

Evaluation of the HER/PI3K/AKT family signaling network as a predictive biomarker of pCR for breast cancer patients treated with neratinib in the I-SPY 2 TRIAL

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Supplementary Methods

DNA sequencing, somatic mutation calling, and copy number alteration analysis

130 patients (N:78; C:52) had pre-treatment tumor-normal pairs DNA sequenced over a ~2000 gene cancer mini-genome by (Illumina x100; Utrecht). Somatic mutations were evaluated using Strelka, Freebayes and VarScan2 and only the high confidence calls were included in the analysis. The copy number variations (CNV) were estimated by the CONTRA 2.0.4. For each gene, samples with at least one non-synonymous mutation were classified as 'mutant' =1, and all others as 'wildtype'=0. Copy number amplification or loss were encoded as +1/-1.

RNA profiling of pre-treatment tumor biopsies

Pre-treatment tumor biopsy cryosections are emulsified in 0.5ml Qiazol solution and sent to Agendia, Inc., for RNA extraction and gene expression profiling on Agilent 44K microarrays. For each array, the green channel mean signal is log₂- transformed and centered within array to its 75th quantile as per the manufacturer's data processing recommendations. A fixed value of 9.5 is added to avoid negative values.

Laser Capture Microdissection and reverse-phase protein microarray

Patient Samples

A total of 168 pre-treatment breast cancer biopsy specimens collected in the I-SPY2 TRIAL (NCT01042379) from the neratinib treatment arm (N=106) and concurrent control patients (N=62) had sufficient tumor epithelium present for this analysis. Of these 168 patients, 52 TN samples (neratinib-treated=31; concurrent controls=21) had both

experimental and response data available.

Enriched epithelial cell populations were isolated from 8µm cryosections (> 95% purity) using an Arcturus Pixcell IIe LCM system (Arcturus, Mountain View, CA, USA) as described.⁴⁹ Approximately 10,000 epithelial cells were captured for each sample in the treatment and concurrent control arms. Microdissected material was stored at -80C and samples were lysed in extraction buffer composed of Tissue Protein Extraction Reagent (TPER; ThermoFisher), 2x SDS-PAGE Sample Buffer (ThermoFisher) mixed 1:1 and 2.5% beta-mercaptoethanol (BME) per 1 mL at a concentration of approximately 500-600 cells per 1 µL of extraction buffer. Samples were heated at 100C for 5min, brought to RT, briefly centrifuged and stored at -20C until printing.

Cell lysates were printed in triplicate spots (approx. 10nL per spot) onto nitrocellulose coated slides (Grace Biolabs, Bend, OR, USA) using an Aushon 2470 Arrayer (Aushon Biosystems, Billerica, MA, USA). Standard curves of control cell lysates were also included for quality assurance purposes.⁵⁰ Proteins and phosphoproteins measured in this study were included for their relationship to the targeted pathway of neratinib, namely EGFR and ERBB2 signaling, and to other pathways known to play a role in breast cancer. Antibodies used on the arrays were validated before use⁵¹, and are listed in Table 1. Only antibodies that showed a single band at the appropriate molecular weight with a panel of cell lysates using conventional western blotting were considered qualified for the analysis.⁵² Significant concordance between RPPA data and western blotting have been previously shown.^{15,53-55} Immunostaining was performed as previously described.⁵⁶ Each slide was probed with one primary antibody targeting the protein of interest. Biotinylated goat anti-rabbit IgG (H+L)(1:7,500, Vector Laboratories Inc, Burlingame, CA) and rabbit anti-mouse IgG (1:10, DakoCytomation, Carpinteria, CA, USA) were used as secondary antibodies. Signal amplification was performed using a tyramide-based avidin/biotin amplification system (DakoCytomation, Carpinteria, CA, USA) followed by streptavidin-conjugated IRDye 680 (LI-COR, Lincoln, NE, USA) for visualization. Total protein was measured using Sypro Ruby protein blot staining per

manufacturer's instructions (Molecular Probes, Eugene, OR, USA). Images were acquired using a Tecan PowerScanner (Tecan, Mannedorf, Switzerland) and analyzed with MicroVigene software Version 5.1.0.0 (Vigenetech, Carlisle, MA, USA).⁵⁶ The final results represent negative control- subtracted and total protein normalized relative intensity values for each endpoint within a given patient sample.