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# Phosphoproteomic analysis reveals PAK2 as a therapeutic target for lapatinib resistance in HER2-positive breast cancer cells

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ABSTRACT

The human epidermal growth factor receptor 2 (HER2)-positive breast cancer with overexpression of HER2 accounts for approximately 25% of breast cancers and is more aggressive than other types of breast cancer. Lapatinib has been widely used as a HER2-targeted therapy, however, a number of patients develop lapatinib resistance and still suffer from poor prognosis. Therefore, it is essential to identify novel therapeutic targets that could overcome lapatinib resistance. In this study, we carried out phosphoproteomic analysis of lapatinib sensitive and resistant cell lines (SKBR3 and SKBR3-LR) using stable isotope labeling with amino acids in cell culture (SILAC). We identified 3808 phosphopeptides from 1807 proteins and then analyzed signaling pathways, Gene Ontology, and protein-protein interaction networks. Finally, we identified PAK2 as a therapeutic target from the network analysis and validated that PAK2 knockdown and PAK inhibitor treatment resensitize the lapatinib resistant cells to lapatinib. This results suggest that PAK2 is a potent therapeutic target to overcome acquired lapatinib resistance in HER2-positive breast cancer cells.



## 1. Introduction

Human epidermal growth factor receptor 2 (HER2, also known as EGFR2) is overexpressed in approximately 25% of breast cancers [1] and regulates survival and proliferation by activating multiple downstream signaling pathways including the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K) pathways by forming kinase-active dimers with HER family members [2–4]. Therefore, targeting HER2 is an effective treatment of HER2 positive breast cancer patients. The HER2 targeted drugs approved by the US Food and Drug Administration for HER2-positive breast cancer are the monoclonal antibodies trastuzumab and pertuzumab, trastuzumab-drug conjugate T-DM1, and the small molecule dual HER1/HER2 inhibitor lapatinib. Although these drugs have initial dramatic clinical responses to these therapies, breast tumors frequently acquire resistance against these targeted therapies.

Lapatinib is the highest selective dual inhibitor of HER1 and HER2 as an ATP competitor and leads to inhibition of cell proliferation by preventing the phosphorylation and subsequent signal transduction of the MAPK and the PI3K pathways [5]. The combination of lapatinib with capecitabine for the treatment of advanced HER2-positive breast cancer was approved [6]. Although lapatinib greatly improved HER2-positive breast cancer prognosis, many patients still suffered from the intrinsic and acquired resistance to lapatinib [7]. Therefore, it is essential to understand the mechanisms involved in resistance to lapatinib and to identify novel therapeutic targets for the resistant patients. Several potential mechanisms of resistance to lapatinib have been proposed to date including activation of: i) receptor tyrosine kinases including HER3 [8], MET [9], and AXL [10]; ii) intracellular kinases including AKT [11], mTOR [12], SRC [13], PTK6 [14]; iii) HER2 by mutation of T798I [15]; iv) estrogen receptor pathway [16]; v) PP2A [17]; vi) reprogramming of glycolysis [18].

In this study, we performed the development, characterization, and stable isotope labeling with amino acids in cell culture (SILAC) [19] based-phosphoproteomic analysis of cell line model to identify altered phosphorylation events associated to lapatinib resistance. We report that phosphoproteome is significantly altered in lapatinib resistant cells and inhibition of PAK2 (p21-activated kinase 2) activity is a novel HER2 targeted therapy to overcome acquired lapatinib resistance.

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## 2. Materials and methods

### 2.1. Establishment of lapatinib-resistant cell lines

SKBR3 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). To establish the lapatinib resistant cell line (SKBR3-LR), SKBR3 cells were treated with 600 nM lapatinib (Selleckchem, Huston, TX, USA) in RPMI 1640 medium with 10% FBS twice weekly for more than 6 months. SKBR3-LR cells were maintained continuously in the presence of 600 nM lapatinib.

### 2.2. Cell culture for SILAC

Basal SILAC medium was made up from RPMI 1640 (WELGENE, Gyeongsan-si, Korea) deficient in L-arginine and L-lysine according to the manufacturer's instructions. For labeling cells, basal SILAC medium was supplemented with 10% dialyzed FBS, antibiotic penicillin/streptomycin solution, light lysine (K) and arginine (R) for light labeling of SKBR3-LR and $^{13}C_6^{15}N_2$-K and $^{13}C_6^{15}N_4$-R (Cambridge Isotope Laboratories, Tewksbury, MA, USA) for heavy isotopic labeling of SKBR3 cells. Biological replicates of cells were grown over seven cell divisions in RPMI1640 based heavy SILAC labeling. The labeling efficiency was confirmed by mass spectrometry analysis.

Cells were washed with ice cold phosphate buffered saline (PBS) before harvesting and lysed with 5 mL of Urea Lysis Buffer (9 M Urea, 20 mM HEPES, pH 8.0, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate) at room temperature. After scraping the cells, lysis buffer was transferred from the first dish to the second dish. All lysates were sonicated at 20% power (3 bursts of 10 s each) with cooling on ice for 1 min between each burst. Lysates were cleared by centrifugation at 13,000 rpm for 15 min at room temperature and supernatants were transferred into a new tube. The protein concentration of the collected supernatant was determined by a standard Bradford protein assay (Thermo Fisher Scientific, San Jose, CA, USA)).

### 2.3. Preparation of enriched phosphopeptides

Equal amount of protein from heavy labeled SKBR3 and light labeled SKBR3-LR cell lysates was mixed and reduced with 5 mM dithiothreitol at 55 °C for 30 min and alkylated with 10 mM iodoacetamide for 15 min at RT in the dark. Lysates were then diluted to less than 2 M urea final concentration using 20 mM HEPES (pH 8) and digested by treatment of trypsin at 1:50 (enzyme: protein ratio) for 16 h at 37 °C. The tryptic peptides were desalted using Sep-Pak Vac $tC_{18}$ (Waters, Milford, MA, USA), dried in vacuum and kept at −20 °C until use.

In order to reduce complexity of peptide samples, they were fractionated (12 fractions) by high pH reserved-phase liquid chromatography (RPLC) using an Agilent 1290 Infinity LC System (Agilent Technologies (Santa Clara, CA, USA). The chromatography performed with XBridge BEH130 $C_{18}$ column (4.6 μm i.d. × 250 mm length; pore size 130 Å and particle size 3.5 μm; Waters) at a flow rate of 0.5 mL/min. For mobile phase, 10 mM ammonium formate (pH 10) set as channel A and 10 mM ammonium formate in 90% acetonitrile (ACN) (pH 10) set as channel B. Peptides dissolved in 105 μL of mobile phase A and then injected into a 100 μL sample loop. Gradient setup was initially 2–5% B for 10 min, 5–40% B for 40 min, 40–70% B for 15 min, holding %B for 10 min, and finally 70-5% for 15min. Fractionation was done by collecting 96 tubes (1 tube/0.8 min) throughout running. Then 96 fractions pooled to 12 concatenated fractions by the following rule. A set of an arithmetic sequence with a common difference of 12 was pooled into one concatenated fraction, for instance, number 1, 13, 25, 37, 49, 51, 63 and 75 fractions were pooled into a first concatenated fraction [20,21]. The chromatogram of each run consists of UV absorbance of 205, 214, 254, 260 and 280 nm. The fractions were dried in a vacuum centrifuge and stored at −20 °C until use.

All phosphopeptide enrichments were conducted as described in Pierce $TiO_2$ phosphopeptide enrichment and clean-up kit (Thermo Fisher Scientific). Dried peptide pellets were resuspended in $TiO_2$ loading buffer (60% ACN, 0.3% trifluoroacetic acid (TFA), 25% lactic acid), and 2 mg of titansphere $TiO_2$ beads was added per 500 μg of peptides. Beads were then sequentially washed with (i) 80% ACN, 0.4% TFA and (ii) 60% ACN, 0.3% TFA, 25% lactic acid. Phosphopeptides were eluted by two buffers (1.5% $NH_4OH$ and 5% pyrolidine). The flow through was collected and subjected to further $TiO_2$ enrichment steps. Samples were acidified with TFA and then desalted with Pierce® Graphite spin columns (Thermo Fisher Scientific) and dried under vacuum.

### 2.4. Liquid chromatography and tandem mass spectrometry

Dried peptide samples were reconstituted in 0.4% acetic acid, and an aliquot containing approximately 1 μg was injected from a cooled (10 °C) autosampler into a reversed-phase $C_{18}$ column (15 cm × 75 μm, in-house packed into the tip with Reprosil-Pur $C_{18}$-AQ 5 μm and 120 Å resin from Dr. Maisch GmbH) on an Eksigent nanoLC 2D system at a flow rate of 300 nL/min. Prior to use, the column was equilibrated with 90% buffer A (0.1% formic acid in water) and 10% buffer B (0.1% formic acid in ACN). The peptides were eluted with a linear gradient from 10% to 35% buffer B over 95 min and 50%–80% buffer B over 10 min followed by an organic wash and aqueous re-equilibration at a flow rate of 300 nL/min with a total run time of 120 min. The high performance liquid chromatography (HPLC) system was coupled to a LTQ OrbitrapXL mass spectrometer (Thermo Fisher Scientific) operated in a data-dependent acquisition (DDA) mode. The electrospray ionization source was operated in the positive ion mode (300–1500 $m/z$) with spray voltage set at 2.1 kV, capillary voltage at 21 V, and the heated capillary temperature at 250 °C. Each scan cycle consisted of node full MS scan in profile mode followed by 10 data dependent MS/MS scans with the following options: isolation width, 2.00 $m/z$; normalized collision energy, 35%; and dynamic exclusion duration, 30s.

### 2.5. Quantification and bioinformatic analysis

MS raw files were analyzed by MaxQuant software version 1.5.5.1, and peptide lists were searched against the UniProtKB SwissProt database (release 2016/04/20) and a common contaminants database by the Andromeda search engine with cysteine carbamidomethylation (+57.02 Da) as a fixed modification and oxidation of methionine (+15.9949 Da) and phosphorylation of serine, threonine, and tyrosine as variable modifications. For the analysis of SILAC samples, variable modifications of [L-$^{13}C_6$, $^{15}N_4$]-Arg (+10.01 Da) and [L-$^{13}C_6$, $^{15}N_2$]-Lys (+8.01 Da) were permitted. Searches were performed with a 10 ppm main search peptide tolerance, 5 ppm for isotope match tolerance, 10 ppm for centroid match tolerance, 40 ppm for centroid half width and checked for MS2 centroids. The false discovery rate was set to 0.01 for both proteins and peptides with a minimum length of seven amino acids. Enzyme specificity was set as trypsin and a maximum of two missed cleavages was allowed in the database search. Peptide identification was performed with an allowed initial precursor mass deviation up to 7 ppm and an allowed fragment mass deviation of 20 ppm. Phosphosite identifications were filtered for greater than 0.75 localization probability and peptides detected

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