Profiling and Cross-Talk of Tyrosine Phosphorylation with Lysine Acetylation and Succinylation in Tyrosine Kinase Inhibitor Resistant Breast Cancer Cells

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Introduction

Human epidermal growth factor receptor 2 (HER2) is a member of the type I transmembrane receptor kinase family and regulates cell growth and differentiation. Overexpression of HER2 is found in over 25% of breast cancer cells, and is indicative of a poor clinical outcome. Selective HER2 inhibitors were developed to treat cancers that become resistant to the monoclonal antibody trastuzumab, which is used as a standard therapy in these cancers. Of these, lapatinib is the one with best specificity and this inhibits both HER2 and EGFR tyrosine kinases. Unfortunately, resistance to lapatinib has also been observed within 12 months of starting therapy. In this case, the inhibition of HER2 kinase is thought to activate redundant survival pathways, which leads to resistance.

Immunoaffinity Purification (IAP) enrichment of post-translational modifications (PTMs) using antibodies against specific PTMs followed by LC-MS/MS analysis has become a popular high-throughput technique to unveil dependent signaling networks. To better understand the signaling pathways and molecular mechanisms involved in acquired resistance to lapatinib, we performed a large-scale quantitative study on phospho-tyrosine (pY), acetylated-lysine (Ac-K) and succinylated-lysine (Suc-K) PTMs in wild-type BT474 breast cancer cells and their lapatinib-resistant counterparts. A substantial increase in the abundance of tyrosine phosphorylation was observed in resistant cells, which provided support for the assumption that multiple survival pathways were activated by the up-regulation of tyrosine phosphorylation. In addition, hundreds of sites of lysine acetylation and succinylation were found to be deregulated in metabolic enzymes, and multiple transcription factors important in signaling pathways such as the PI3K-Akt, Rap1, and insulin-mediated pathways. By combining the quantitative data from the multiple PTM profiling study, we illustrate for the first time, evidence for cross-talk between tyrosine phosphorylation with lysine modifications in breast cancer cells resistant to tyrosine kinase inhibitors.

Methods

Cells and Lyophilized Peptides

Wild-type and lapatinib-resistant BT474 cells were from Dr. Neil Spector's lab cultured as previously described (1). Biological duplicates for each cell line were prepared, and the cells were harvested and lysed in urea lysis buffer. Lysates (6 mg of total soluble protein) were reduced by DTT, alkylated by iodoacetamide, and digested by trypsin. Crude peptides were purified by C18 Sep-Pak (Waters Corp, Milford MA) column and subject to lyophilization.

Immunoaffinity Purification of Tyrosine Phosphorylated, Lysine Acetylated and Succinylated Peptides

IAP of pY, Ac-K, and Suc-K containing tryptic peptides from wild-type BT474 and lapatinib-resistant cells was performed using the PTMScan® protocol as described previously (2). Briefly, antibodies corresponding to each PTM were conjugated to Protein A beads (Roche) overnight at 4˚C and then washed extensively with PBS. A total of 6 mg of tryptic peptides were dissolved in 1.4 ml of IAP buffer, mixed with phospho-tyrosine antibody beads, and incubated for 2 hours at 4˚C. Flow-through of the IAP was subject to IAPs of lysine acetylation and lysine succinylation in sequence. After IAP, the beads were washed twice with 1 ml of IAP buffer and three times with 1 ml of HPLC grade water. Peptides were eluted from beads using 0.15% TFA. Eluted peptides were desalted over tops packed with Empore™ C18 and eluted with 40% acetonitrile in 0.1% TFA. Eluted peptides were dried under vacuum (**Figure 1**).

LC-MS/MS Analysis and Database Searching

Enriched peptides for each PTM were separated on a 100 µm X 15 cm reversed-phase column and eluted using a 90-min linear gradient of 5%–30% acetonitrile in 0.125% formic acid delivered at 300 nl/min using an Easy-nLC™ (Thermo Fisher Scientific Inc). Tandem mass spectra were collected in a data-dependent manner with an Orbitrap Velos™ mass spectrometer (3). All MS/MS spectra were then exported as individual DTA files and searched using SEQUEST® (v. 28 (rev. 12), 1998–2007) against the NCBI human database and their reversed complements. A precursor mass tolerance of 50 ppm and a product ion tolerance of 1.0 Da (CID) were allowed. One tryptic terminus was required, and four missed cleavages were allowed. Static carbamidomethylation of cysteine (+57.02146374) was required, and appropriate variable modification [pY (+79.9663304104), Ac-K (+42.0105646863), Suc-K (+100.0160439947)] and methionine oxidation (+15.9949146221) were dynamically allowed with a maximum of four modifications of one type per peptide. Peptide spectral matches were filtered to a 5% false discovery rate using linear discriminant analysis in combination with the target-decoy strategy.

Peptide Quantification and KEGG Pathway Analysis

For each PTM IAP study, filtered pepXML files containing modified peptides only and raw data were processed by Skyline v2.5 according to the online tutorial. Quantitative data was evaluated and clustered in TIBCO Spotfire® DecisionSite v 9.1.2. Pathway analysis of identified proteins with modifications combined from all three PTM IAPs was done in KEGG Mapper module by importing the accession numbers of the identified proteins.

References

1. Chen, F., Xia, W., and Spector, N. (2008) *Clin. Cancer Res.* 14, 6730–6734.
2. Rush, J., Moritz, A., Lee, K.A., Guo, A., Goss, V.L., Spek, E.J., Zhang, H., Zha, X.M., Polakiewicz, R.D. and Comb, M.J. (2005) *Nat. Biotechnol.* 23, 94–101.
3. Gu, H., Stokes, M., Guo, A., Lee, K., Ren, J.M., Jia, X., Suri, V. and Silva, J. (2013) *61st Annual American Society for Mass Spectrometry* (ASMS), Minneapolis, MN.

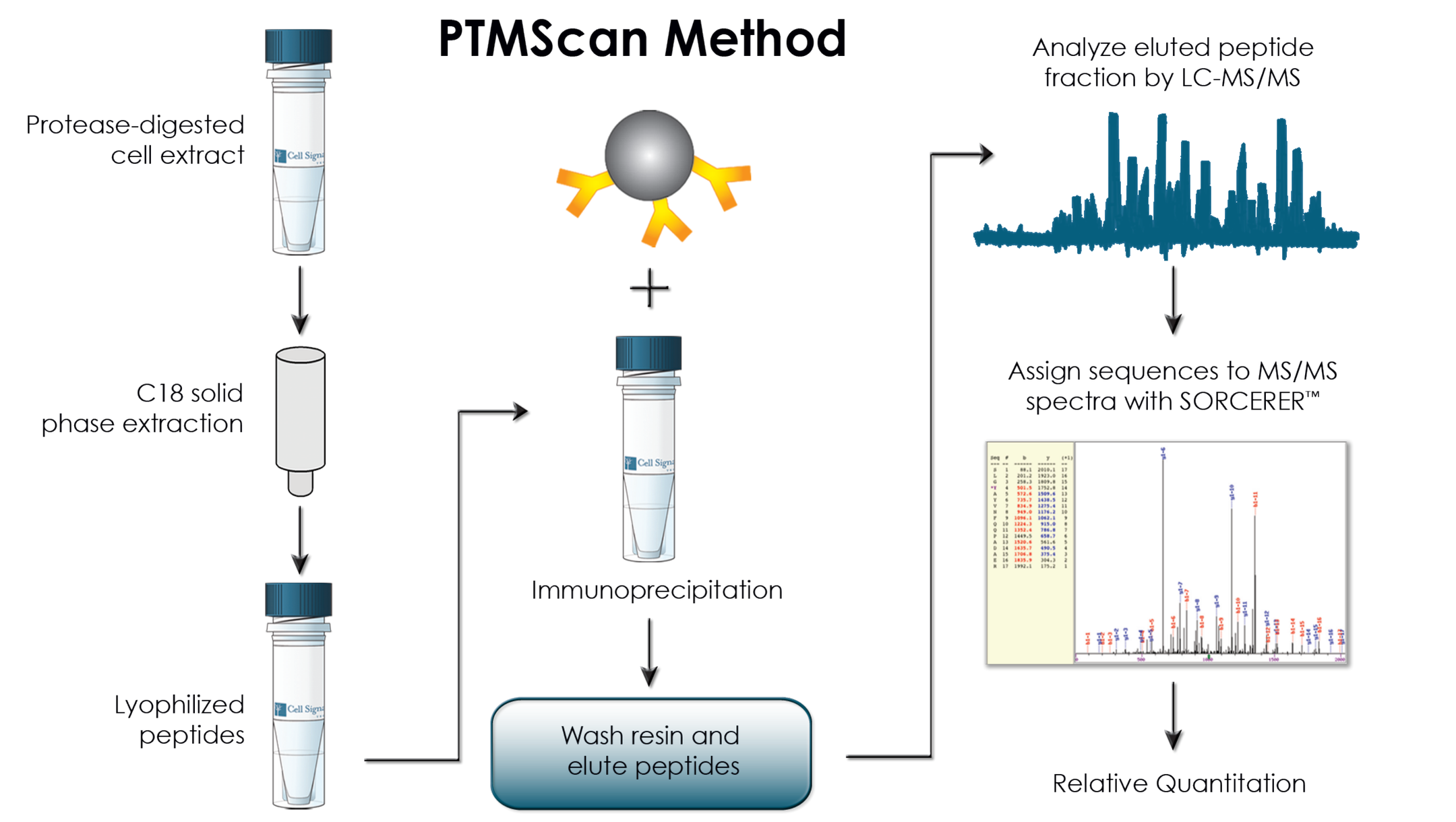
PTMScan Method
Protease-digested cell extract
C18 solid phase extraction
Lyophilized peptides
Immunoprecipitation
Wash resin and elute peptides
Analyze eluted peptide fraction by LC-MS/MS
Assign sequences to MS/MS spectra with SORCERER™
Relative Quantitation

Figure 1: PTMScan Method. Immunoaffinity LC-MS/MS using Acetyl-lysine or Succinyl-lysine Motif Antibodies.

Results

	Unique Peptides	Sites	Proteins	CST Reagent
Acetylation	3,719	2,549	1,098	#13416
Succinylation	4,552	2,986	948	#13764

Table 1: Identification of lysine acetylation and succinylation in mouse liver by IAP-LC-MS/MS. Modified peptides were enriched from tryptic peptides of mouse liver (5 mg) using the appropriate antibodies and then analyzed using an Orbitrap-Velos™ over a 120 min gradient.

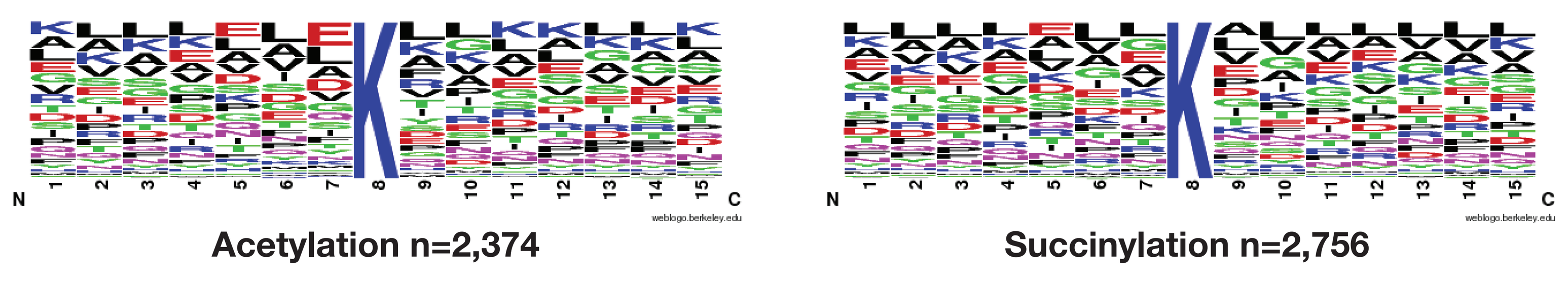

Figure 2: Motif logo analysis of lysine acetylation and succinylation reagents.

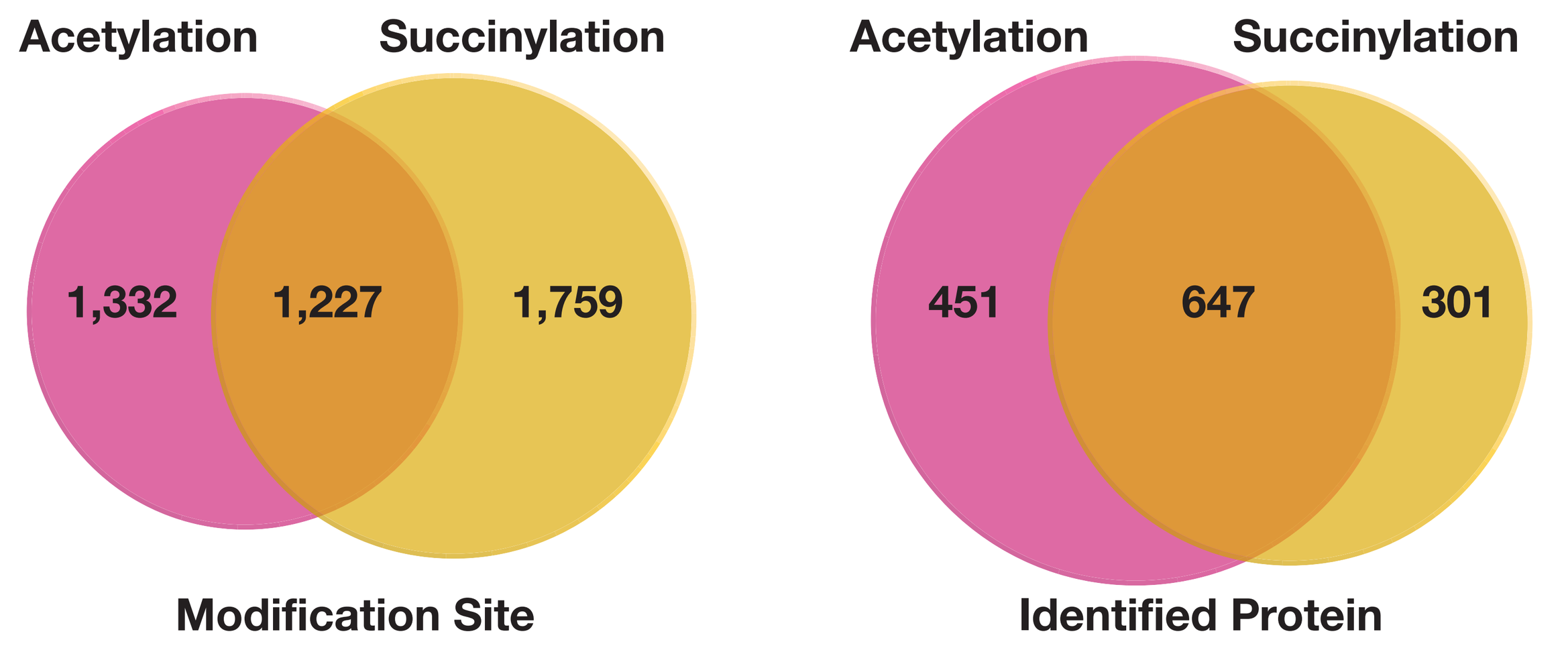

Figure 3: Overlap between lysine acetylation and succinylation at the modification site and identified protein levels.

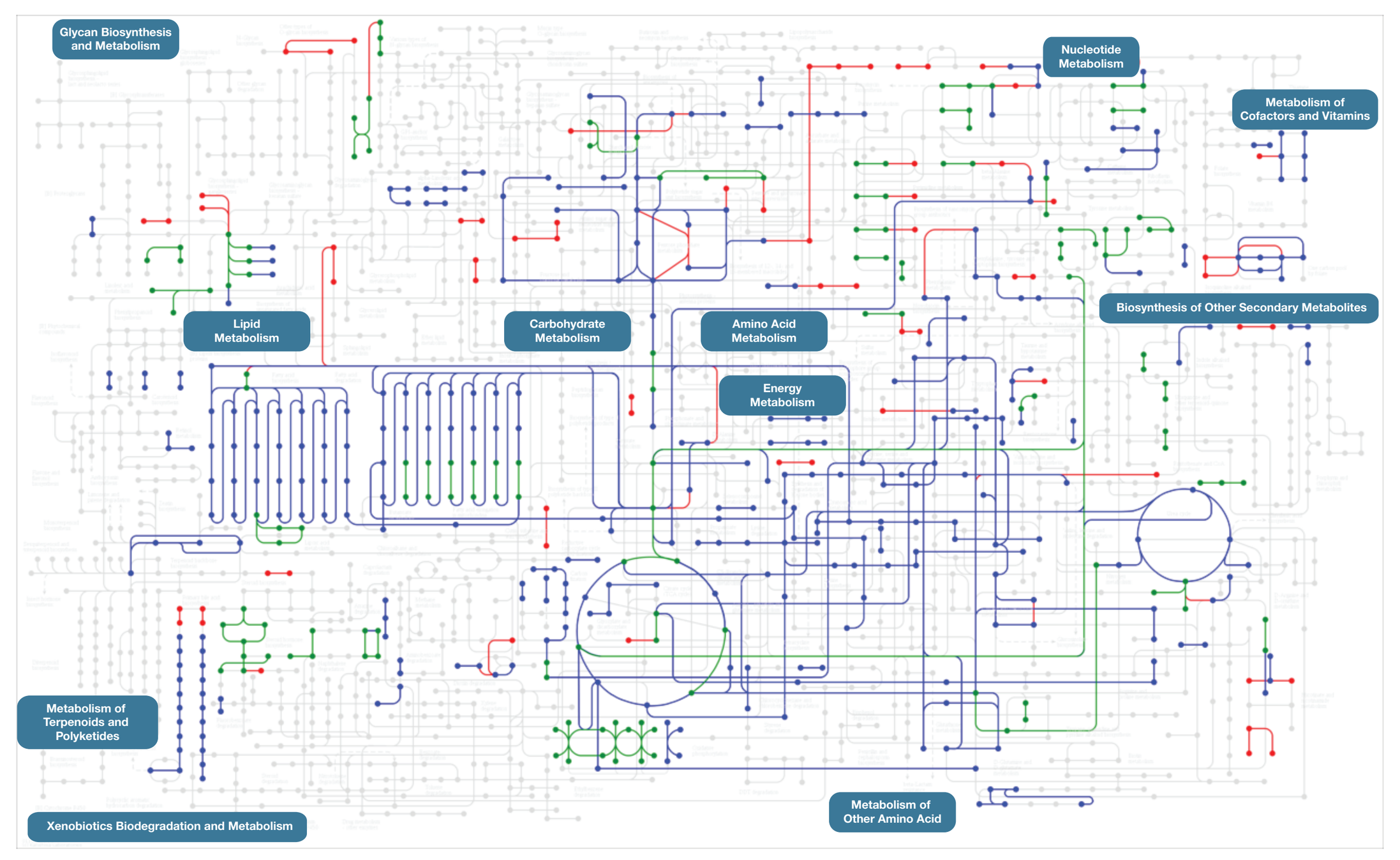

Figure 4: Coverage of metabolic enzymes identified by combining lysine acetylation and succinylation IAPs. Enzymes identified by acetylation, succinylation and both are labeled in red, green and blue, respectively.

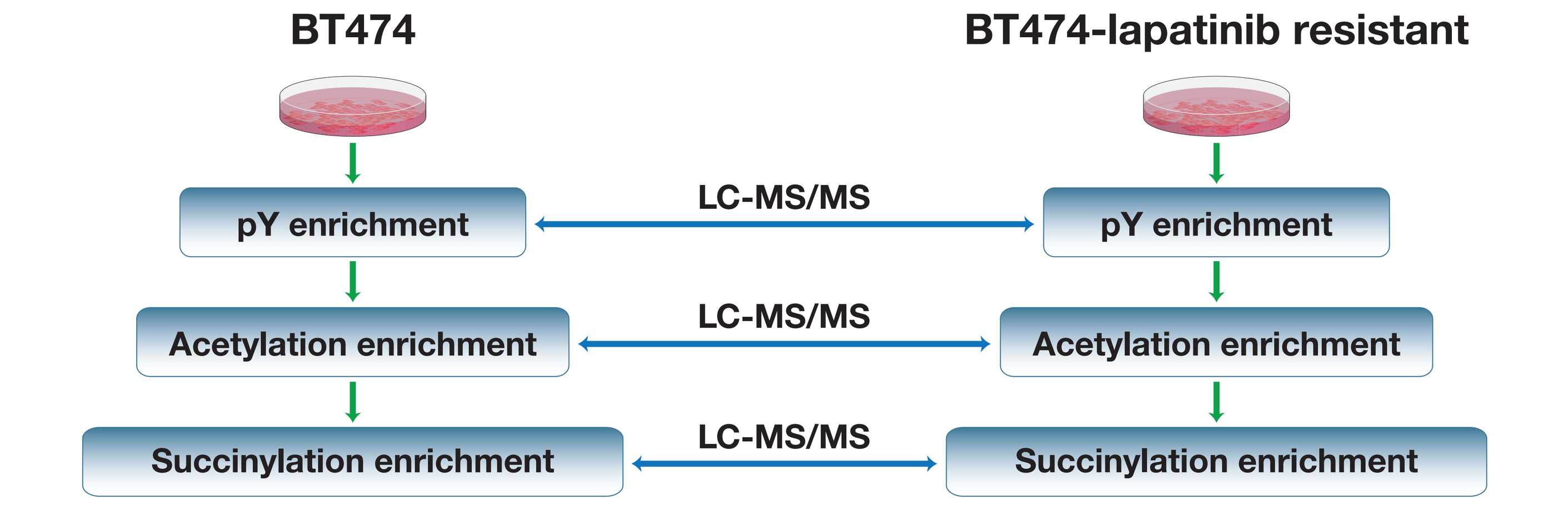

Figure 5: Experimental flow chart of PTM cross-talk in BT474 resistant cells. Tryptic peptides of BT474 and BT474 lapatinib-resistant cells were subject to phospho-tyrosine (pY) IAP followed by IAPs of lysine acetylation and succinylation sequentially using the flow-through from the previous IAP. Enriched peptides from each IAP were analyzed by LC-MS/MS. Raw data was processed by label-free quantification using Skyline for quantitative fold change of each identified modification site.

	Unique Peptides	Sites	Proteins	CST Reagent
pY	3,707	1,635	1,380	#8803
Acetylation	6,377	3,094	1,857	#13416
Succinylation	3,250	1,320	1,320	#13764

Table 2: Identification of phospho-tyrosine, lysine acetylation and lysine succinylation. Enriched peptides from BT474 and BT474-lapatinib-resistant cells were analyzed by Orbitrap-Velos™ over a 90 min gradient. The raw data was searched against the NCBI human database and merged for label free quantification.

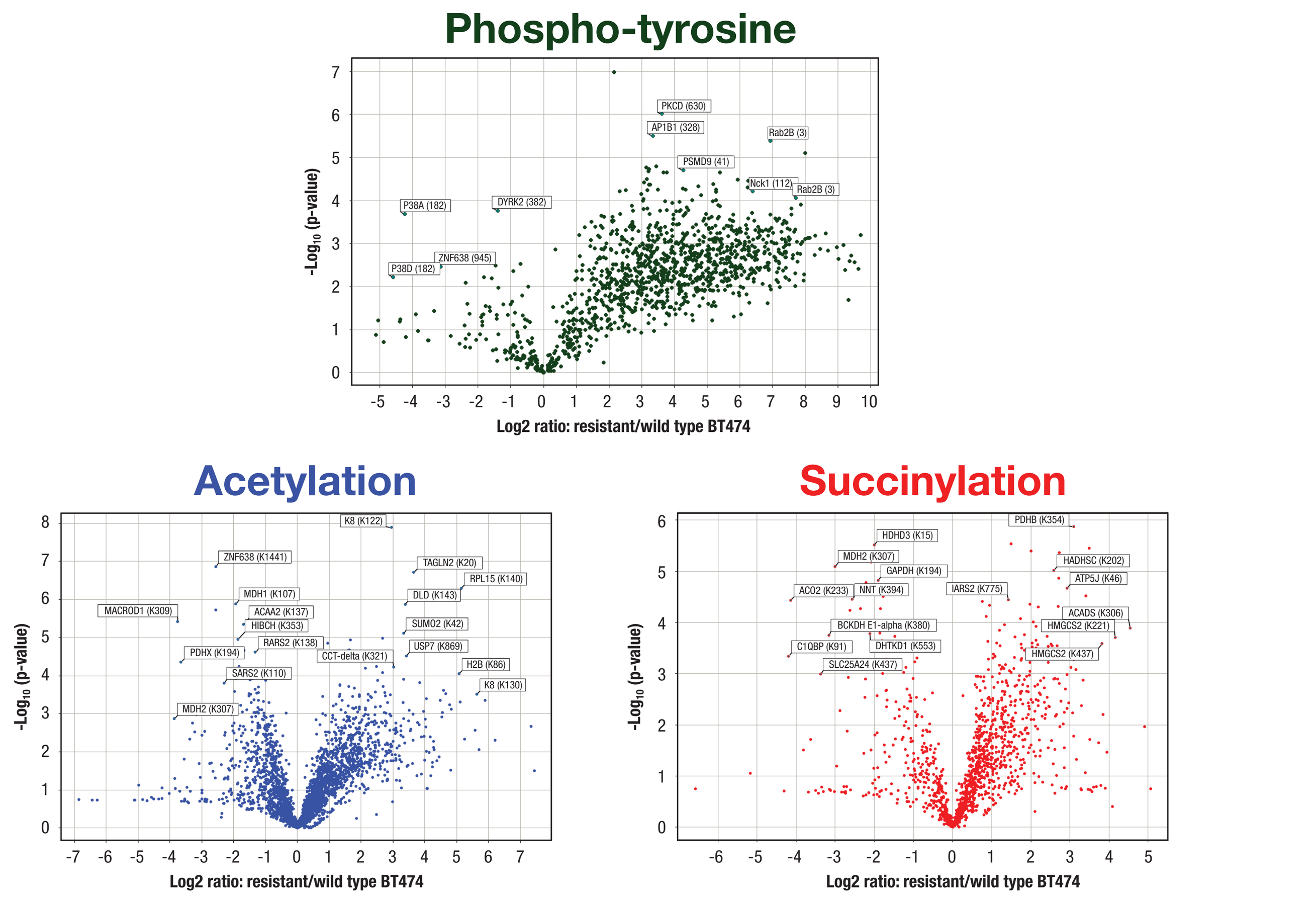

Figure 6: Volcano plots of PTM sites quantified by comparative analysis between wild-type and lapatinib-resistant BT474 cells. Fold change of each site represents the ratio average intensity of the site in resistant over wild-type BT474 cells. Enriched peptides from two independent culture batches (two biological replications) were analyzed in duplicate (two technical replications). Two-tail t-test was performed to calculate p-value for each ratio.

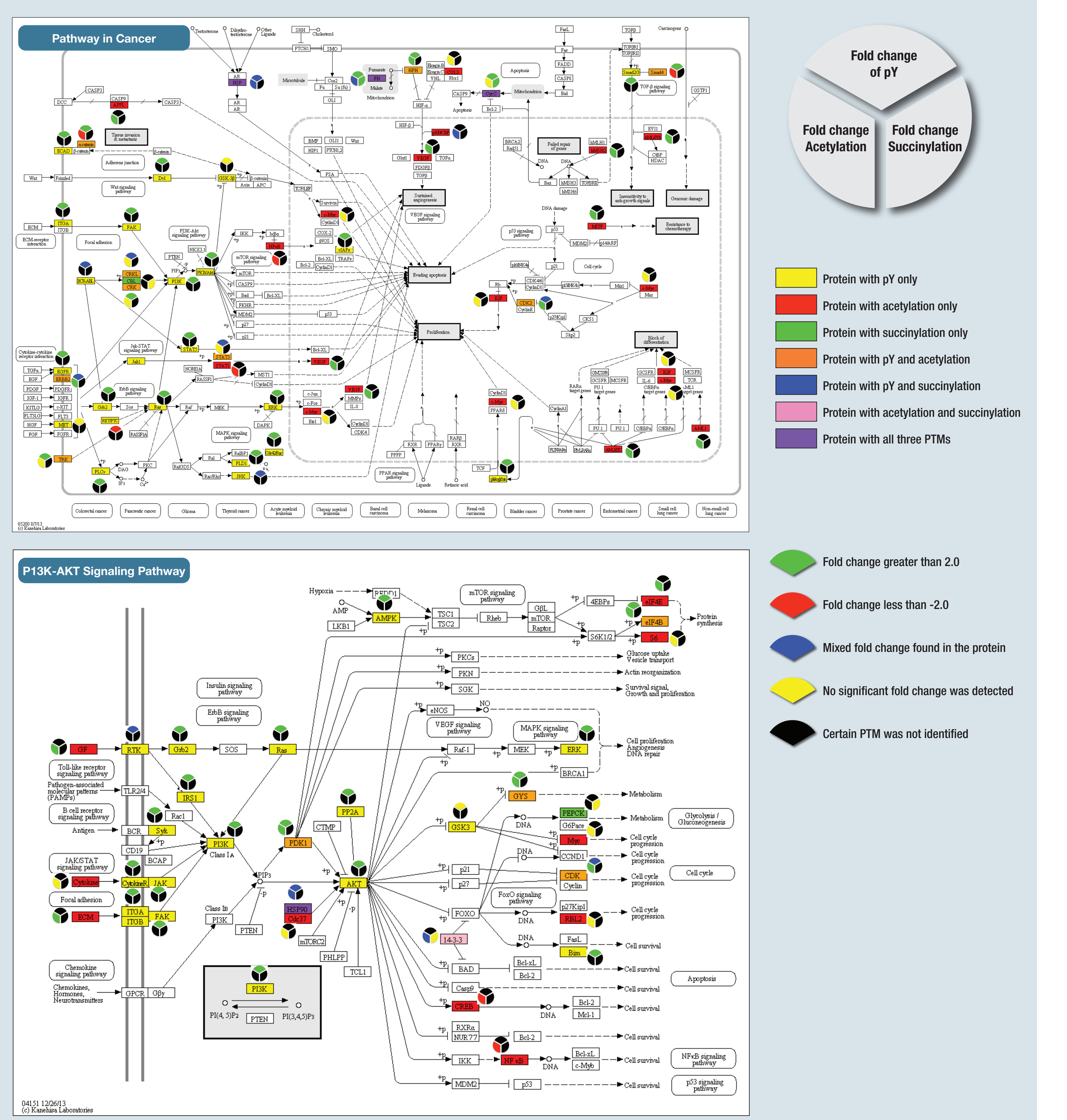
Pathway in Cancer
PI3K-AKT Signaling Pathway
Fold change of pY
Fold change Acetylation
Fold change Succinylation
Protein with pY only
Protein with acetylation only
Protein with succinylation only
Protein with pY and acetylation
Protein with pY and succinylation
Protein with acetylation and succinylation
Protein with all three PTMs
Fold change greater than 2.0
Fold change less than -2.0
Mixed fold change found in the protein
No significant fold change was detected
Certain PTM was not identified

Figure 7: Pathway analysis of identified and quantified sites of tyrosine phosphorylation, lysine acetylation and lysine succinylation. Identification of proteins carrying PTMs is indicated by background colors of squares. Fold changes for each PTM on the protein are represented by the pie charts next to protein symbols.

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