR was expressed at uniformly low levels in KRAS wild type cells, whereas the KRAS mutant cell lines SW480 and LOVO expressed EGFR at highest abundance. However, the KRAS mutant cell lines LS174T and HCT15 expressed EGFR at levels similar to KRAS wild type cells. Immunoblot analysis confirmed these differences in EGFR expression (Figure 3B).

Six of the CRC cell lines (Caco2, COLO205, HT29, SW480, DLD1, and HCT15) have TP53 mutations, whereas four (HCT116, LOVO, LS174T, and RKO) do not. Comparison of PTK profiles for these cells revealed substantial variation in expression of the non-receptor PTK LYN. Figure 3C and Figure S3 shows that LYN which was expressed at uniformly low levels in TP53 mutated cell lines, but at high levels in the TP53wt cell lines LOVO and RKO. Immunoblot analysis of LYN were consistent with the PRM measurements (Figure 3D).

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We further compared PTK profiles for the 10 CRC cell lines based on their classification as microsatellite stable (MSS) (Caco2, COLO205, HT29, and SW480) and MSI (DLD1, HCT116, HCT15, LOVO, LS174T, and RKO) cells. Three PTKs, EPHA4, IGF1R, and SYK displayed significant lower average expression ($p < 0.05$) in MSI than in MSS cells (Figure S4). However, protein abundances of these PTKs were highly variable within MSI and MSS cells. Six CRC cell lines (HT29, DLD1, HCT116, HCT15, LS174T, and RKO) have PIK3CA mutations. We found that PIK3CA mutant cells express on average higher ERBB3 and lower EPHA4, IGF1R, and NTRK3 than in PIK3CA wild type cells (Figure S5), although expression patterns of these three PTKs differed dramatically between PIK3CA mutant and wild type cells. Three of the CRC cell lines (COLO205, HT29, and RKO) have BRAF V600E mutations and displayed elevated ABL1, EPHB4, FAK1, and SRC (Figure S6). Six of the CRC cell lines (HT29, SW480, DLD1, HCT15, LOVO, and RKO) had CTNNB1 mutations and displayed decreased FGFR2 and elevated EPHB2, FGFR4, and HCK compared to CTNNB1 wild type CRC cells (Figure S7), although protein abundance of these three PTK was highly variable between cell lines.

**Study 4: PTK alterations related to erlotinib resistance in lung tumor cells**

A potentially important application of PTK profiling is to identify mechanisms of drug resistance involving alterations in PTK signaling pathways. We explored this application in a lung tumor cell model of acquired resistance to the PTK inhibitor erlotinib (20). The parental cell line 11-18 displays sensitivity to erlotinib, which blocks EGF-stimulated activation of ERK. Resistant 11-18R cells display activated ERK in the presence of erlotinib, which has been attributed to an activating NRAS Q61K mutation (20). Analyses with the PTK PRM panel quantified 21 proteins in both cell lines, of which 13 displayed significant

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differences (p < 0.05). EGFR, FLK2, and MET were increased in 11-18R cells, whereas EPHA1, FAK1, FGFR2, IGF1R, LYN, PGFRA, PTK7, SRC, VGFR2, and YES were decreased (Figure 4A). PRM analyses also were consistent with measurements for several of the PTKs that were detected in shotgun proteomic analyses (see Supplemental Methods and Table S6). These results also were confirmed by immunoblot analysis (Figure 4B).

Our analyses also detected increased MET abundance in 11-18R. Although MET amplification has not been reported as a characteristic of this particular cell line (20), several studies have demonstrated that in lung adenocarcinoma–derived cells, EGFR inhibition can be overcome by signaling through hepatocyte growth factor (HGF) and MET (37, 38). Moreover, MET amplification is associated with acquired resistance to anti-EGFR treatment in patients (39).

To better assess the role of PTK abundance changes versus PTK activation, we performed a phosphotyrosine profiling analysis of the 11-18 and 11-18R cells with a 4-plex iTRAQ reagent (Figure S8). This analysis identified 226 tyrosine phosphorylation sites on 133 proteins, with 64 phosphorylation sites exhibiting greater than 1.5-fold differences in tyrosine phosphorylation between 11-18 and 11-18R cells (Table S7). Annotated MS/MS spectra of all assigned phosphotyrosine sequences are presented in Supplementary Dataset 1. Phosphotyrosine sites quantified on the PTKs targeted by our PRM panel are shown in Table 2. Erlotinib resistance in 11-18R cells resulted in multiple phosphorylation changes, with EPHB4, ERBB3, MET, and FAK1 phosphorylation sites increasing and IGF1R phosphorylation sites decreasing. Phosphorylation of MET is consistent with activation of HGF/MET as an adaptation associated with resistance to EGFR TK inhibitors.

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Comparison of protein and mRNA for PTKs

Because our PTK assay panel was developed from a list of known human PTKs, some proteins may not have been detected either because of low protein abundance or because of lack of transcription of the corresponding genes. We performed transcriptome sequencing (RNA-seq) analyses of the cell lines in Studies 1, 2 and 4. In Study 1, 27 PTK proteins were detected in the A431 cell model, whereas 64 PTKs were detected as the corresponding mRNA transcripts (Figure S9). In Study 2, 28 PTK proteins were detected in the SW480APC model, whereas 71 PTKs were detected as the corresponding mRNA transcripts (Figure S9). In Study 4, 21 PTK proteins were detected in the 11-18/11-18R model, whereas 66 PTKs were detected as the corresponding mRNA transcripts (Figure S10).

These analyses demonstrated a high correspondence between detected proteins and mRNA for PTKs, although approximately 70% of the transcripts in all three models were not detected as proteins. This may reflect either low protein abundance or inefficient translation of these mRNAs.

Concluding remarks

PTKs are among the most intensively pursued superfamilies of enzymes as targets for anti-cancer drugs. PTK expression varies between different types and stages of cancer and alterations in PTK expression are an important mechanism of resistance to targeted cancer therapeutics. These considerations suggest that multiplexed, targeted analysis of PTK expression profiles could be valuable in studying mechanisms of drug susceptibility and resistance. Here, we provide proof of concept for this approach with a PRM-based assay to quantify up to 83 PTKs in mammalian cells. This PTK assay panel detects selective

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alterations in PTK expression in multiple cellular model systems and the PRM data are highly consistent with data from western blot and shotgun LC-MS/MS analyses. Although we employed the labeled reference peptide method as a low-cost alternative to stable isotope dilution, other strategies could be employed, such as the intermediate-cost alternative of using low purity stable isotope labeled peptide standards for all analytes (40).

We observed distinct PTK expression changes in all of the cell model systems we studied. Moreover, our analyses of colon tumor cell lines illustrated the unexpected associations of many cancer-associated mutations with PTK expression, as KRAS, BRAF, TP53, PIK3CA and CTNNB mutations all exerted distinct effects in different cells. Our analyses detected previously reported PTK expression changes induced by EGFR ligand stimulation, APC...

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Although our studies were limited to cell culture models, PTK assay panels could be extended to studies of tissues. Signaling networks can be analyzed through measurements of phosphosites, but recent studies indicate that phosphorylation status in tissues is highly sensitive to tissue ischemia (42, 43), whereas protein abundance remains unaffected. PTK profiling at the protein expression level thus may provide a robust alternative to study adaptation of signaling networks in human tumors.
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### Table 1. Coefficients of variation for PRM peak areas from labeled reference peptide standards

| Study | CV (%) | AP | BG | ACTB |
|-------|--------|----|----|------|
| 1     | A431 EGF | 5.91 | 8.83 | 6.53 |
| 2     | SW480 APC | 6.76 | 7.39 | 6.49 |
| 3     | CRC-10 cell lines | 8.83 | 9.32 | 12.92 |
| 4     | 11-18/11-18R | 5.55 | 7.44 | 6.24 |

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Table 2. Phosphotyrosine sites quantified on PTKs

| PTK   | Phosphosite | Phosphotyrosine Ratio (1118R/1118) | Protein abundance |
|-------|-------------|-----------------------------------|-------------------|
|       |             |                                   | Ratio (1118R/1118) | p-value          |
| EGFR  | Y1172       | 1.26                              |                   | 1.56 <0.001      |
| EGFR  | Y1110       | 1.04                              |                   | 1.56 <0.001      |
| EGFR  | Y1197       | 0.86                              |                   | 1.56 <0.001      |
| EPHB4 | Y574        | 1.53                              |                   | 1.00 0.973       |
| EPHB4 | Y774        | 1.75                              |                   | 1.00 0.973       |
| ERBB2 | Y1248       | 1.25                              | ND                | ND               |
| ERBB3 | Y1307       | 1.99                              | ND                | ND               |
| ERBB3 | Y1328       | 1.16                              | ND                | ND               |
| FER   | Y402        | 1.20                              | ND                | ND               |

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FAK1  | Y577 | 1.36 | 0.73 | <0.001 |
FAK1  | Y397 | 1.87 | 0.73 | <0.001 |
TNK2  | Y827 | 1.22 | ND   | ND     |
TNK2  | Y284 | 1.07 | ND   | ND     |
TYK2  | Y292 | 0.87 | ND   | ND     |

1 ND, not detected.
FIGURE LEGENDS

Figure 1. PTKs related to EGF stimulation in A431 cells. A, Quantitative comparison of PTK expression in proliferating and EGF stimulated A431 cells. EGF stimulation results in increased expression of RET, FAK2 and SRC (red symbols) and decreased expression of EGFR, IGF1R, PTK7, EPHA1 and UFO (green symbols). B, Confirmation of expression changes of EGFR, EPHA2, PTK7 and LYN by immunoblot analysis. Four replicate cultures of proliferating (P) and EGF stimulated A431 (E) cells were analyzed.

Figure 2. PTKs related to APC mutation in a colorectal cancer model. A, Quantitative comparison of PTK expression in SW480Null and SW480APC cells. APCnull status results in increased expression of EGFR, IGF1R, EPHA2, EPHA4, RON and JAK1 (red symbols) and decreased expression of TNK1, SYK and ZAP70 (green symbols). B, Confirmation of the expression of EGFR, EPHA2, SYK, Zap70 and PTK7 by immunoblot analysis. Three replicate cultures of SW480 (N) and SW480APC (A) were analyzed.

Figure 3. PTKs related to different genomic abnormalities in 10 CRC cell lines. A, Representative quantitative results by PRM assay for peptide YLVIQGDER from EGFR in CRC cell lines. Plotted values are mean ± SD of the LRP-normalized peptide peak areas from three replicate cultures of each cell line. B, Confirmation of the expression of EGFR by immunoblot analysis. C, Representative quantitative results by PRM assay for peptide QLLAPGNSAGAFLIR from LYN in CRC cell lines. Plotted values are mean ± SD of the LRP-normalized peptide peak areas from three replicate cultures of each cell line. D, Confirmation of the expression of LYN by immunoblot analysis.

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Figure 4. PTKs related in erlotinib resistance in lung cancer cells. A, Quantitative comparison of PTK expression for 11-18 and 11-18R cells. Acquired resistance resulted in increased expression of EGFR, FLK2 and MET (red symbols) and decreased expression of IGF1R, PDGFRα, VEGFR2, FGR2, PTK7, EPHA1, LYN, FAK1, SRC and YES (green symbols). B, Conformation of the expression of EGFR, PTK7, LYN and FYN by immunoblot analysis. Four replicate cultures of proliferating (11-18, P) and erlotinib resistant (11-18R, R) cells were analyzed.

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