Mapping deregulated pathways downstream of RTKs in cell line models

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Abstract

In virtually all epithelial tumors, growth factor receptor activity is deregulated by activated mutations, genomic amplification, and autocrine loops. The dependence of tumor cell survival upon the driving oncogene has been called "oncogene addiction" and demonstrates the acute sensitivity of cancer cells to inhibition of the pathways driving their proliferation, growth and survival. Major questions regarding the sensitivity of solid tumors to targeted kinase inhibitors are why some tumors respond while others do not and why tumors which respond still are able to develop resistance through the activation of other pathways. Robust, but not durable, response to receptor tyrosine kinase inhibitors (RTKIs) highlights the need to inhibit pathway activity at multiple levels.

In this study, we identify extensive signaling networks downstream of receptor tyroine kinases (RTKs) across multiple spaces, including phosphorylation, acetylation, and methylation. We used the established method of TMT labeling coupled with serial peptide immunoprecipitation to profile drug-treated and untreated RTK pathway-addicted cancer cell lines. We also examined the ratio of protein levels in treated cell lines. Quantitative analysis was performed to look at the effects of drugs on RTK-dependent cell lines: MKN45 (Met) with Crizotinib, H1703 (PDGFRα) with Gleevec, and H3255 (EGFR) with Iressa. Phosphotyrosine, acetyl-lysine, methyl arginine, ATM/ATR substrate (s/tQ), and AGC/CAMK/STE family kinase motif were all selected for use in peptide immunoprecipitation which allowed us to characterize and quantify a broad PTM space. In this study we were able to identify and quantify hundreds of modification sites for phosphorylation, arginine methylation, and lysine acetylation. Those sites found to be drug-sensitive, including all PTM types, were used for pathway analysis of signaling downstream of each RTK disease driver.

Materials and Methods

Cells were cultured in DMEM medium or RPMI 1640 medium supplemented with 10% fetal bovine serum and processed in the appropriate amount of a 8 M urea lysis buffer. Cells were starved overnight and then treated with the following drugs for 1, 3, 6, and 24 hrs: MKN45 (Met) with Crizotinib, H1703 (PDGFRα) with Gleevec®, and H3255 (EGFR) with Iressa®. Samples were then reduced and alkylated before being digested with trypsin. After digestion, samples were purified over Sep-Pak® C18 columns and eluted peptides were lyophilized. After peptide quantitation 2 mg of each sample was labeled using TMT reagents with 100 µg used for total proteome analysis and the rest used to profile PTM spaces. Six plex were organized the following way:

Labeled samples were then combined and subjected to sequential immunoprecipitations using motif antibodies for PTMs and in parallel basic reverse phase (bRP) chromatography was performed to profile the full proteome. Samples were purified over Sep-Pak® C18 columns before being processed by LC-MS/MS/MS analysis. Data generated from the MS analysis were processed through several modules in CORE, ending with quantitated data.

Sample	TMT Channel
H3255 Control	126
H3255 Control	127
H3255 1 hr Iressa	128
H3255 3 hr Iressa	129
H3255 6 hr Iressa	130
H3255 24 hr Iressa	131

Sample	TMT Channel
H1703 Control	126
H1703 1 hr Gleevec	127
H1703 3 hr Gleevec	128
H1703 6 hr Gleevec	129
H1703 24 hr Gleevec	130
H1703 24 hr Gleevec	131

Sample	TMT Channel
MKN 45 Control	126
MKN 45 Control	127
MKN 45 1 hr Criz.	128
MKN 45 3 hr Criz.	129
MKN 45 6 hr Criz.	130
MKN 45 24 hr Criz.	131

Ab Target	Clone #	Motif
pY-1000	D1G10/D2D1	Phospho Tyr
AGC/PSD Akt substrate AMPK ATM/ATR substrate ATM/ATR substrate	D3E5/D8D9/D4E2/D8B11 100B7 D72H3/D78G9 D23/D69 D14/D86	AGC/CAMK/STE kinase acitivation loop RXX(s/t) LXRXXT(S) (s/t)Q (s/t)Q
Ac-K K-Me R-Me	D10G3/11D/16E D4P3J, D3Z9J, D8R1C Me-R4-100	Acetyl lysine K-Me R-Me

Figure 1. Motif antibodies chosen for PhosphoScan® profile (MS/MS).

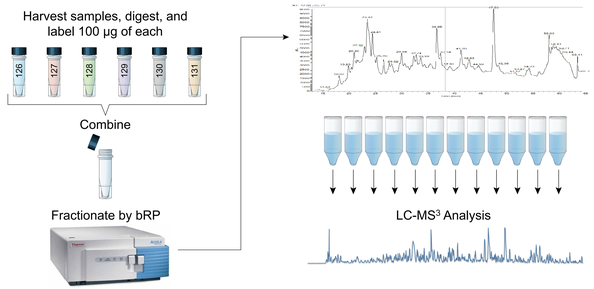

Figure 2. Full proteome. Samples are harvested in 8M urea, homogenized if necessary, reduced, and alkylated before being digested. Samples were purified over Sep-Pak® C18 columns before being subjected to peptide quantification. 100 µg of each sample was then labeled with the appropriate TMT reagent according to the optimized protocol, combined at a 1:1:1:1:1:1 ratio, and again purified over a Sep-Pak® C18 column. Samples were then fractionated over an ACCELA® HPLC using a 4.6X150 mm ZORBAX® 300 Extend-C18 (5 µm particle size with 300 A-pore). 96 fractions were collected over the 60-minute gradient. The fractions were then combined to give a total of 12 and these fractions were again purified over a C18 micro column. Each purified fraction was then subjected to MS/MS/MS analysis. Data processing was performed using CORE. (Protein Sieve, Protein Assembler, Protein Quant.)

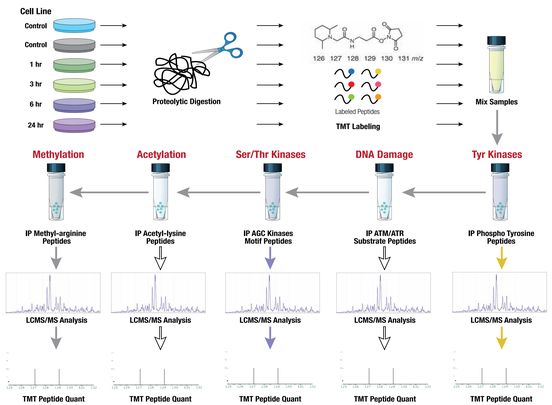

Figure 3. Experimental flowchart. Cell lysates were prepared from RTK inhibitor treated and untreated cell lines, proteins were digested with trypsin, peptides were TMT-labeled, and samples were mixed for 6-plex analysis. The peptide mixtures were serially fractionated through immunoprecipitation with antibodies specific for phosphotyrosine, ATM/ATR substrate motif, AGC/CAMK/STE family kinase motif, acetyl-lysine, and methyl-arginine, though any motif-recognizing antibody could be used with this protocol. The enriched peptides from each immunoprecipitation were analyzed by LC-MS/MS using an LTQ® Orbitrap® Elite, with quantification of the TMT labels enabled by HCD fragmentation.

Results

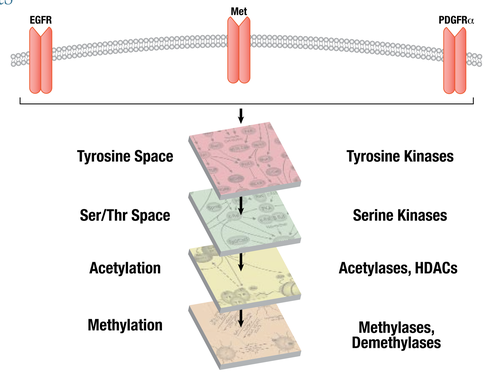

Figure 4. RTK driven cell lines profiled through the following spaces: phosphotyrosine, phosphoserine/phosphothreonine, acetylation, methylation.

	A549 Iressa®, Gleevec®	H1703 Iressa®	H3255 Gleevec®	MKN45 Crizotinib
pY				
AGC/PSD Akt/AMPK ATM/ATR				
Ac-K				
K-Me				
R-Me				
Total Proteome				

unchanged
x > = 2.5
x < = -2.5

Figure 5. Number of sites affected by RTK inhibitors across spaces

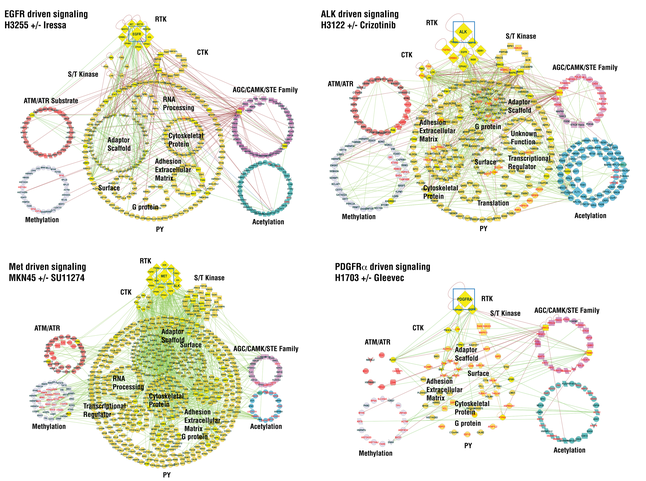

Figure 6. Pathway diagrams for each cell line were generated using Cytoscape®. These diagrams include all proteins with modification sites increasing or decreasing at least 2x upon relevant drug treatment. Proteins with PTM site peptides decreasing upon treatment (dark green protein names) are distinguished from those with sites increasing upon drug treatment (red protein names). Proteins identified in different PTM classes (phosphotyrosine, ATM/ATR substrate phosphorylation, AGC/CAMK/STE kinase family motif phosphorylation, lysine acetylation, and arginine methylation) are distinguished by different colored protein symbols. Shapes of protein symbols signify protein functionality (cytoskeletal, adaptor, kinase, adhesion protein, etc.). First order interacting partners with the driving RTK are presented in bright yellow.

Summary

- **Intelligent fractionation through sequential immunoprecipitation can be used to probe numerous PTM spaces, allowing in-depth pathway mapping of valuable samples**
- **Deep data sets generated through enrichment of different types of modified peptides allow elucidation of novel biological relationships**

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