Towards the Mechanism of EGFR Inhibitor Resistance in Non-Small Lung Cancer Cells

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Overview

Purpose: To determine the mechanism of tyrosine kinase inhibitor (TKI) resistance through analysis of differential changes in protein pathway expression levels by liquid chromatography-mass spectrometry (LC-MS).

Methods: Parental and tyrosine-kinase inhibitor (Erlotinib) resistant cell lines were treated with Epidermal Growth Factor (EGF), Erlotinib, Erlotinib and EGF, or left untreated. Samples from each condition were labeled with TMT8plex isobaric tags and analyzed/quantified by LC-MS on two hybrid instruments based on the Thermo Scientific™ Orbitrap™ mass analyzer.

Results: The increased multiplexing capabilities of Tandem Mass Tag® (TMT®) labeling enables simultaneous comparison of 8 different treatment conditions. Changes in protein expression ratios between parent and TKI-resistant cell lines were observed in several important cell signaling pathways with improved quantitative accuracy using synchronous precursor selection (SPS) acquisition methods compared to higher-energy collision dissociation (HCD) MS^2 methods.

Introduction

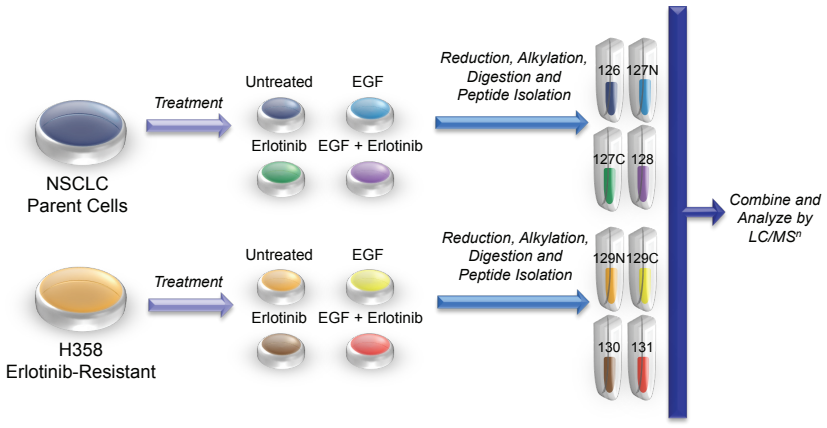
While tyrosine kinase inhibitors against Epidermal Growth Factor Receptor (EGFR) have positive therapeutic effects in a subset of Non-Small Cell Lung Cancer (NSCLC) patients, their clinical efficacies are limited due to the development of TKI resistance. To study the mechanism of TKI resistance, two NSCLC Erlotinib-resistant cell lines were established. To identify differences in protein expression between parental and drug-resistant cells after EGF and/or Erlotinib treatment, the high multiplexing capabilities of TMT reagents and MS analysis were utilized. Various pathways implicated in drug-resistance were examined in the context of expression differences in key proteins using ion trap/Orbitrap mass spectrometer hybrids with a new synchronous precursor selection (SPS) MS^3 fragmentation/quantitation method.

Methods

Sample Preparation

Two NSCLC cell lines (H2170 and H358) were established with resistance to the EGFR-tyrosine kinase inhibitor, Erlotinib, exhibiting a 4-5 fold higher IC50 than the parent cell line[1]. Parental and drug-resistant NSCLC cell lines were left untreated or treated with EGF or 10μM Erlotinib alone, or treated with both EGF and Erlotinib, for 2.5 minutes. After reduction and alkylation, proteins from the eight conditions were digested with trypsin and then labeled with Thermo Scientific TMT6plex reagents plus two 13C/15N isotope variants of the TMT6-127 and TMT6-129 reagents according to the manufacture's protocol. The peptides were then combined and analyzed by LC/MS (Figure 1). Immunoblotting using antibodies against phospho-EGFR, EGFR, mTOR, phospho-mTOR, S6 kinase, phospho-S6 kinase, phospho-ERK and ERK was also performed.

FIGURE 1. Sample treatment and peptide labeling scheme of both Parental and H358 Erlotinib Resistant NSCLC cell lines. Following treatment, cellular proteins are extracted, reduced, alkylated, and digested before being labeled with TMT8plex reagents. Labeled peptides from each treatment condition are combined and analyzed by LC/MS^n.



Liquid Chromatography

Samples were eluted by RP-HPLC using a Thermo Scientific™ EASY-nLC™ 1000 nano-flow system connected to a Thermo Scientific™ EASY-Spray™ column, 25 cm × 75 µm, Thermo Scientific™ Acclaim™ PepMap™ C18 Column and a 2 cm Acclaim PepMap100 trap column. Mobile phase A consisted of 0.1% formic acid in water while mobile phase B was 0.1% formic acid in acetonitrile. A gradient beginning with 5% B increasing to 25% B over 180 minutes followed by 25% to 40% B over a further 30 minutes was employed with a flow rate of 300 nL/min.

Mass Spectrometry

Spectra were acquired on a an Thermo Scientific™ Orbitrap Elite™ hybrid mass spectrometer using data-dependent MS/MS acquisition mode. The 15 most intense precursors selected from the FT MS1 full scan (resolution 120,000 FWHM) were isolated and fragmented by FT MS^2 (HCD) at resolution of 30,000@*m/z* 400. SPS MS^3 quantification on the Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer was performed using an FTMS full scan at 120,000 @ *m/z* 200 resolution followed by IT MS^2 CID and FT MS^3 HCD (resolution 60000 @ *m/z* 200) on a total of 10 fragments from the MS^2 spectra[2].

Data Analysis

Spectral data files were analyzed using Thermo Scientific™ Proteome Discoverer™ 1.4 software using the SEQUEST®HT search engine, constrained with a precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.02 Da (0.8 Da for CID identification using SPS). Carbamidomethylation (+57.021 Da) for cysteine and TMT isobaric labeling (+229.162 Da) for lysine and N-terminus residues were treated as static modifications while methionine oxidation (+15.996 Da) and deamidation of asparagine and glutamine (+0.984 Da) were considered as variable peptide modifications. Data was searched against a Swiss-Prot® complete human database with a 1% FDR criteria using Percolator[3].

Pathway analysis/protein profiling was performed using Thermo Scientific™ ProteinCenter™ 3.9 software. The TMT8plex quantification method within Proteome Discoverer 1.4 software was used to calculate the reporter ratios with mass tolerance ±10 ppm without applying the isotopic correction factors. A protein ratio was expressed as a median value of the ratios for all quantifiable spectra of the peptides pertaining to that protein[5].

Results

Immunoblot Analysis

Targeted antibodies revealed little change in the degree of mTOR, EGFR, S6 kinase, ERK, and β-actin expression between the parental and drug-resistant cell lines under all treatment conditions (Figure 2A). Autophosphorylation of EGFR was noticeably suppressed in the H358 cell line, while p-mTOR (S2448) and its downstream signaling proteins (for example, p70S6 kinase,S371) were upregulated in the H358 cell line even under Erlotinib treatment conditions (Figure 2B).

FIGURE 2. A) Western blot analysis for mTOR, EGFR, S6 kinase, ERK, and β-actin in parental and drug resistant cell line H358. B) Similar analysis for the phosphorylated isoforms of mTOR, EGFR, S6 kinase, ERK, β-actin, and 4E-BP1.

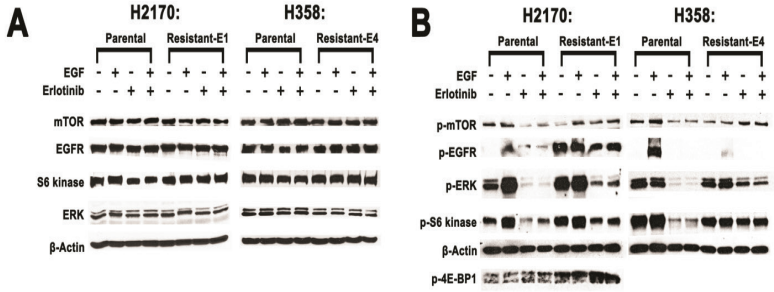
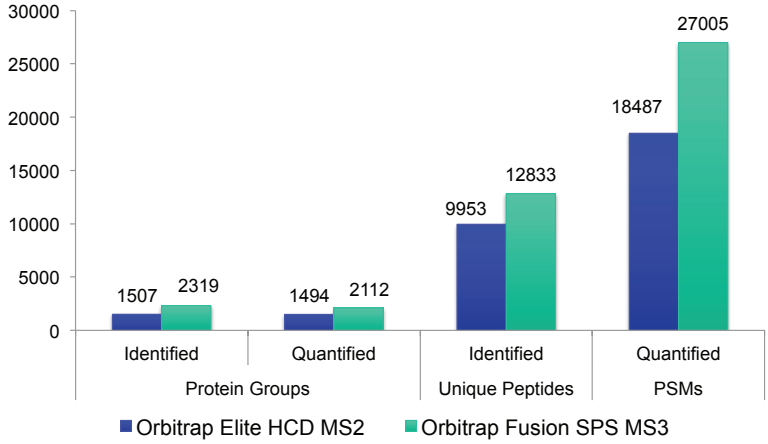


FIGURE 3. Quantitative performance of the SPS MS³ fragmentation method on the Orbitrap Fusion compared to MS² HCD fragmentation on the Orbitrap Elite. Results are the MUDPIT search of two replicate files



Relative Quantitation Using TMT8plex Reagents

The use of TMT6plex reagents with two additional isotope variants of the TMT6-127 and TMT6-129[4,5] enabled the simultaneous comparison of relative protein abundances in response to EGF stimulation, exposure to a tyrosine-kinase inhibitor, or both. Using the Orbitrap Elite mass spectrometer, almost 1500 protein groups and 10,000 unique peptides were able to be identified, with a quantitation rate exceeding 99% at the peptide level employing HCD MS² quantitation/fragmentation (Figure 3). This enabled the partial mapping of numerous important extra-cellular signaling pathways as well as those associated with cancer from the KEGG™ reference set with corresponding relative abundance information (Figure 4A).

Repeating the MS analysis with the enhanced capabilities of the Orbitrap Fusion mass spectrometer resulted in a substantial increase in identified protein groups using the new SPS functionality (Figure 3). We identified nearly13,000 unique peptides corresponding to over 2300 protein groups (a 40% increase over previous performance) with a quantitation rate above 90%. This resulted in increased coverage of the pathways of interest to better understand of the impact of EGF stimulation and Erlotinib treatment upon the H358 NSCLC cell line.

Even without treatment, we found evidence of marked upregulation of HER2/erbB2[6] in the H358 erlotinib-resistant cell line. HER2/erbB2 is a closely related receptor tyrosine kinase to EGFR/erbB1 which can activate the classical MAPK pathway or other proliferation and survival pathways. Interestingly, we found the relative abundance of Ras in the H358 cell line is substantially less compared to the parent cell line corresponding with an observed increase in the abundance of Ras suppressor protein 1. Also, in addition to MEK, there are several other kinases and kinase receptors such as CNK1 and STRAP that are noticeably more prevalent in the H358 cell line. (Figures 4 and 5).

As expected both methods report no change in cyctoplasmic actin expression between the two cell lines and are in good agreement about many of the other important protein expression ratio changes, previously observed using SILAC™ labeled cell lines (data not shown). Consistent with immunoblotting results (Figure 2), S6 kinase shows upregulation indicating yet another alternative mechanism to resistance(Figure 5).

Additional modulations in protein expression ratio are apparent between H358 and parental cells. Rho A and Rac are also more abundant in H358 cells, and both play a role in stimulating JNK initiating gene transcription as part of the WnT pathway. Of note, a C-Jun kinase interacting protein, which works in combination with MAP kinase 8 and is strongly implicated in tumor growth and development, is heavily upregulated. Finally, another kinase, p38 MAPK, a crucial element of the p38 signaling pathway, is also expressed to a much greater degree in H358.

The use of the SPS method produced a marked expansion in the range of expression ratios compared to HCD MS² with numerous additional proteins displaying a significant change in relative abundance under one or more of the treatment conditions (Figure 5 and 6). Proteins involved in apoptosis inhibition and translation initiation factors had increased expression in H358 cell line. These proteins and many others exhibited a broad range of regulation changes in response to the treatment conditions as demonstrated by behavioral clustering.

FIGURE 4. Elements of the Erb (A) and WnT (B) Signaling Pathways (KEGG). Highlighted in blue are proteins identified and quantified proteins by either MS² or SPS methods. Pathway maps are generated by Protein Center 3.9 software

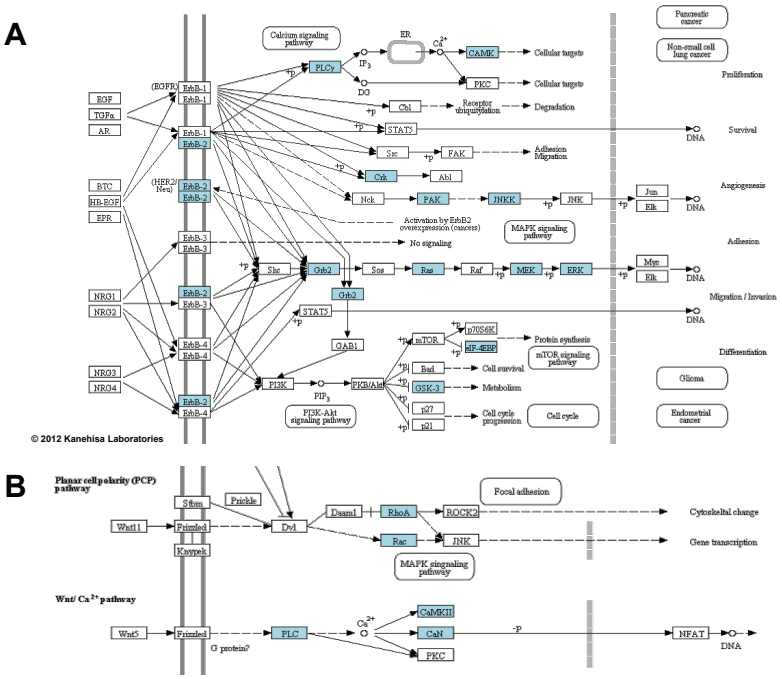


TABLE 1: Response of the H358 and Parental cells to treatment versus the untreated controls using quantitative information from the SPS MS³ method on the Orbitrap Fusion mass spectrometer. A 1.5-fold change up or down was used as the threshold for differences in regulation.

		Quantifiable Proteins					
		Parental			H358		
EGF	Erlotinib	*Down Regulated*	*No Change*	*Up Regulated*	*Down Regulated*	*No Change*	*Up Regulated*
+	-	102	1804	126	62	1801	248
-	+	337	1541	84	95	1683	325
+	+	51	1667	288	158	1777	145

FIGURE 5. Differential Expression of key proteins in H358 vs. Parental cells as determined by MS² or SPS MS³ methods on the Orbitrap Fusion mass spectrometer.

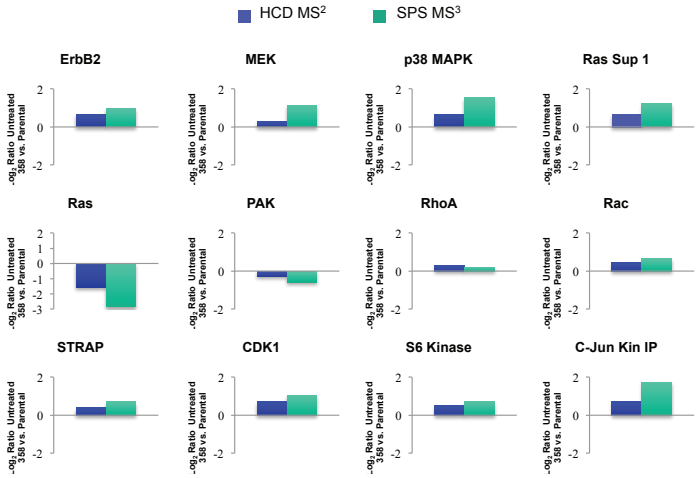
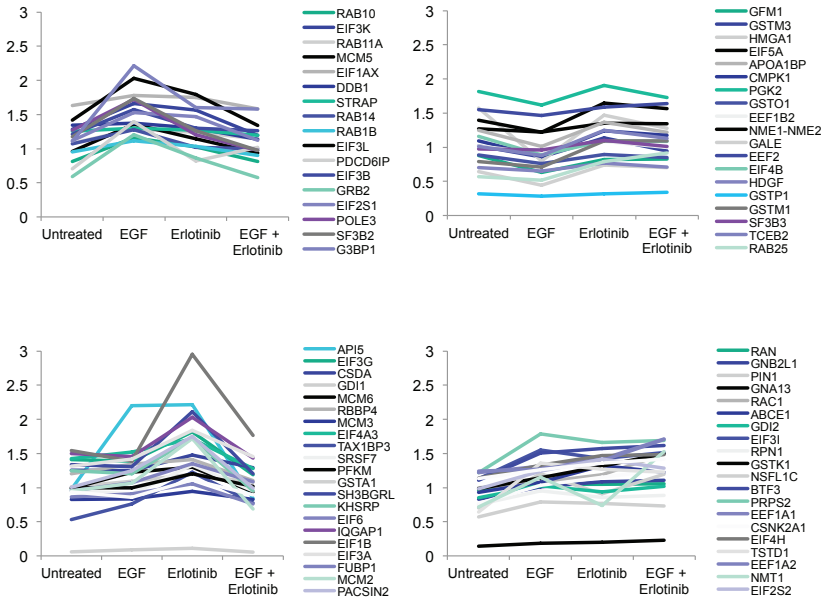


FIGURE 6. Clustering analysis based on SPS MS^3 TMT quantitation highlighting several patterns in the changes in protein expression between the two cell lines: H358 versus parental for the four treatment conditions.



Conclusion

- TMT labeling with additional isobaric tags allows for the simultaneous quantification of protein expression ratios under numerous experimental conditions when coupled with the high resolution/accurate mass of the Orbitrap instrument.
- The enhanced identification rate of the Orbitrap Fusion instrument coupled with the improved accuracy of quantitation using the SPS MS^3 method enables markedly improved pathway profiling.
- MS^2 and SPS MS^3 methods resulted in similar protein expression trends, but the SPS method yields a larger dynamic range of protein expression ratios resulting in significantly more identified up or down regulated proteins.
- Substantial upregulation of the erbB2 signaling receptor and other downstream proteins in H358 may indicate subsequent activation of the p38 or WnT signaling pathways.

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