Profiling EGFR Kinase Inhibitor Resistance Pathways in Non-Small Lung Cancer Cells

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Overview

Purpose: To determine the mechanism of tyrosine kinase inhibitor (TKI) resistance through quantitative LC/MS research analysis of differential changes in protein pathway levels.

Methods: Parental and TKI-resistant cell lines were treated with and without growth factors (EGF or HGF) and TKI inhibitors erlotinib or SU11274. Samples from each treatment condition were labeled with Thermo Scientific™ TMT10plex™ reagents and analyzed/quantified by LC/MS using a Thermo Scientific™ Orbitrap™ Fusion™ Tribrid™ mass spectrometer.

Results: Changes in protein expression and/or protein phosphorylation between parent and TKI-resistant cell lines were observed in several key signaling pathways.

Introduction

Tyrosine kinase inhibitors (TKIs) against epidermal growth factor receptor (EGFR) have positive therapeutic effects in a subset of non-small cell lung cancer (NSCLC) patients; however, their clinical efficacies are limited due to the development of TKI resistance. To study the potential mechanism of TKI resistance, two NSCLC erlotinib-resistant cell lines were established by exposing the cells to progressively increasing concentrations of inhibitor. Differences in protein expression and signal transduction pathway activation between parental and drug-resistant cells after EGF and/or erlotinib treatment were assessed by western blotting (Figure 1) and mass spectrometry (MS). For MS analysis, Thermo Scientific™ Tandem Mass Tag™ (TMT™) reagents were used for sample multiplexing and measuring differences in relative protein abundance. Various pathways implicated in drug-resistance were examined for differences in protein expression using an Orbitrap Fusion Tribrid instrument.

Methods

Sample Preparation

NSCLC cell lines (H2170 and H358) were established with resistance to the EGFR-TKI, 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 10 μM erlotinib alone, or treated with both EGF and erlotinib, for 2.5 minutes. Parental and resistant H358 cells were also treated with HGF and SU11274. After reduction and alkylation, proteins from the up to ten different conditions were digested with trypsin, labeled with TMT10plex reagents, and combined before LC/MS analysis (Figure 2). Magnetic Fe-NTA resin was also used for phospho-peptide enrichment of TMT-labeled samples. Combined peptide samples (unenriched or phospho-enriched) were also fractionated by high pH reverse phase in a novel spin column format containing a polystyrene divinyl benzene resin to generate eight fractions for LC/MS analysis. Western blotting using antibodies against p-EGFR, EGFR, p-mTOR, mTOR, p-S6 kinase, S6 kinase, p-ERK, and ERK was also performed.[2]

Liquid Chromatography

Samples were separated by RP-HPLC using a Thermo Scientific™ Dionex™ UltiMate™ 3000 system connected to a Thermo Scientific™ EASY-Spray™ column, 25 cm × 75 µm or a Thermo Scientific™ Acclaim™ PepMap™ C18 column over a 3 hr 5–30% gradient (A: water, 0.1% formic acid; B: acetonitrile, 0.1% formic acid) at a 300 nL/min flow rate.

Mass Spectrometry

Spectra were acquired using an Orbitrap Fusion Tribrid mass spectrometer using top speed FT MS^2 (HCD) at resolution of 60,000 at *m/z* 200.

Data Analysis

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

Proteome Discoverer software was used to calculate TMT 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.[4] Hierarchical clustering and heat map analysis was performed using Spotfire® analytics software.

Results

Characterization of H358 cells by Western Blot Analysis

Initial characterization of erlotinib-resistant (ER) cell lines using phospho-specific antibodies showed p-EGFR to be constitutively auto-phosphorylated (upregulated 19-fold) in H2170 cells, but downregulated 6-fold in ER H358 cells. Interestingly, c-Met/EGFR downstream signaling proteins p-mTOR, p-S6 kinase, and p-ERK were found to be 2–4-fold upregulated in ER H2170 and H358 cells (Figure 1A). Targeted antibodies revealed little change in the degree of total mTOR, EGFR, S6 kinase, ERK, and β-actin expression between the parental and drug-resistant cell lines under all treatment conditions (Figure 1B).

FIGURE 1. Western blot analysis for the phosphorylated isoforms (A) and total protein (B) levels of mTOR, EGFR, ERK, S6 kinase, and β-actin in parental and drug resistant H2170 and H358 cell lines

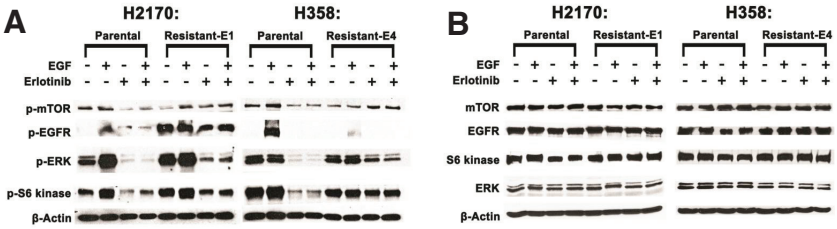
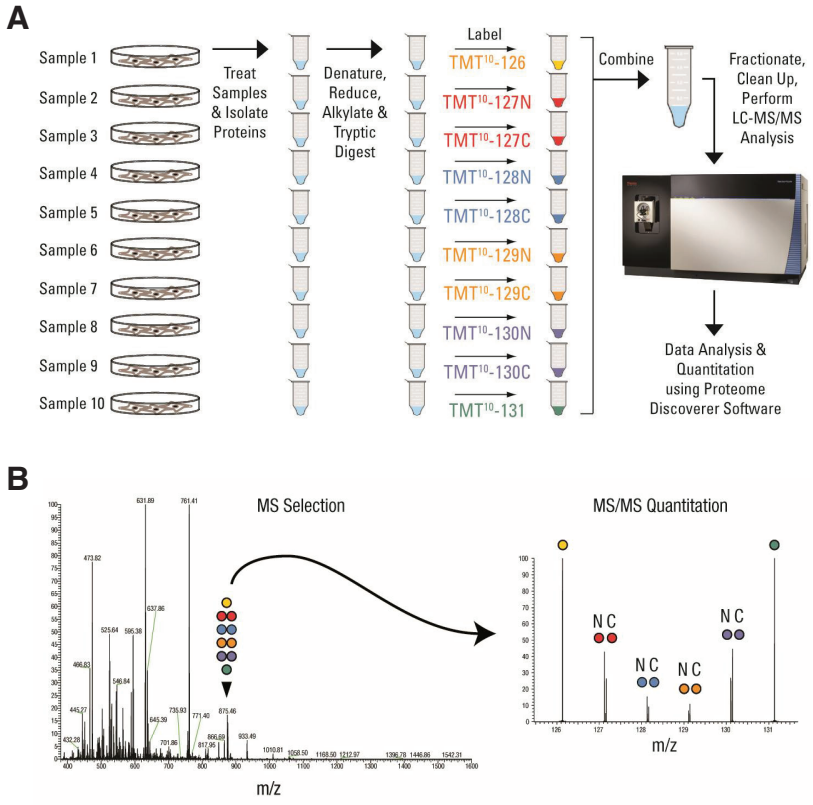


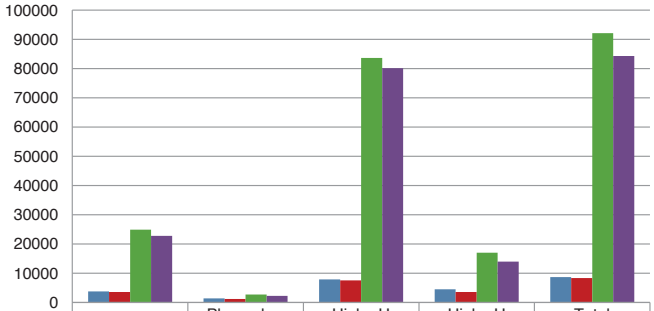
FIGURE 2. Schematic of TMT10plex reagent sample preparation and LC/MS analysis. A) Proteins from parental and H358 erlotinib-resistant NSCLC cell lines treated with diluent, EGF, Erlotinib, or both EFG/Erlotinib were extracted, reduced, alkylated, and digested before being labeled with TMT10plex reagents. Labeled peptides from each treatment condition are combined, enriched for phospho-peptides, fractionated by high pH reversed-phase chromatography, and analyzed by LC/MS analysis. B) TMT10plex-labeled peptides co-elute during MS acquisition but generate 10 unique reporter ion masses during MS^n for relative quantitation.



Relative Quantitation of Protein Abundance Using TMT10plex Reagents

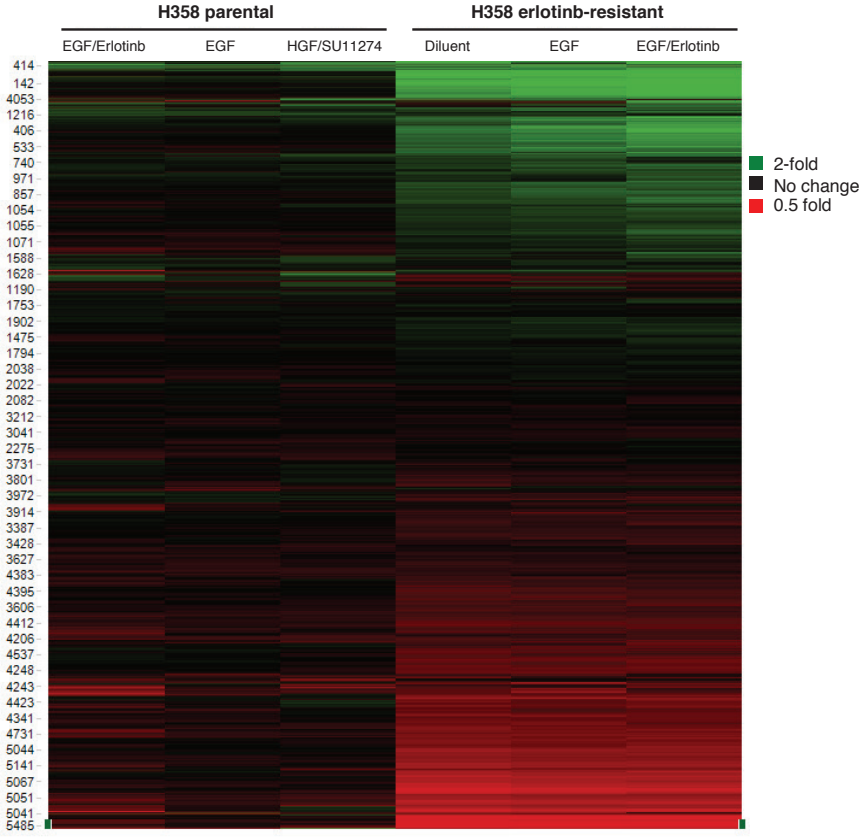
TMT10plex reagents enabled the simultaneous comparison of relative protein abundances in response to EGF stimulation, exposure to erlotinib, or both. Using the Orbitrap Fusion mass spectrometer, almost 7,800 protein groups and 90,000 unique peptides were able to be identified from samples, with a quantitation rate exceeding 90% at the peptide level employing HCD MS^2 quantitation/fragmentation (Figure 3). High pH reversed-phase fractionation of unenriched and phospho-peptide enriched sample increased the number of quantified proteins almost 3-fold compared to unfractionated samples. Hierarchical cluster analysis revealed numerous changes in relative protein abundance between parental and resistant cell lines (Figure 4).

FIGURE 3. Number of quantifiable proteins and peptides using MS^2 HCD fragmentation on the Orbitrap Fusion MS after high pH fractionation and phospho-peptide enrichment. Results are the MudPIT search of two replicate files.



	Load (L)	Phopspho-enriched (P)	High pH fractions (L)	High pH fractions (P)	Total (Unique)
Protein Groups (ID)	3770	1399	7865	4493	8689
Protein Groups (Quan)	3553	1220	7528	3592	8328
Total peptides (ID)	24897	2734	83626	17017	92099
Total peptides (Quan)	22796	2252	80091	13987	84287

FIGURE 4. Clustering analysis based on TMT quantitation showing changes in protein expression for 5,485 proteins (2 peptides per protein) between H358 parental versus erlotinib-resistant cells for different treatment conditions. Data is normalized to untreated H358 parental cells.



Pathway Analysis of H358 Cells

Protein expression profiling studies of H358 erlotinib-resistant (ER) and H2170 SU11274-resistant (SR) cells using SILAC™ have shown that β-catenin was downregulated in ER cells but upregulated SR cells.[2] TMT10plex quantitation in this study also showed ~2 fold lower expression of β-catenin for H358 ER cells (Figure 5A). Epidermal growth factor receptor (EGFR/Erb2, 5B) and SHC-transforming protein 1 (SHC1, 5C) were both phosphorylated after EGF and HGF treatment but showed partial inhibition to erlotinib treatment in ER cells. Staphylococcal nuclease domain-containing protein 1 (SND1, 5D) and glucosamine 6-phosphate N-acetyltransferase (GNA1, 5E) both were phosphorylated in ER cells compared to parental cells.

FIGURE 5. Differential expression of key proteins in H358 cell lines determined by TMT reporter ion quantitation using the Orbitrap Fusion mass spectrometer. Phospho-peptide signals were normalized to relative protein abundance.

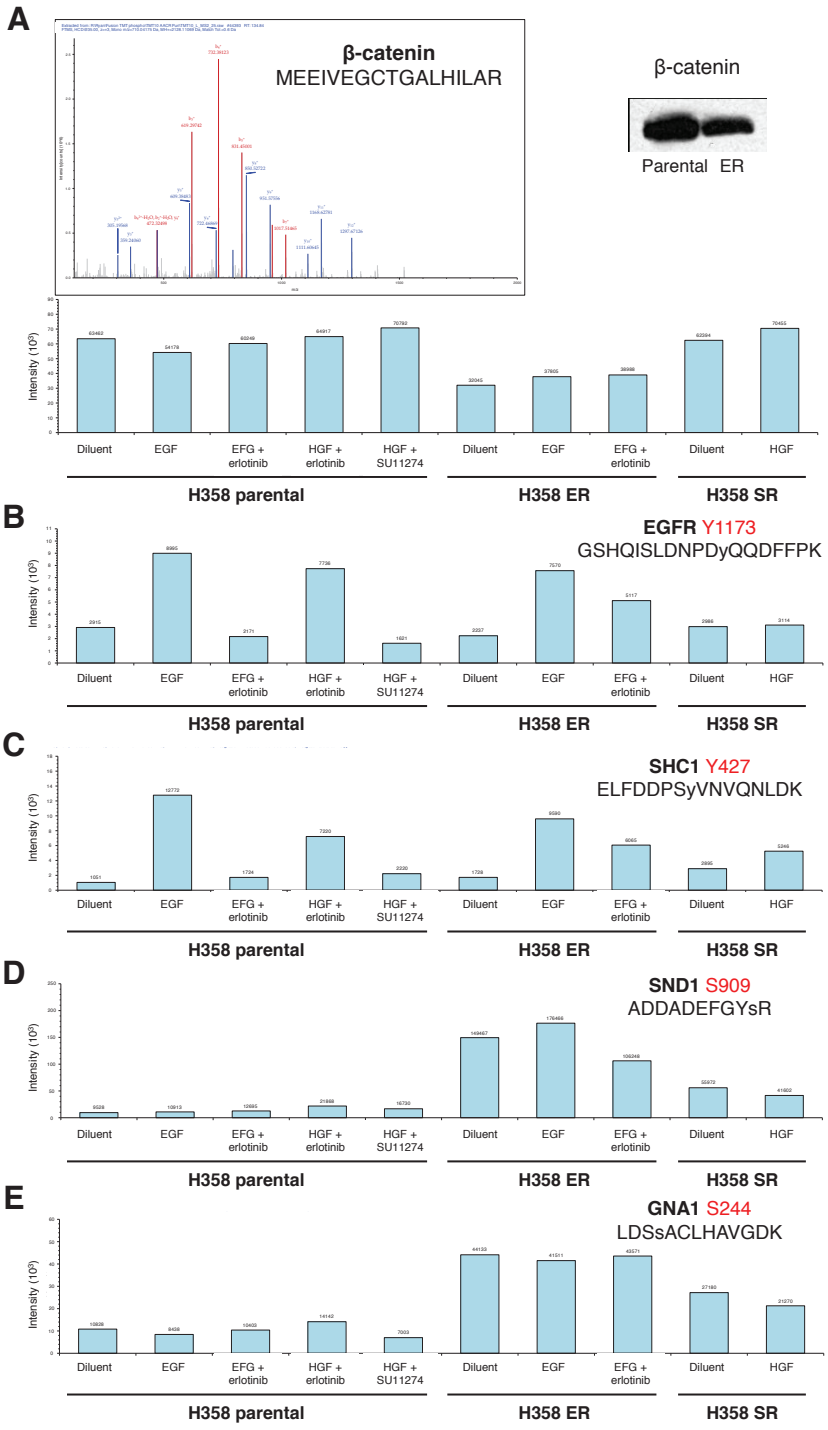
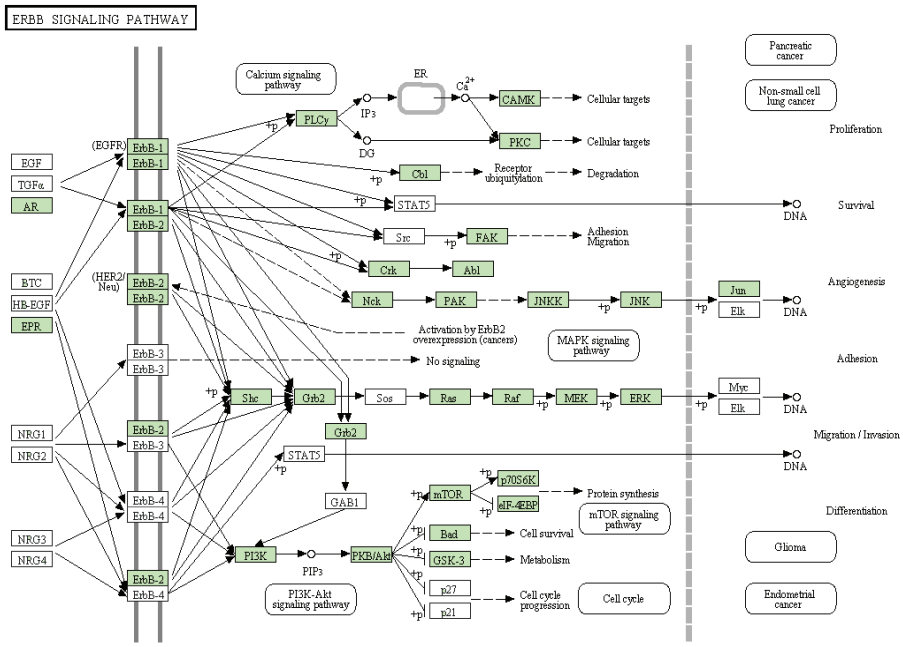


FIGURE 6. Elements of the ErbB signaling pathway. Proteins identified and quantified by LC/MS are highlighted in green. KEGG™ pathway maps were generated by Thermo Scientific™ ProteinCenter™ software version 3.9.



Conclusion

- TMT labeling enables the simultaneous quantification of protein expression from multiple experimental conditions when coupled with the high resolution/accurate mass of the Orbitrap instruments.
- High pH reversed-phase fractionation of unenriched and phospho-peptide enriched samples resulted in over 7,800 protein groups identified and over 5,500 proteins quantified from ten different experimental conditions.
- Protein expression ratios from TMT ion reporters correlated well with previous SILAC studies and western blot analysis.
- Cluster analysis revealed differences in regulation of key ErbB pathway signaling proteins.

References

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2. Jacobs, R.; Fong, J.; Moravec, D.; Botting, G.; Bomgarden, R.; Rogers, J.; Viner, R.; Blank, M.; Puri, N. 2014. Proceedings of the 106th Annual Meeting of the American Association for Cancer Research. Apr 6-10, Washington, D.C.
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