Published in final edited form as:

Electrophoresis. 2014 December ; 35(24): 3463–3469. doi:10.1002/elps.201400022.

Tissue phosphoproteomics with PolyMAC identifies potential therapeutic targets in a transgenic mouse model of HER2 positive breast cancer

Adam C. Searleman¹, Anton B. Iliuk², Timothy S. Collier^{1,*}, Lewis A. Chodosh³, W. Andy Tao², and Ron Bose¹

¹Division of Oncology, Department of Medicine, Washington University School of Medicine, St. Louis, MO, USA

²Department of Biochemistry, Purdue University Center for Cancer Research, Purdue University, West Lafayette, IN, USA

³Department of Cancer Biology, Department of Cell and Developmental Biology, Department of Medicine, Abramson Family Cancer Research Institute, University of Pennsylvania School of Medicine, Philadelphia, PA, USA

Abstract

Altered protein phosphorylation is a feature of many human cancers that can be targeted therapeutically. Phosphopeptide enrichment is a critical step for maximizing the depth of phosphoproteome coverage by MS, but remains challenging for tissue specimens because of their high complexity. We describe the first analysis of a tissue phosphoproteome using polymer-based metal ion affinity capture (PolyMAC), a nanopolymer that has excellent yield and specificity for phosphopeptide enrichment, on a transgenic mouse model of HER2-driven breast cancer. By combining phosphotyrosine immunoprecipitation with PolyMAC, 411 unique peptides with 139 phosphotyrosine, 45 phosphoserine, and 29 phosphothreonine sites were identified from five LC-MS/MS runs. Combining reverse phase liquid chromatography fractionation at pH 8.0 with PolyMAC identified 1571 unique peptides with 1279 phosphoserine, 213 phosphothreonine, and 21 phosphotyrosine sites from eight LC-MS/MS runs. Linear motif analysis indicated that many of the phosphosites correspond to well-known phosphorylation motifs. Analysis of the tyrosine phosphoproteome with the Drug Gene Interaction database uncovered a network of potential therapeutic targets centered on Src family kinases with inhibitors that are either FDA-approved or in clinical development. These results demonstrate that PolyMAC is well suited for phosphoproteomic analysis of tissue specimens.

© 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Correspondence: Dr. Ron Bose, Division of Oncology, Department of Medicine, Washington University School of Medicine, St. Louis, MO 63110, USA, rbose@dom.wustl.edu.

*Current address: Cleveland Heart Lab, 6701 Carnegie Avenue Suite 500, Cleveland, OH, USA

A.C.S., T.S.C., L.A.C. and R.B. declare no conflicts of interest. A.B.I. and W.A.T. are principals at Tymora Analytical Operations LLC, the company which currently sells PolyMAC-Ti and was formed after the research in this manuscript was conducted.

Additional supporting information may be found in the online version of this article at the publisher's web-site

Keywords

Breast Cancer; Drug identification; HER2; Phosphoproteomics; PolyMAC

1 Introduction

Reversible phosphorylation of proteins by kinases is a common mechanism to regulate protein function, localization, binding, and signaling. Many of the signaling pathways that contribute to cancer development depend on tyrosine kinase activity and have been successfully targeted with small-molecule inhibitors. Examples include BCR-ABL inhibitors for chronic myelogenous leukemia [1], EGFR inhibitors for lung adenocarcinomas carrying mutated EGFR [2, 3], and HER2 inhibitors for breast cancers with gene amplification or, potentially, activating mutations of HER2 [4, 5]. Hence, the study of the phosphoproteome, or the set of all phosphorylated proteins, can reveal potential therapeutic targets.

Enrichment of phosphopeptides has been proven to be an effective approach to improving phosphoproteome coverage by MS (see review in this issue by Iliuk, Arrington, and Tao [6]). Animal tissues and clinical specimens present several challenges for efficient phosphopeptide enrichment. Tissues are composed of a mixture of cell types, each with different protein expression and phosphorylation profiles, as well as extracellular components including the extracellular matrix, lymph, and blood. Moreover, cells in situ are subject to modulating signals from their microenvironment, resulting in more tightly controlled protein phosphorylation. These attributes increase the complexity and decrease phosphopeptide abundance in tissue lysates compared to the cell line lysates generally used to evaluate phosphopeptide enrichment methodologies.

Polymer-based metal ion affinity capture (PolyMAC) is a polymer-based adaptation of immobilized metal ion affinity capture with superior phosphoproteome coverage and recovery [7]. PolyMAC is a soluble nanopolymer designed to interact with phosphopeptides in solution with subsequent capture on a solid phase support for washing and phosphopeptide elution. PolyMAC-Ti is the most extensively studied formulation of PolyMAC and is composed of Ti-functionalized dendrimers to efficiently chelate phosphopeptides and an aldehyde moiety for covalent attachment to hydrazine-agarose beads. The high local concentrations of Ti(IV) cations and homogeneous reaction environment contribute to rapid and reproducible phosphopeptide chelation. PolyMAC-Ti had superior reproducibility and phosphopeptide enrichment than the commonly used TiO₂ and immobilized metal ion affinity capture methods, with phosphopeptide selectivity approaching 95% and recovery >90% [7–9]. Importantly, PolyMAC-Ti has very good recovery and selectivity for samples with low total levels of protein phosphorylation [7, 10].

We reasoned that PolyMAC-Ti would provide sufficient phosphoproteome coverage and depth for general use with tissue specimens. Here, we describe a phosphoproteomic methodology using PolyMAC-Ti for the identification of potential therapeutic targets using a mouse model of breast cancer driven by a highly activated receptor tyrosine kinase.

2 Materials and methods

2.1 Mouse model

The LTR-MMTV-rtTA (MTB) and TetO-NeuNT-IRES-luc (TAN) transgenic mouse lines were described previously [11]. Both lines were originally derived from the FVB/N background. The TAN transgenic line was maintained by homozygous inbreeding, and the MTB transgenic line was maintained by breeding MTB⁺ hemizygous males with FVB/N females newly obtained from Taconic Farms for each breeding. Experimental bitransgenic MTB⁺; TAN⁺ mice were generated by crossing MTB⁺ males with TAN^{+/+} females. All mice were housed under standard barrier conditions with a 12 h light/dark cycle, and all mouse experiments were approved by the Washington University Animal Studies Committee.

2.2 Tumor formation and preparation of tumor lysates

Six to eight week old female MTB⁺; TAN⁺ littermates were provided with drinking water containing 1 mg/mL doxycycline (Sigma-Aldrich) with 5% sucrose ad libitum for a total of 6 wk, with doxycycline water changes every four days. This dose and timing of doxycycline administration was chosen because it gave the most rapid and reproducible tumor formation during optimization experiments (data not shown). The mice were anesthetized by an intraperitoneal injection of 600 μ L of 100 mM 2,2,2-tribromoethanol (Sigma-Aldrich) and 1.5% *t*-amyl alcohol in HBSS prewarmed to 37°C. The thoracic mammary glands were exposed and any gland with visible or palpable tumors was removed and washed three times in ice-cold HBSS. Small representative pieces of the glands were removed and fixed overnight in neutral-buffered formalin at 4°C for histology. Each gland was individually minced with a clean razor and then homogenized with a Polytron PT 1200E (Kinematica) rotor-stator in 5 $\times$ the tissue weight of ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-Cl pH 7.5, 1% Igepal CA-630 (Sigma-Aldrich)) with freshly added 1 mM sodium orthovanadate, 10 mM NaF, and 1% Phosphatase Inhibitor Cocktail 3 (Sigma-Aldrich, P0044). The homogenized lysates were incubated on ice for 20 min. Due to the high adiposity of mouse breasts, lysates were centrifuged twice at 16 100 $\times g$ for 10 min at 4°C to remove the floating lipid layer and insoluble debris. An aliquot of each supernatant was removed for quantification by bicinchonic acid protein assay (Thermo Fisher Scientific) and phosphotyrosine Western blotting, and the remainder snap frozen in liquid nitrogen and stored at -80°C.

2.3 Histology

Formalin-fixed mammary biopsies were embedded in paraffin, and 5 μ m sections were stained with Harris hematoxylin and eosin. Each specimen was examined for the extent of neoplasia, histology of invasive foci, and contamination with muscle, immune cells, or other extraneous tissue. Specimens with a large extent of neoplasia consisting mostly of epithelial sheets with minimal stroma, inflammation, or contaminating tissues were considered for phosphoproteomic analysis.

2.4 Immunoprecipitation and phosphotyrosine Western blot

For each tissue lysate, 1 mg clarified protein lysate was pre-cleared with protein A-agarose beads and added to 20 μ L of a 50% slurry of 4G10-agarose (EMD Millipore) overnight at 4°C. The beads were washed three times with 1 mL ice-cold lysis buffer and boiled in 40 μ L of 2 $\times$ loading buffer (100 mM Tris-Cl pH 6.8, 4% SDS, 20% glycerol, 10% DTT, 0.2% bromophenol blue). From each eluate, 35 μ L was separated on a 16 cm 8% SDS-PAGE gel and transferred onto a nitro-cellulose membrane in 20% methanol, 25 mM Tris, 192 mM glycine, 0.1% SDS. The membrane was blocked with 5% BSA in TBST (TBS + 0.1% Tween 20), incubated with 1:10 000 4G10 antiphosphotyrosine antibody (EMD Millipore) for 1 h, washed three times with TBST, incubated with 1:10 000 α -mouse IgG-horseradish peroxidase (Cell Signaling) for 1 h, washed three times with TBST, and developed using ECL.

2.5 Tyrosine phosphopeptide enrichment

Five biological replicates were prepared by combining equal protein quantities from two mice each for samples #1–4 and from three mice for sample #5. Total protein input was 3 mg for sample #1 and 15 mg for samples #2–5. The samples were denatured and reduced in 50 mM trimethylammonium bicarbonate, 0.1% RapiGest (Waters), and 5 mM DTT for 30 min at 37°C, then alkylated by adding iodoacetamide to a final concentration of 15 mM for 1 h at room temperature while protected from light. The samples were digested with proteomics-grade trypsin at a 1:100 ratio at 37°C overnight. Removal of the RapiGest was accomplished by a 40-min incubation at a pH value below 3 followed by centrifugation at 16 100 $\times$ g. To immunoprecipitate phosphotyrosine containing peptides, the sample pH values were first adjusted to pH 7.4 using 100 mM Tris-HCl buffer, pH 8.0, then the samples were incubated with 200 μ L of a PT66 antibody bead slurry (Sigma-Aldrich) overnight at 4°C with gentle agitation. The beads were washed twice with 500 μ L lysis buffer (lacking phosphatase inhibitors) for 10 min each and twice with 500 μ L water for 2 min each. Tyrosine phosphopeptides were recovered by vigorously agitating the beads three times in 100 μ L of 0.1% TFA for 10 min, twice in 100 μ L of 0.1% TFA in 50% ACN for 10 min, and twice in 50 μ L of 100 mM glycine, pH 2.5, for 30 min. All eluates were combined and dried completely in a SpeedVac for subsequent PolyMAC-Ti enrichment under high recovery conditions [8].

2.6 Total phosphopeptide enrichment

A total of 3 mg protein from sample #1 was denatured, alkylated, and digested with trypsin as described in Section 2.5. The sample was adjusted to pH 8.0 and loaded onto a C18 column (XBridge, Waters) for separation into eight fractions. This fractionation method, according to our experience, is orthogonal to the reverse phase liquid chromatography (RPLC) separation used during LC-MS/MS for phosphopeptides. Each fraction was subjected to PolyMAC-Ti enrichment under high selectivity conditions [8].

2.7 PolyMAC-Ti enrichment

The balance between recovery and selectivity of the PolyMAC-Ti enrichment procedure is controlled by the capture and washing conditions. High recovery conditions were used for

the phosphotyrosine-immunoprecipitated samples from Section 2.5. These samples were resuspended in 300 μ L of high recovery loading buffer (150 mM HEPES, pH 6.8) containing 5 nmol of the PolyMAC-Ti reagent and incubated for 5 min at room temperature. The mixture was transferred into a spin column containing Affi-Gel hydrazide beads (Bio-Rad), gently agitated for 10 min, and then centrifuged at $2300 \times g$ for 30 s. The gel was washed once with 200 μ L high recovery loading buffer, twice with 200 μ L washing buffer (100 mM acetic acid, 1% TFA, 80% ACN), and once with 200 μ L MS-grade water for 5 min each under gentle agitation. Phosphopeptides were eluted by gently agitating the gel twice in 100 μ L of elution buffer (400 mM ammonium hydroxide) for 5 min. The combined eluates were dried completely in a SpeedVac. High selectivity conditions, used for the HPLC pH 8.0 fractions from Section 2.6, differed from high recovery conditions as follows. Instead of high recovery loading buffer, the fractions were resuspended in 100 μ L of loading buffer (100 mM glycolic acid, 1% TFA, 50% ACN) containing 5 nmol of the PolyMAC-Ti reagent and incubated for 5 min at room temperature. Before adding the samples to the spin column containing the Affi-Gel hydrazide beads, the sample pH values were raised to above pH 6.3 using 200 μ L of capture buffer (300 mM HEPES, pH 7.7). During the washing step, 200 μ L loading buffer was used instead of high recovery loading buffer.

2.8 MS

Peptide samples were redissolved in 8 μ L of 0.1% formic acid and injected into an Agilent nanoflow 1100 HPLC system with an RPLC capillary column packed in-house with a 5- μ m C₁₈ Magic bead resin (Michrom; 75- μ m id and 12-cm bed length) and with an ESI emitter tip generated with a laser puller (Model P-2000, Sutter Instrument). An eluting buffer of 100% ACN was run with a shallow linear gradient over the mobile phase buffer of 0.1% formic acid in MS-grade water. A 60-min gradient was run for the phosphotyrosine enriched samples, and a 90-min gradient was run for the total phosphoproteome fractions. The Agilent 1100 HPLC system was coupled in line with an LTQ-Orbitrap XL (Thermo Fisher Scientific) operated in a data-dependent mode in which a full MS scan (from m/z 300 to 1700 with a resolution of 30 000 at m/z 400) was followed by four MS/MS scans of the most abundant ions. The dynamic mass exclusion time was 180 s and ions with a charge state of +1 were excluded.

2.9 Phosphoproteome analysis

Peptide identification, phosphorylation detection and localization, and protein inference were accomplished using MaxQuant version 1.4.1.2 by searching the Uniprot mouse proteome, last updated on 12 November 2013, with the Ne-uNT protein sequence and common MS contaminants added. Phospho-S,T,Y, *N*-terminal acetylation, and M oxidation were allowed as variable modifications, and carbamidomethylation of cysteines was the only fixed modification. The precursor mass tolerance was set at 20 ppm for the initial search and 4.5 ppm after recalibration in MaxQuant [12]; the fragment mass tolerance was 0.5 Da. Trypsin/P cleavage rules were used allowing for up to two missed cleavages. A minimum score of 40 and delta score of 17 was required for all modified peptides. Both peptide and protein false discovery rates were set at 1% and estimated using MaxQuant's REWARD algorithm, which is designed to give better performance when multiple variable modifications are possible. The list of peptides was manually curated to remove redundancy

due to missed cleavages and unstable phosphosite localization, arbitrarily defined as localization probability <75% [13]. MS/MS spectra matching common MS contaminants were also excluded. Tyrosine, serine, and threonine phosphosites were independently searched for motifs using motif-x with a width of 13 and cutoffs of $p = 0.0000001$ and at least 5% occurrence [14, 15]. The list of tyrosine phosphoproteins were input into Drug Gene Interaction database (DGIdb) to identify phosphoprotein–drug interactions [16]. Potential therapeutic targets were identified by a three step sequential filter: (i) available inhibitors FDA-approved or in clinical development, (ii) known or suspected oncogene for breast cancer of any subtype, and (iii) a literature search reveals that the observed phosphosite is activating.

3 Results and discussion

3.1 Description of NeuNT-driven mammary tumors

We reasoned that the MTB+; TAN+ model would provide an ideal model to examine the analytical performance of PolyMAC-Ti for phosphopeptide enrichment of tissue specimens and to study the phosphoproteome of breast cancer driven by the HER2 tyrosine kinase. These inbred mice have been engineered to express an activated HER2 ortholog, Ne-uNT, in the mammary epithelium when administered doxycycline, leading to tumors that develop in a highly reproducible manner and with a uniform genetic background.

MTB+; TAN+, and MTB–; TAN+ littermates were induced with 1 mg/mL doxycycline in their drinking water for 6 wk. All of the 12 MTB+; TAN+ mice developed mammary tumors as previously described [11], and none of the nine MTB–; TAN+ mice had any discernible histological abnormalities (Fig. 1A and B). As expected, differences could be seen in the tyrosine phosphoproteome of MTB+; TAN+ tumors compared to MTB–; TAN+ control mammary tissue as determined by phosphotyrosine immunoprecipitation and immunoblotting (Fig. 1C). Consistency was verified for each tumor by histological analysis and the phosphotyrosine banding pattern on Western blot. Variation between tumors was minimal, permitting more accurate technical assessment of the PolyMAC-Ti method with tissue specimens.

3.2 Description of total and phosphotyrosine-enriched phosphoproteomic workflows

Two phosphoproteomic workflows using PolyMAC-Ti were examined: a phosphotyrosine-enriched workflow and a total phosphoproteome workflow (Fig. 2). A specific phosphotyrosine-enriched workflow greatly improves coverage of the tyrosine phosphoproteome, which represents only 1–2% of the total phosphoproteome [17–19]. The tyrosine phosphoproteome is of particular interest because most tyrosine kinases function in known signaling pathways and have known inhibitors that are FDA-approved or in clinical trials. Hence, analysis of the tyrosine phosphoproteome from MTB+; TAN+ specimens could suggest novel therapeutic options for HER2-driven breast cancer.

The phosphotyrosine-enriched workflow was based on prior studies demonstrating efficient phosphotyrosine enrichment using immunoprecipitation with an antiphosphotyrosine antibody followed by phosphopeptide enrichment [17–19]. We have previously demonstrated improved tyrosine phosphopeptide yield and specificity using phosphotyrosine

immunoprecipitation followed by PolyMAC-Ti compared to phosphotyrosine immunoprecipitation on its own or in conjunction with phosphopeptide enrichment using TiO₂ resin [7]. Tryptic peptides from a total input of 15 mg protein were subjected to immunoprecipitation using the antiphosphotyrosine antibody PT66, followed by PolyMAC-Ti enrichment. High recovery conditions are used for PolyMAC-Ti enrichment because the immunoprecipitation step has already reduced sample complexity. In the total phosphoproteome workflow, 3 mg protein was fractionated into eight fractions by RPLC under neutral conditions (pH 8.0). Each fraction was subjected to PolyMAC-Ti enrichment under high selectivity conditions.

3.3 Performance of PolyMAC-Ti peptide enrichment from NeuNT-driven mammary tumors

Five biological replicates were analyzed using the phosphotyrosine-enriched workflow with a single LC-MS/MS run each. Biological replicate #1 used 3 mg total protein input, but this gave only 43 unique peptides. Therefore, the total protein input was increased to 15 mg for biological replicates #2–5, which yielded 94–292 unique peptides each (Fig. 3E). In total, 411 unique peptides were observed with 182 (44.3%) singly, 11 (2.7%) doubly, and 3 (0.7%) triply phosphorylated peptides (Fig. 3A and Supporting Information Table 1). Additionally, 158 (78.6%) phosphopeptides had phosphorylation localization probabilities >75%, a commonly used cutoff for high confidence [13]. As expected, phosphotyrosines were greatly overrepresented, with 139 (65%) phosphotyrosine, 45 (21%) phosphoserine, and 29 (14%) phosphothreonine sites identified (Fig. 3C). The predominance of peptides with a single phosphotyrosine is consistent with performance of PolyMAC-Ti after phosphotyrosine immunoprecipitation in cell line studies [7, 10]. The selectivity of PolyMAC-Ti under high recovery conditions was very consistent between biological replicates, with phosphorylation detected for 47% of identified peptides (Fig. 3E). Biological replicate #2 was an outlier with 95% selectivity, but had fewer peptide identifications than biological replicates #3–5.

Biological replicate #1 above was also analyzed using the total phosphoproteome workflow. Combined, the eight RPLC fractions identified 1571 unique peptides, including 1020 (64.9%) singly, 218 (13.9%) double, and 19 (1.2%) triply phosphorylated peptides (Fig. 3B and Supporting Information Table 2). Additionally, 1022 (79.9%) phosphopeptides had phosphorylation localization probabilities >75%. The proportion of phosphorylated serine, threonine, and tyrosine residues matched their relative abundance [13], with 1279 (84.5%) phosphoserine, 213 (14.1%) phosphothreonine, and 21 (1.4%) phosphotyrosine sites identified (Fig. 3D). Overall, the high-selectivity condition had very high phosphopeptide enrichment, with phosphorylation detected in 80% of identified peptides (Fig. 3F). The middle of the RPLC gradient, fractions 3–7, showed the greatest consistency and highest selectivity in their phosphopeptide enrichment.

To find overrepresented linear phosphorylation motifs, the phosphotyrosine, phosphoserine, and phosphothreonine sites were independently analyzed with the program motif-x (Fig. 4 and Supporting Information Fig. 1). Three phosphotyrosine motifs were identified, two of which were the highly acidic DXXY and EXXY motifs, and a YXXP motif. Tyrosine kinases tend to have poorly informative linear motifs; these motifs could correspond to Src

family kinases, A-type ephrin receptor kinases, EGFR family (including HER2), ABL, SYK, or others [20]. Among the serine and threonine phosphosites, many of the motifs included (S/T)P, RRX(S/T), or RXX(S/T), which are common to several serine/threonine kinase families. Additionally, the PX(S/T)P motif is characteristic of the mitogen activated protein kinase (MAPK) family [20], which is downstream of HER2 activation. The TPP and TS motifs enriched in the phosphothreonine sites are of unknown significance.

3.4 Identification of potential inhibitors of HER2-driven breast cancer

The MTB+; TAN+ tumors are driven by tyrosine kinase activity, hence either the active tyrosine kinases or their substrates are potential therapeutic targets. The DGIdb is an effective way to query drug–protein interactions from a variety of sources and was used to determine which tyrosine phosphoproteins have known inhibitors or are potentially druggable [16]. Priority was given to targets that have (i) observed activating phosphosites, (ii) inhibitors that have successfully passed phase I clinical studies, and (iii) known or suspected roles in breast cancer.

The potential therapeutic targets could be assembled into a network centered on HER2 signaling through the Src family of nonreceptor tyrosine kinases, which includes SRC, FYN and LYN (Fig. 5 and Supporting Information Table 3). HER2 associates with SRC through its kinase domain and leads to SRC activation [21]. An FDA-approved inhibitor of Src family kinases, dasatinib, has shown activity against NeuNT-driven mammary tumors [21, 22]; however, dasatinib may also be working through inhibition of EPHA2, a receptor tyrosine kinase associated with breast cancer metastasis [23]. Additionally, HER2 directly phosphorylates and activates receptor-type protein tyrosine phosphatase epsilon on Y695, which subsequently dephosphorylates an autoinhibitory site on Src family kinases. Interestingly, protein tyrosine phosphatase epsilon can be inhibited by the FDA-approved bisphosphonate alendronate, which has been linked to a decreased incidence of breast cancer through an unknown mechanism [24]. Src family kinases also phosphorylate STAT3 on Y705, PTK2 (FAK) on Y407, and PTK2B (FAK2/PYK2) on Y579 [25, 26]. STAT3, a transcription factor essential for HER2-dependent tumor formation in mice, can be directly inhibited with ISIS-STAT3. PTK2 and PTK2B further activate SRC and integrate HER2 and integrin signaling pathways [26]; PTK2 is inhibited by masitinib and PTK2B is inhibited by leflunomide or genistein. BTK is a nonreceptor tyrosine kinase important for B-cell activation with a specific, FDA-approved inhibitor, ibrutinib. BTK Y551 is a direct target of LYN and other Src family kinases [27], but may also have been detected due to infiltrating inflammatory cells. MAPK1 (ERK2) and MAPK3 (ERK1) are downstream of HER2 signaling as critical components of the oncogenic RAF/RAS/MAPK pathway and can be inhibited with the FDA-approved drug sorafenib. Collectively, these results demonstrate the ability of the phosphotyrosine-enriched workflow to identify novel therapeutic avenues for further evaluation, such as potential synergy between Src inhibitors and alendronate for HER2-driven breast cancer.

4 Concluding remarks

We describe the first analysis of tissue specimens using Poly-MAC and we find that that PolyMAC is well suited for this purpose. PolyMAC was readily coupled with either

phosphotyrosine immunoprecipitation or sample fractionation by RPLC and large numbers of phosphopeptides were identified from a modest number of LC-MS/MS runs. These phosphopeptides demonstrate activation of many families of protein kinases, including Src family kinases and both basic and proline-directed serine/threonine kinases, in a model of HER2-driven breast cancer. Analysis of the tyrosine phosphoproteome with DGIdb revealed an oncogenic network of tyrosine kinases with known inhibitors for future investigation.

Acknowledgments

This work was supported by grants from Susan G. Komen for the Cure (R. B.), the Cancer Frontier Fund Research Development Award from the Siteman Cancer Center and Foundation for Barnes-Jewish Hospital (R. B.) and NIH grant R01GM088317 (W. A. T.).

Abbreviations

DGIdb	Drug Gene Interaction database
MTB	LTR-MMTV-rtTA
PolyMAC	polymer-based metal ion affinity capture
TAN	TetO-NeuNT-IRES-luc
TBST	TBS + 0.1% Tween 20

References

1. Druker BJ, Guilhot F, O'Brien SG, Gathmann I, Kantarjian H, Gattermann N. *N Engl J Med.* 2006; 355:2408–2417. [PubMed: 17151364]
2. Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S. *Science.* 2004; 304:1497–1500. [PubMed: 15118125]
3. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW. *N Engl J Med.* 2004; 350:2129–2139. [PubMed: 15118073]
4. Geyer CE, Forster J, Lindquist D, Chan S, Romieu CG, Pienkowski T. *N Engl J Med.* 2006; 355:2733–2743. [PubMed: 17192538]
5. Bose R, Kavuri SM, Searleman AC, Shen W, Shen D, Koboldt DC. *Cancer Discov.* 2013; 3:224–237. [PubMed: 23220880]
6. Iliuk AB, Arrington JV, Tao WA. *Electrophoresis.* 2014; 3510.1002/elps.201400153
7. Iliuk AB, Martin VA, Alicie BM, Geahlen RL, Tao WA. *Mol Cell Proteomics.* 2010; 9:2162–2172. [PubMed: 20562096]
8. Iliuk A, Jayasundera K, Schluttenhofer R, Tao WA. *Methods Mol Biol.* 2011; 790:277–285. [PubMed: 21948422]
9. Xue L, Wang WH, Iliuk A, Hu L, Galan JA, Yu S. *Proc Natl Acad Sci USA.* 2012; 109:5615–5620. [PubMed: 22451900]
10. Walls CD, Iliuk A, Bai Y, Wang M, Tao WA, Zhang ZY. *Mol Cell Proteomics.* 2013; 12:3759–3777. [PubMed: 24030100]
11. Moody SE, Sarkisian CJ, Hahn KT, Gunther EJ, Pickup S, Dugan KD. *Cancer Cell.* 2002; 2:451–461. [PubMed: 12498714]
12. Cox J, Michalski A, Mann M. *J Am Soc Mass Spectrom.* 2011; 22:1373–1380. [PubMed: 21953191]
13. Olsen JV, Blagoev B, Gnäd F, Macek B, Kumar C, Mortensen P. *Cell.* 2006; 127:635–648. [PubMed: 17081983]
14. Schwartz D, Gygi SP. *Nat Biotechnol.* 2005; 23:1391–1398. [PubMed: 16273072]

15. Chou MF, Schwartz D. Curr Protoc Bioinformatics. 2011; 13:15–24. [PubMed: 21901740]
16. Griffith M, Griffith OL, Coffman AC, Weible JV, McMichael JF, Spies NC. Nat Methods. 2013; 10:1209–1210. [PubMed: 24122041]
17. Salomon AR, Ficarro SB, Brill LM, Brinker A, Phung QT, Ericson C. Proc Natl Acad Sci USA. 2003; 100:443–448. [PubMed: 12522270]
18. Ficarro S, Chertihin O, Westbrook VA, White F, Jayes F, Kalab P. J Biol Chem. 2003; 278:11579–11589. [PubMed: 12509440]
19. Zhang Y, Wolf-Yadlin A, Ross PL, Pappin DJ, Rush J, Lauffenburger DA. Mol Cell Proteomics. 2005; 4:1240–1250. [PubMed: 15951569]
20. Miller ML, Jensen LJ, Diella F, Jørgensen C, Tinti M, Li L. Sci Signal. 2008; 1:ra2. [PubMed: 18765831]
21. Marcotte R, Zhou L, Kim H, Roskelley CD, Muller WJ. Mol Cell Biol. 2009; 29:5858–5871. [PubMed: 19704002]
22. Karim SA, Creedon H, Patel H, Carragher NO, Morton JP, Muller WJ. J Pathol. 2013; 230:430–440. [PubMed: 23616343]
23. Tandon M, Vemula SV, Mittal SK. Expert Opin Ther Targets. 2011; 15:31–51. [PubMed: 21142802]
24. Chlebowski RT, Chen Z, Cauley JA, Anderson G, Rodabough RJ, McTiernan A. J Clin Oncol. 2010; 28:3582–3590. [PubMed: 20567009]
25. Chung SS, Giehl N, Wu Y, Vadgama JV. Int J Oncol. 2014; 44:403–411. [PubMed: 24297508]
26. Wang SE, Xiang B, Zent R, Quaranta V, Pozzi A, Arteaga CL. Cancer Res. 2009; 69:475–482. [PubMed: 19147560]
27. Lin L, Czerwinski R, Kelleher K, Siegel MM, Wu P, Kriz R. Biochemistry (Mosc). 2009; 48:2021–2032.

Figure 1.

Representative hematoxylin and eosin stained sections of (A) normal mammary epithelium from a MTB⁻; TAN⁺ control and (B) a tumor from a MTB⁺; TAN⁺ mouse. (C) To highlight differences in the tyrosine phosphoproteome between MTB⁺; TAN⁺ tumor and normal MTB⁻; TAN⁺ mammary epithelium, tyrosine phosphoproteins were immunoprecipitated with the 4G10 antiphosphotyrosine antibody from 1 mg protein input, separated by 8% SDS-PAGE and detected by Western blot with 4G10. Arrows highlight prominent phosphoproteins not discernible in normal mammary epithelium.

Figure 2.

Schematic of phosphotyrosine-enriched and total phosphoproteomic workflows.

Phosphotyrosine enrichment was accomplished by immunoprecipitation with the PT66 antiphosphotyrosine antibody followed by PolyMAC-Ti in high recovery conditions. The total phosphoproteome was fractionated on a C18 column under neutral conditions followed by PolyMAC-Ti in high selectivity conditions.

Figure 3.

Performance of PolyMAC-Ti with phosphotyrosine enrichment (A, C, and E) or RPLC fractionation at pH 8 for total phosphoproteome identification (B, D, and F). (A and B) The number of unique peptides detected at a 1% false discovery rate. (C and D) The number of unique phosphotyrosine, phosphoserine, and phosphothreonine sites detected. (E and F) The selectivity of phosphopeptide enrichment for each LC-MS/MS run from (A–D). Error bars represent 95% confidence intervals, and *n* is the total number of unique peptides within each run.

Figure 4.

Linear motifs identified for phosphotyrosines, phosphoserines, and phosphothreonines. The motifs were simplified from motif-x analysis, available in the Supporting Information.

Figure 5.

Connections between potential therapeutic targets for HER2-driven breast cancer. SFK: Src family kinases, which includes SRC, FYN, and LYN. Proteins shown in gray were observed with tyrosine phosphosites known to be activating. All shown inhibitors are FDA-approved or in clinical development, and were identified using DGIdb.