Quantitative Phosphoproteomic Phenotyping of Acquired Resistance to HER2 Kinase Inhibitors in Breast Cancer using Multimodal Phosphopeptide Enrichments

Erik J. Soderblom[1]; Hongbo Gu[2]; Jeffrey Sliva[2]; J. Will Thompson[1]; Wenle Xia[1]; Neil Spector[1]; M. Arthur Moseley*[1]
[1]Duke University School of Medicine, Durham, NC; [2]Cell Signaling Technology, Danvers, MA

Introduction

Analytical Significance - At ASMS in 2013, Soderblom et al. reported a comparison of phosphopeptide pulldowns from mouse brain and mouse embryo samples, using both TiOx and a mixture of motif-specific antibodies (pST motifs). These data sets showed a surprisingly small overlap (<5%) between the phosphopeptides enriched by these two methods, suggesting that a more complete understanding of the underlying biology requires the use of multiple phosphopeptide enrichment strategies. This current work extend the original studies, now using three methods for phosphopeptide enrichment – pST motif specific antibody mixture, pY motif specific antibody, and TiOx. Moreover, the relationship between the phosphopeptide enrichment method and the biological information thus obtained has been studied in detail. Two ErbB2 positive (HER2+) breast cancer cell lines were quantitatively analyzed– BT474 and SKBR3 – both as 'wild-type' and as stable tyrosine kinase inhibitor resistant forms. Ingenuity Pathway Analysis has been used to determine the biological information revealed by each enrichment strategy. As would be expected, the low overlap between phosphopeptides enriched by each of the methods leads to a correspondingly low overlap in the resultant biological information, highlighting the need to use multiple phosphopeptide enrichment strategies to understand the underlying biology.

Biological Significance - The development of resistance to HER2 targeted therapies remains a significant obstacle limiting the clinical efficacy of this important class of cancer drugs, particularly in the advanced stage setting. To study the underlying molecular mechanisms involved in resistance, we have developed several models acquired therapeutic resistance using HER2-overexpressing (HER2+) human breast cancer cell lines including the BT474 and SKBR3 cell lines. Treatment naïve, parental BT474 and SKBR3 cell lines are highly sensitive to the antitumor effects of a HER2 tyrosine kinase inhibitor, with an IC50 in the 100 nM range. Higher concentrations of the HER2 tyrosine kinase inhibitor (~1 uM) acutely kills BT474 and SKBR3 cells within 72 hours. To generate HER2 kinase inhibitor-resistant cells, we continuously treated BT474 and SKBR3 cells with clinically relevant concentrations of the tyrosine kinase inhibitor. After several weeks, we began to see the outgrowth of resistant clones. After several months of culture in the continuous presence of the tyrosine kinase inhibitor, we established stable HER2 kinase inhibitor resistant BT474 and SKBR3 cell lines (rBT474; rSKBR3) that maintained >90% viability in the presence of 1 uM of the inhibitor. The methodology used to develop these resistant cell lines was previously published (Xia W., et al. PNAS, 2006). We have further characterized resistant cells from a functional standpoint. They appear to have the same growth kinetics as their parental cell counterparts. However, resistant BT474 exhibit increased migration and invasion in cell-based assays compared with parental controls. Therefore, there are functional differences in tumor behavior that occur with the development of resistance. This work seeks to understand these differences in the context of phosphorylation signaling.

Experimental

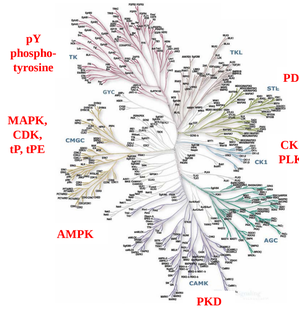
Cells and Lyophilized Peptides - Wild type and HER2 kinase inhibitor-resistant BT474 and SKBR3 cells were cultured. Biological triplicates for each cell line were prepared. The cells were harvested, and lysed in 8M urea lysis buffer. After dilution to 6M urea, Bradford assays were performed, and then all samples were diluted with 50 mM ammonium bicarbonate to give equal protein concentrations. From each sample, an 8.2 mg aliquot (total soluble protein) was reduced by DTT, alkylated by iodoacetamide, and digested by TPCK trypsin. (overnight, 37C). Crude peptides were purified by C18 Sep-Pak (Waters Corp, Milford MA) column, aliquoted, lyophilized and stored at -80C until use.

Immunoaffinity Purification of pY and pST Phosphopeptides - IAPs of pY and pST peptides from wild-type and resistant cells were performed using the PTMScan protocol. Briefly, the antibody for each PTM was conjugated to Protein A bead (Roche) (overnight, 4 ˚C) and then washed extensively by PBS. For each sample, a total of 6 mg of tryptic peptides were dissolved in 1.4 ml of IAP buffer and mixed with pST 'SuperMix' (mixture of 17 different pST motif-specific antibodies) Ab beads, and incubated for 2 hours at 4 ˚C. The flow-through of the pST IAP was subject to IAP using a pY motif-specific antibody. After each IAP, each set of beads was independently washed twice with 1 ml of IAP buffer and three times with 1 ml of HPLC grade water. Peptides were eluted from beads with 0.15% TFA. Eluted peptides were desalted (Empore C18) and eluted with 40% acetonitrile in 0.1% TFA. The eluted peptides were dried under vacuum.

TiOx Enrichment of Phosphopeptides – For each sample, a 2 mg aliquot of the tryptic peptides was resuspended in TiOx enrichment buffer (80% acetonitrile, 1% trifluoroacetic acid, 1M gylcolic acid) along with 60 picomoles of pre-digested casein as a surrogate standard, and enriched on GL Sciences Titansphere TiO2 Spin-Columns. Elution was accomplished using 20% acetonitrile with 5% aqueous NH3 (pH 10), followed by lyophilization.

pST 'SuperMix' of 17 Motif-Specific Antibodies

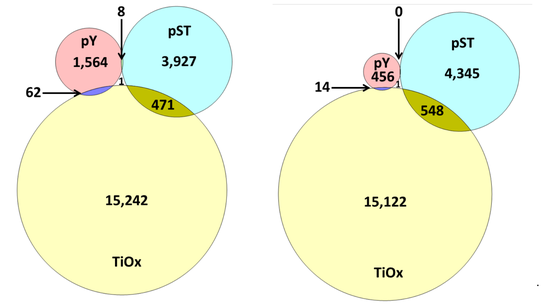
	Motif Antibody	Motif
Basophilic	Akt Substrate	RXX(s/t)
	Akt Substrate	RXRXX(s/t)
	PKA Substrate	(K/R)(K/R)X(s/t)
	PKC Substrate	(K/R)XsX(K/R)
	PKD Substrate	LXRXP(s/t)
	CDK Substrate	(K/R)sPX(K/R)
	AMPK	LxRXX(s/t)
Proline Directed	MAPK Substrate	PXsP
	tPE Motif	tPE, tP
	PLK Binding motif	StP
	tXR Motif	tXR, tPR
	14-3-3	(R/K)XXsXP
Atypical	Phosphotyrosine	y
	ATM/ATR Substrate	(s/t)QG
	ATM/ATR Substrate	sQ
	CK Substrate	t(D/E)X(D/E)
	PDK1 Docking Motif	(F/Y)(s/t)(F/Y)



Phosphopeptide Analysis

LC-MS/MS Analysis and Database Searching - Peptides from each enrichment strategy were separated on a 75 µm X 10 cm Pico-Frit reversed-phase column packed with Magic C18 AQ and eluted using a 90-min linear gradient of 5%–30% acetonitrile in 0.125% formic acid delivered at 280 nl/min (Easy nLC (Thermo Fisher). Tandem mass spectra were collected in a Top 20 data-dependent manner with an LTQ-Orbitrap Velos mass spectrometer. All MS/MS spectra were then exported as individual DTA files and searched using SEQUEST 3G in SORCERER 2 (Sage-N Research) against the NCBI human database and their reversed sequences. A precursor mass tolerance of 50 ppm and a product ion tolerance of 1.0 Da (CID) was allowed. One tryptic terminus was required, and four missed cleavages were allowed. Static carbamidomethylation of cysteine was required, and appropriate variable modification - phosphorylation and methionine oxidation - 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 with the target-decoy strategy. Each sample was analyzed in duplicate.

Very Low Overlap in Phosphopeptides Enriched by Three Methods



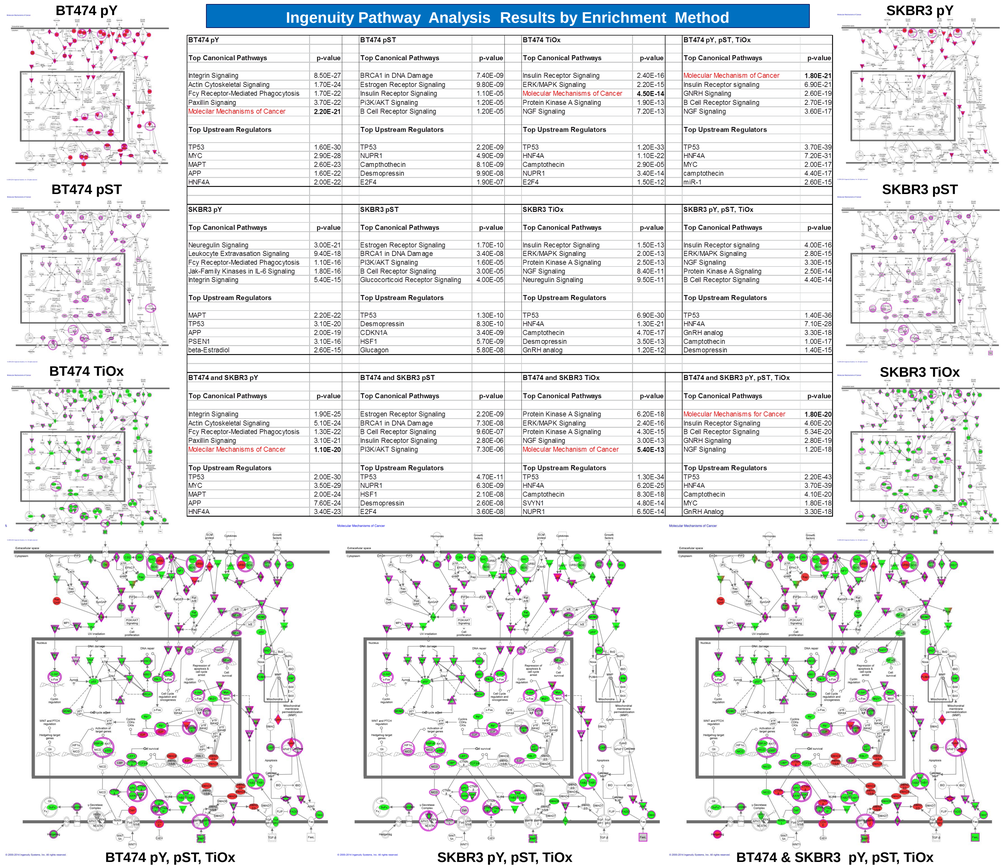
Increases in Biological Information Content via Multimodal Phosphopeptide Enrichments

Ingenuity Pathway Analysis Results by Enrichment Method

BT474 pY		BT474 pST		BT474 TiOx		BT474 pY, pST, TiOx	
Top Canonical Pathways	**p-value**	**Top Canonical Pathways**	**p-value**	**Top Canonical Pathways**	**p-value**	**Top Canonical Pathways**	**p-value**
Integrin Signaling	8.50E-27	BRCA1 in DNA Damage	7.40E-09	Insulin Receptor Signaling	2.40E-16	Molecular Mechanism of Cancer	**1.80E-21**
Actn Cytoskeletal Signaling	1.70E-24	Estrogen Receptor Signaling	9.80E-09	ERK/MAPK Signaling	2.20E-15	Insulin Receptor signaling	6.90E-21
Fcy Receptor-Mediated Phagocytosis	1.70E-22	Insulin Receptor Signaling	1.10E-05	Molecular Mechanisms of Cancer	**4.50E-14**	GNRH Signaling	2.60E-19
Paxillin Signaing	3.70E-22	PI3K/AKT Signaling	1.20E-05	Protein Kinase A Signaling	1.90E-13	B Cell Receptor Signaling	2.70E-19
Molecular Mechanisms of Cancer	**2.20E-21**	B Cell Receptor Signaling	1.20E-05	NGF Signaling	7.20E-13	NGF Signaling	3.60E-17
Top Upstream Regulators		**Top Upstream Regulators**		**Top Upstream Regulators**		**Top Upstream Regulators**	
TP53	1.60E-30	TP53	2.20E-09	TP53	1.20E-33	TP53	3.70E-39
MYC	2.90E-28	NUPR1	4.90E-09	HNF4A	1.10E-22	HNF4A	7.20E-31
MAPT	2.60E-23	Camptothecin	8.10E-09	Camptothecin	2.90E-05	MYC	2.00E-17
APP	1.60E-22	Desmopressin	9.90E-08	NUPR1	3.40E-14	camptothecin	4.40E-17
HNF4A	2.00E-22	E2F4	1.90E-07	E2F4	1.50E-12	miR-1	2.60E-15

SKBR3 pY		SKBR3 pST		SKBR3 TiOx		SKBR3 pY, pST, TiOx	
Top Canonical Pathways	**p-value**	**Top Canonical Pathways**	**p-value**	**Top Canonical Pathways**	**p-value**	**Top Canonical Pathways**	**p-value**
Neuregulin Signaling	3.00E-21	Estrogen Receptor Signaling	1.70E-10	Insulin Receptor Signaling	1.50E-13	Insulin Receptor signaling	4.00E-16
Leukocyte Extravasation Signaling	9.40E-18	BRCA1 in DNA Damage	3.40E-08	ERK/MAPK Signaling	2.00E-13	ERK/MAPK Signaling	2.80E-15
Fcy Receptor-Mediated Phagocytosis	1.10E-16	PI3K/AKT Signaling	1.60E-05	Protein Kinase A Signaling	2.50E-13	NGF Signaling	3.30E-15
Jak-Family Kinases in IL-6 Signaling	1.80E-16	B Cell Receptor Signaling	3.00E-05	NGF Signaling	8.40E-11	Protein Kinase A Signaling	2.50E-14
Integrin Signaling	5.40E-15	Glucocorticoid Receptor Signaling	4.00E-05	Neuregulin Signaling	9.50E-11	B Cell Receptor Signaling	4.40E-14
Top Upstream Regulators		**Top Upstream Regulators**		**Top Upstream Regulators**		**Top Upstream Regulators**	
MAPT	2.20E-22	TP53	1.30E-10	TP53	6.90E-30	TP53	1.40E-36
TP53	3.10E-20	Desmopressin	8.30E-10	HNF4A	1.30E-21	HNF4A	7.10E-28
APP	2.00E-19	CDKN1A	3.40E-09	Camptothecin	4.70E-17	GnRH analog	3.30E-18
PSEN1	3.10E-16	HSF1	5.70E-09	Desmopressin	3.50E-13	Camptothecin	1.00E-17
beta-Estradiol	2.60E-15	Glucagon	5.80E-08	GnRH analog	1.20E-12	Desmopressin	1.40E-15

BT474 and SKBR3 pY		BT474 and SKBR3 pST		BT474 and SKBR3 TiOx		BT474 and SKBR3 pY, pST, TiOx	
Top Canonical Pathways	**p-value**	**Top Canonical Pathways**	**p-value**	**Top Canonical Pathways**	**p-value**	**Top Canonical Pathways**	**p-value**
Integrin Signaling	1.90E-25	Estrogen Receptor Signaling	2.20E-09	Protein Kinase A Signaling	6.20E-18	Molecular Mechanisms for Cancer	**1.80E-20**
Actn Cytoskeletal Signaling	5.10E-24	BRCA1 in DNA Damage	7.30E-08	ERK/MAPK Signaling	2.40E-16	Insulin Receptor Signaling	4.60E-20
Fcy Receptor-Mediated Phagocytosis	1.30E-22	B Cell Receptor Signaling	9.60E-07	Protein Kinase A Signaling	4.30E-15	B Cell Receptor Signaling	5.34E-20
Paxillin Signaing	3.10E-21	Insulin Receptor Signaling	2.80E-06	NGF Signaling	3.00E-13	GNRH Signaling	2.80E-19
Molecular Mechanisms of Cancer	**1.10E-20**	PI3K/AKT Signaling	7.30E-06	Molecular Mechanism of Cancer	**5.40E-13**	NGF Signaling	1.20E-18
Top Upstream Regulators		**Top Upstream Regulators**		**Top Upstream Regulators**		**Top Upstream Regulators**	
TP53	2.00E-30	TP53	4.70E-11	TP53	1.30E-34	TP53	2.20E-43
MYC	3.50E-29	NUPR1	6.30E-09	HNF4A	6.20E-25	HNF4A	3.70E-39
MAPT	2.00E-24	HSF1	2.10E-08	Camptothecin	8.30E-18	Camptothecin	4.10E-20
APP	7.60E-24	Desmopressin	2.60E-08	SIVYN1	4.80E-14	MYC	1.80E-18
HNF4A	3.40E-23	E2F4	3.60E-08	NUPR1	6.50E-14	GnRH Analog	3.30E-15



Conclusions

Phosphopeptides were enriched from two HER2+ breast cancer cell lines (BT474 and SKBR3), both in their wild-type and tyrosine kinase inhibitor resistant forms. Three enrichment strategies were used – TiOx, motif-specific pST antibody mixture (17 different antibodies) and a pY antibody. Samples were prepared in triplicate and were analyzed in duplicate. These three phosphopeptide enrichment strategies each created essentially unique phosphopeptide samples, with very little overlap either in phosphopeptide sequence or in biological information. **This work shows that multiple phosphopeptide enrichment methods are required to understand phenotypic variation in biological systems.**

Acknowledgements

Meredith Mayer-Salman, Duke
Duke Translational Research Institute
Duke Cancer Institute
Duke University School of Medicine