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Biomarkers for Predicting Response to HER2-targeted Therapies

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Biomarkers for Predicting Response to HER2-targeted Therapies

by

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DISSERTATION

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Dedication

I would like to dedicate this dissertation to my mother, who has always been my role model and pillar of strength. Thank you for supporting my decision to fly halfway around the world to pursue my dream though it would mean that I could not be with you at the times when you need me the most. Thank you for your always believing in me even when I didn't believe in myself. Thank you for always encouraging Sis and me to the best that we can be. You may not understand the contents of this dissertation but I want you to know that this dissertation would not have been possible without you.

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Abstract

Biomarkers for Predicting Response to HER2-targeted Therapies

Pei Rong Evelyn Lee

HER2-targeted therapies have been the mainstay of treatment of HER2-positive breast cancer. To date, the selection of patients most likely to respond to HER2-targeted agents is based primarily on HER2 amplification and/or overexpression. However, the correlations among current clinical methods of detecting HER2 amplification and/or overexpression are imperfect with regards to both prognostication and the prediction of drug response to many of the HER2-targeted therapies, and therefore, there is a critical need for the discovery and translation of additional biomarkers that predict patient response to a specific HER2-targeted therapy. Here, we evaluated BluePrint molecular subtypes – a gene expression-based molecular subtype classification – as a predictor of response to HER2-targeted therapies using patient data from the I-SPY 2 TRIAL. We demonstrated the potential clinical utility of BluePrint molecular subtyping in identifying a subset of HER2-positive, estrogen receptor-positive (HER2+/HR+) patients who are less likely to benefit from HER2-targeted therapies. In addition, gene expression analysis of this subset of patients reveal lower immune signaling and higher estrogen receptor expression, and thus may potentially benefit from alternative strategies, such as endocrine therapy or immunotherapy.

In a second study, we evaluated the baseline activation state of 104 key signaling phosphoproteins/ proteins from prosurvival, mitogenic, apoptotic, and growth regulatory pathways as predictors of response to neratinib – an irreversible pan-HER tyrosine kinase inhibitor of EGFR/HER2 – in HER2-positive breast cancer cell line models with differential neratinib sensitivity. We identified 13 phosphoproteins/ proteins, representing a multitude of

pathways, in particular the HER family signaling pathway, that are associated with neratinib sensitivity. We also demonstrated in HER2-positive breast cancer cell line models that acquired resistance to neratinib could potentially be mediated through adaptive kinome reprogramming, and that the combination of neratinib and BET bromodomain inhibitor appears to be a promising therapeutic strategy to overcome such resistance.

In conclusion, the work presented here provide insight into mechanisms underlying differential drug responses and resistance to HER2-targeted therapies, and highlight novel genomic and proteomic biomarker candidates that could potentially complement HER2 overexpression and/or amplification in predicting patient response to HER2-targeted therapies.

Table of Contents

List of Figures

Chapter 3

List of Tables

Chapter 2

Chapter 3

Chapter 1:

General Introduction

HER/ ErbB transmembrane receptor tyrosine kinase family

Human epidermal growth factor receptor 2 (HER2/ ErbB-2) is a member of the HER/ErbB family of transmembrane receptor tyrosine kinases (RTKs) that also includes EGFR (ErbB-1, HER1), HER3 (ErbB-3) and HER4 (ErbB-4). Together, the HER family of RTKs normally regulate cell growth and survival, as well as adhesion, migration, differentiation and other cellular responses in a variety of tissues[1,2]. The relevance of the HER family in cancer was first recognized over three decades ago when it was found that EGFR had close sequence homology to avian erythroblastosis virus (AEV)[3,4]. The rodent homologue of HER2, neu, was first discovered in a rat carcinogen-induced brain tumor model[5–7], and the HER2 human homologue was later found to be amplified in a human breast cancer cell line[8,9]. HER3[10] and HER4[11] were subsequently identified due to their sequence homologies to EGFR.

The HER family proteins are type I transmembrane growth factor receptors that function to activate intracellular signaling pathways in response to extracellular signals. Each member of the HER RTK family consists of an extracellular ligand-binding domain, a transmembrane lipophilic segment, and (with the exception of HER3[12]) a functional intracellular tyrosine kinase domain. Signaling in the HER family is typically initiated by the binding of ligand binding to the extracellular domain, triggering conformational changes that allow homo- or heterodimerization with other HER family members[1,13](Figure 1.1). Dimerization of the HER proteins activate the intracellular tyrosine kinase domains resulting in trans- and autophosphorylation of tyrosine

residues in the cytoplasmic tails[14]. These phosphorylated tyrosine residues dock numerous intracellular signaling molecules, leading to activation of a plethora of downstream signaling cascades. The 2 major downstream signaling pathways activated by HER receptors are the PI3K-AKT and the RAS/RAF/MEK/MAPK pathways[1,13](Figure 1.1). The signaling events triggered by the HER receptors promote cellular proliferation and survival, which are the driving forces of malignant transformation.

In contrast to the extracellular domain of the three other HER receptors, the extracellular domain of HER2 is unable to bind any known natural ligand. Instead, it adopts a fixed conformation resembling a ligand-activated state i.e. active conformation[15,16], permitting HER2 to homodimerize in the absence of a ligand or engage its signaling function through its ligand-bound heterodimeric partners[17]. The specific combination of HER receptors within a homo- or heterodimer appears to be a key determinant of downstream signaling activity. The general paradigm is that heterodimers have stronger signaling functions than homodimers. Among the 4 HER family receptors, HER2 has the strongest catalytic kinase activity and HER2-containing heterodimers have the strongest signaling functions[18,19]. In particular, HER2-HER3 heterodimers appears to be the most potent signaling complex[18,20,21] and they favor downstream signaling via the PI3K-AKT pathway due to the presence of six consensus phosphotyrosine sites on the C-terminal tail of HER3 that bind to the PI3K p85 subunit[22–24].

HER2 overexpression in human breast cancer

Breast cancer is the most common form of cancer in women, and it is responsible for the second highest number of cancer-related deaths in women in the United States[25]. Overall breast

cancer death rates have decreased rapidly (~39%) from 1989 to 2015; the decline in breast cancer mortality has been attributed to improvements in treatment, early detection and increased awareness[25].

Several distinct molecular subtypes of breast cancer have been defined based on gene expression patterns[26,27]. In the clinic, the major subtypes of breast cancer are approximated by the joint expression of three tumor markers/ receptors: estrogen receptor (ER), progesterone receptor (PR), and HER2. The most common subtypes are hormonal receptor (ER or PR) positive i.e. ER+ or PR+, comprising the luminal A and luminal B molecular subtypes; triple-negative tumors (ER-/PR-/HER-), most of which are of the basal-like phenotype; and HER2-positive (HER2+) tumors, which are mostly HER2-enriched (50-60%) by molecular subtyping[26–28]. HER2-positive breast cancer, which is characterized by the amplification of the HER2 gene and/or overexpression of HER2 at the protein level, occurs in about 15-20% of patients with early stage breast cancer[29]. Before the advent of HER2-directed therapies, HER2-positive breast cancer was associated with high recurrence rates and increased mortality in patients with node-negative and node-positive disease[30].

In contrast to normal cells which express approximately 20,000 HER2 receptors on the cell membrane, each HER2-positive breast cancer cell expresses approximately one to two million HER2 receptors at the surface[31–33]. Overexpression of HER2 changes the composition of HER family dimers, significantly increasing HER2-containing heterodimers and HER2-homodimers, resulting in constitutive signaling of downstream pathways that drive proliferation and survival in HER2-positive breast cancer. HER2 overexpression and/or gene amplification is

also seen in subsets of gastric, esophageal, bladder and gallbladder cancers, and also seen albeit rarely in cancers of the oropharynx, lung, colorectal, pancreas and ovary[34].

HER2 activating mutations in breast cancer

Large scale sequencing efforts to screen human tumors for somatic mutations in recent years have led to the identification of activating somatic mutations of HER2, which can occur in the absence of gene amplification[35–37]. Although these HER2 mutations are found in a variety of cancers, such as breast, colorectal and lung cancers, the overall prevalence of HER2 somatic mutations is less than 5% of all cancers[38]. Mutations in HER2 are clustered in the extracellular, transmembrane and kinase domains. Unlike other mutant oncogenes, such as BRAF or KRAS, no single mutant allele predominates and the precise distribution of HER2 mutations varies by tumor type. The most prevalent HER2 activating mutations found in breast cancer are kinase domain mutations L755S and V777L[38].

Functional characterization of HER2 mutations[36,39–41] revealed that a subset of these mutations induce ligand-independent constitutive HER2 receptor signaling and promote oncogenesis. The mechanism of these oncogenic effects seem to differ by mutation/variant, with some causing enhanced HER2 kinase activity and others causing receptor dimerization. However, one potential limitation is that most of the preclinical data that explore the functional consequences of HER2 mutations have been generated using engineered models that overexpress the mutation, thus the results may be confounded by the known oncogenic effects of HER2 overexpression. This is further enforced by recent studies[41,42] which demonstrate that HER2 mutants, when expressed at endogenous levels by gene-editing, demonstrate weak oncogenic

properties and require additional cooperating mutations in oncogenes, such as PIK3CA[41] and HER3[42], to transform cancer cells.

HER2-targeted therapies and their mechanisms of action

Given the compelling nature of HER2 as a drug target in HER2-positive breast cancer, several strategies have been adopted to target the HER2 oncogene. Currently, there are five HER2-targeted therapies that have been approved by the FDA for the treatment of early stage and/or metastatic HER2-positive breast cancer: trastuzumab and pertuzumab, both of which are monoclonal antibodies that bind to the extracellular domain of HER2; trastuzumab emtansine (also known as T-DM1), an antibody-drug conjugate comprising trastuzumab that is linked to the cytotoxic agent emtansine; lapatinib, a reversible tyrosine kinase inhibitor of EGFR/ HER2 that competes with ATP the ATP-binding site of the catalytic domain; and neratinib, an irreversible pan-HER tyrosine kinase inhibitor that interacts covalently with a conserved cysteine residue in HER family receptors (EGFR, HER2 and HER4).

Monoclonal antibodies: Trastuzumab & Pertuzumab

Trastuzumab was the first HER2-targeted therapy to be approved for adjuvant treatment for patients with HER2-positive early stage breast cancer as a monotherapy or in combination with chemotherapy[43]. The antitumor action of monoclonal antibody trastuzumab is mediated by several mechanisms following binding of the antibody to the subdomain IV of the HER2 receptor; these mechanisms include antibody-dependent cell-mediated toxicity (ADCC), inhibition of proteolytic cleavage of the extracellular domain of the HER2 receptor (to prevent formation of a residual truncated but constitutively active form of HER2, p95-HER2), inhibition

of ligand-independent HER2 receptor dimerization, inhibition of downstream PI3K-AKT and MAPK signal transduction pathways, induction of apoptosis, inhibition of angiogenesis and interference with DNA repair[44,45].

Pertuzumab binds to the extracellular dimerization domain (subdomain II) of HER2 and prevents the ligand-dependent dimerization of HER2 with EGFR or HER3 by steric hindrance[46,47]. Pertuzumab is more effective than trastuzumab in disrupting HER2-HER3 complex formation. However, unlike trastuzumab, pertuzumab is unable to inhibit ligand-independent receptor dimerization[46]. Given their complementary mechanisms of action, most preclinical studies on pertuzumab[48,49], and subsequent clinical studies[50,51], have focused on co-treatment with trastuzumab. Combining trastuzumab and pertuzumab has demonstrated synergistic inhibitory effects in clinical trials[50–52] – inhibiting cell proliferation and survival and inducing apoptosis to a greater degree than either agent alone – and has been approved for use in combination with chemotherapy in early stage (neoadjuvant and adjuvant) and metastatic HER2-positive breast cancer[43].

Antibody-drug conjugate: Trastuzumab-emtansine (T-DM1)

Trastuzumab emtansine (T-DM1) is an antibody–drug conjugate that incorporates the HER2-targeted antitumor properties of trastuzumab with the cytotoxic activity of the microtubule-inhibitory agent DM1 (derivative of maytansine); the antibody and the cytotoxic agent are conjugated by means of a stable linker[53]. T-DM1 allows intracellular drug delivery specifically to HER2-overexpressing cells, thereby improving the therapeutic index and minimizing exposure of normal tissue. T-DM1 is indicated for treatment of metastatic HER2-

positive breast cancer patients who have previously received trastuzumab and a taxane[43]. The promising results seen with the combination of pertuzumab and trastuzumab has led to clinical studies evaluating the combination of T-DM1 and pertuzumab in the neoadjuvant[54] and frontline metastatic setting[55].

Tyrosine kinase inhibitors: Lapatinib & Neratinib

Lapatinib reversibly inhibits the intracellular tyrosine kinase domains of EGFR and HER2, down-regulating phospho-HER2, phospho-HER3 and downstream PI3K-AKT and MAPK signaling to induce cell cycle arrest and apoptosis[56,57]. Lapatinib has demonstrated activity in trastuzumab-resistant cell lines in preclinical studies[57,58]. It has been approved by the FDA as a second-line treatment of patients with metastatic HER2-positive breast cancer with disease progression on trastuzumab[43] and further studies are underway to evaluate the efficacy of dual HER2 blockade by a combination of trastuzumab and lapatinib in the neoadjuvant setting[59–62].

Unlike lapatinib, neratinib exerts its anti-tumor action by binding irreversibly to the tyrosine kinase domain of EGFR, HER2 and HER4[63] through a covalent bond with a conserved cysteine residue (Cys-773 in EGFR/ Cys-805 in HER2/ Cys-803 in HER4)[64]. Its binding mode renders neratinib highly selective for HER family members[63]. Neratinib has been approved for extended adjuvant treatment of early-stage HER2-positive breast cancer following adjuvant trastuzumab treatment[65,66] and its role in the neoadjuvant[67] and metastatic[68] settings are currently under clinical investigation. Notably, neratinib has also demonstrated anti-tumor activity in breast cancers with oncogenic activating HER2 mutations in recent preclinical[36] and clinical[69] studies.

Biomarkers of response to HER2-targeted therapies

Aside from being the drug target, amplification and/or overexpression of HER2 also serve as a predictive biomarker for HER2-targeted therapies. However, the correlations among the current clinical methods of detecting HER2 amplification and/or overexpression – by fluorescent *in situ* hybridization (FISH) and immunohistochemistry (IHC) respectively – are imperfect with regards to both prognostication and the prediction of drug response to many of the HER2-targeted therapies. Given the increasing number of HER2-targeted therapy options and the innate heterogeneity of HER2-positive breast cancer, there is a critical need for the discovery and translation of additional biomarkers that predict patient response to a specific HER2-targeted therapy so as to improve patient outcomes and limit toxicity from inappropriate selection of drug therapy.

PIK3CA mutations/ Loss of PTEN

Genomic alterations along the PI3K/AKT signaling pathway leading to constitutive activation of PI3K/AKT signaling has been associated with poor response to trastuzumab[70,71]. In particular, mutations of phosphatidylinositol-4,5- bisphosphate 3-kinase catalytic subunuit alpha isoform (PIK3CA) and PTEN mutation/ loss of expression – which occurs in 42% and 19% of HER2-enriched tumors respectively[35] – have been associated with trastuzumab resistance in the metastatic setting[72]. In the neoadjuvant setting, patients without PIK3CA mutations appear to have better pathologic complete response rates (pCR) to dual HER2 blockade by a combination of trastuzumab and pertuzumab[73] or trastuzumab and lapatinib[74,75].

Overexpression/ activation of other receptor tyrosine kinases

Overexpression of other receptor tyrosine kinases outside the HER family, such as IGF1R[49] and MET[76], has been associated with trastuzumab resistance in HER2-positive breast cancer. *In vitro* experiments[49] demonstrated that overexpressed IGF1R could be recruited into signaling complexes with HER2 and HER3, offering a bypass mechanism to activate PI3K-AKT signaling despite HER2 inhibition by trastuzumab. The association between overexpression of IGF1R and trastuzumab resistance was also observed in HER2-positive metastatic breast cancer patients[77].

Intrinsic molecular subtypes

Accumulating evidence suggests that the intrinsic molecular subtypes might provide predictive value to HER2-targeted therapies. The PAM50 gene expression classifier[26,27] identified all 4 main intrinsic molecular subtypes of breast cancer (Luminal A, Luminal B, HER2-enriched, Basal-like) in HER2-positive breast cancer[78,79]. Among which, the HER2-enriched (HER2-E) molecular subtype predominates (~50-60%)[78]. Given that HER2-positive tumors of the HER2-E subtype have the highest activation of HER2/ EGFR signaling pathway[78], HER2+/HER2-E tumors have been postulated to derive the most benefit from HER2-targeted therapies. This was demonstrated in 4 neoadjuvant trials (NeoALTTO[80], CALGB40601[81], NOAH[82] and CHER-LOB[83]), in which HER2+/HER2-E subtypes achieved a higher pCR to HER2-targeted therapies in combination with chemotherapy compared to other subtypes. The predictive value of HER2+/HER2-E subtype was further supported by findings in the PAMELA phase II neoadjuvant trial[84] designed specifically to test this hypothesis. In the PAMELA study[84], 151 patients with stage I-III HER2-positive disease were treated with neoadjuvant trastuzumab

and lapatinib. Patients with HER2+/HER2-E tumors achieved a higher pCR rate than patients with HER2+/non-HER2-E tumors (41% vs. 10%; OR: 6.2), confirming the higher sensitivity of HER2+/HER2-E to HER2-targeted therapies. Larger clinical studies[82] are currently underway to validate HER2-E subtype as a predictive biomarker. These findings highlight the potential of further investigating molecular subtypes within HER2-positive breast cancer to identify subsets of patients who are more likely to benefit from HER2-targeted therapies.

Immune-related biomarkers

The relevance of the immune system in the activity of HER2-targeted monoclonal antibodies trastuzumab and pertuzumab has prompted investigation of the use of immune status to identify patients who are likely to benefit from these therapies. In the NCCTG N9831 trial[55], patients with tumors that had increased expression of a subset of 14 immune-related genes – enriched in genes related to chemokine signaling and inflammation – were associated with increased relapse-free survival when treated with adjuvant trastuzumab. Subsequent application of this 14-gene immune signature in the NeoALTTO trial also associated with higher pCR in patients receiving a combination of trastuzumab and lapatinib in the neoadjuvant setting[80]. The potentially critical role that the immune system plays in modulating response to HER2-targeted therapies was further supported by findings in the NeoSphere trial[85], in which low expression of immune genes and metagenes such as MHC1 – known to inhibit antibody-dependent cell-cytoxicity – was associated with higher pCR to the neoadjuvant treatment with the trastuzumab and pertuzumab combination.

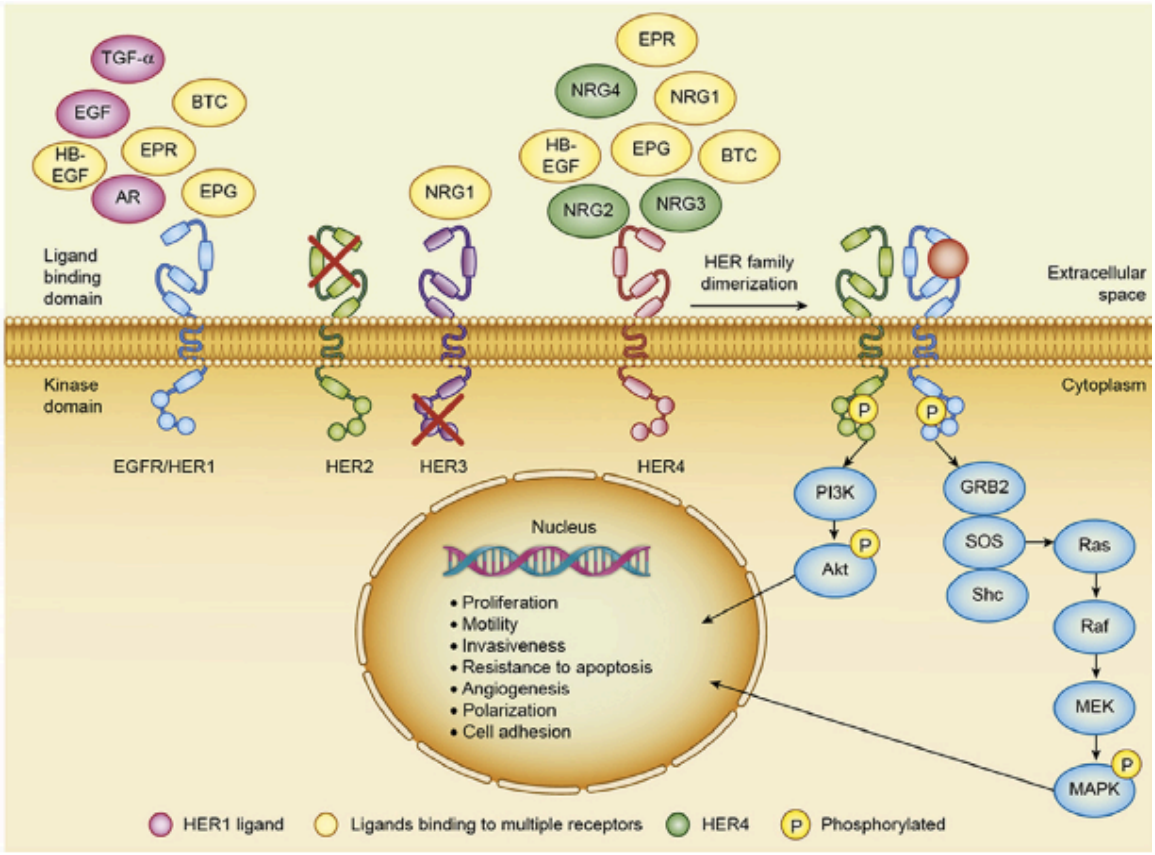
Conflicting findings have been observed in neoadjuvant and adjuvant studies investigating the value of tumor infiltrating lymphocytes (TILs) in prediction of response to HER2-targeted therapies. While high TILs were significantly associated with a higher pCR to neoadjuvant trastuzumab and/or lapatinib in combination with chemotherapy in the CHER-LOB study[83], this association was not observed in the NeoALTTO[80] and NeoSphere[85] trials. A possible explanation could be that TILs do not recapitulate the complex interplay between the immune system and tumor as comprehensively as immune gene signature, and are thus less effective in predicting drug response[86]. Many ongoing in-depth evaluations into specific types or composition of immune cells in the TILs may further shed light on the complexity of tumor-immune interactions.

Overarching goal of thesis research

In the following chapters of this thesis, we will report our findings from our studies designed to evaluate BluePrint molecular subtypes (Chapter 2) and phosphoproteomic/proteomic markers (representing different hallmarks of cancer) (Chapter 3) as potential biomarkers of response to different HER2-targeted therapies. The overarching goal of my research described in this thesis is to identify novel genomic and proteomic biomarkers that would complement HER2 overexpression and/or amplification in predicting patient response to HER2-targeted therapies, thereby enabling better patient selection of patients who are most likely to respond and benefit from HER2-targeted therapies, and also prevent adverse events from unnecessary drug exposure in non-responders. The transcriptomic and proteomic/ phosphoproteomic techniques employed in the studies also provide a unique opportunity to explore a broad multi-omic view to identify predictive markers that could transcend conventional subtypes or protein networks/pathways

linked to the HER family, and thus allow us to expand our knowledge of the biological mechanisms underlying differential drug responses and resistance to HER2-targeted therapies.

Figure 1.1 | Heterodimer formation of members of the HER family and downstream signaling via the PI3K/AKT and MAPK pathways. Signaling downstream of HER family activation is dependent on heterodimerization/ homodimerization of the HER family member(s) triggered by ligand binding to the extracellular ligand-binding domain of EGFR, HER3 or HER4. The extracellular domain of HER2 is unable to bind any known natural ligand and it adopts a fixed conformation resembling a ligand-activated state that allows for dimerization. Phosphorylation of the HER intracellular kinase domains (with the exception of HER3 which does not have a functional kinase domain) initiates a downstream cascade. The signaling events by the HER receptors promote cellular proliferation and survival, and other physiological processes required for carcinogenesis. Figure is adapted from Parakh, S. et. al. Evolution of anti-HER2 therapies for cancer treatment. *Cancer Treatment Reviews*; **59**: 1-21.



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Chapter 2:

Luminal subtype predicts non-response to HER2-targeted therapies in HER2+HR+ (triple positive) I-SPY 2 breast cancer patients

Introduction

The human epidermal growth factor receptor 2 (HER2) oncogene is amplified and/or overexpressed in 15-20% of invasive breast cancers[1]. Several HER2-targeted agents such as trastuzumab, lapatinib, pertuzumab, ado-trastuzumab emtansine (T-DM1) and neratinib have been developed; these agents have gained FDA approval for clinical use either as a single HER2-targeted agent or, in the case of pertuzumab, for use in combination with trastuzumab in early-stage and/or metastatic HER2-positive (HER2+) breast cancer[2].

To date, the selection of patients most likely to respond to HER2-targeted agents is based primarily on the detection of overexpression of HER2 at the protein level by immunohistochemistry (IHC), and/or amplification of the number of HER2 gene copies at the chromosome level by fluorescent *in situ hybridization* (FISH)[3,4]. However, not all patients with HER2 gene amplification and/ or protein overexpression respond to HER2-targeted therapies.

An increasing amount of genomic and clinical data suggests that HER2+ tumors have distinct molecular and clinical profiles according to estrogen receptor (ER) status. In several neoadjuvant clinical trials[5–7], HER2+/hormone receptor-positive (HR+) patients had lower rates of pathologic complete response (pCR) to single and/or dual HER2-targeting therapies compared to HER2+/HR-negative (HR-) patients. The interplay between HER2 and ER pathways in

HER2+/HR+ tumors could lead to dominance of one signaling pathway over the other, influencing drug response. This is evident in the HERA study[8] in which HER2+/HR+ patients with lower HER2 FISH ratios and high ER expression derived less benefit from adjuvant trastuzumab, suggesting that HER2+/HR+ patients with dominant estrogen signaling may be less likely to benefit from these HER2-targeted therapies.

Aside from expression of conventional receptors, the heterogeneity of HER2+/HR+ patients may manifest in different molecular subtypes. Using the 80-gene BluePrint (BP) gene expression subtype signature[9], the NBRST neoadjuvant breast cancer study identified two BP molecular subtypes within the HER2+/HR+ population with differential response to dual HER2-targeting with pertuzumab and trastzumab; BP-Luminal subtype patients had a more pronounced improvement in response rate with addition of pertuzumab to trastuzumab compared to BP-HER2 subtype patients[10]. This suggests that BP molecular profile may have clinical utility in identifying a subset of HER2+/HR+ patients who may benefit from the addition of pertuzumab to trastuzumab-based regimen.

In this study, we used patient data from the I-SPY 2 TRIAL to investigate whether further stratification of HER2+/HR+ patients by BP subtype might lead to improved response prediction across 6 HER2-targeted treatment arms. The I-SPY 2 TRIAL is a multi-center Phase 2 adaptive trial for women with high-risk clinical stage II or III breast cancer[11]. The trial is designed to screen multiple experimental regimens in addition to standard neoadjuvant chemotherapy, with complete pathologic response (pCR) i.e. no invasive cancer left in the breast or lymph nodes as the primary endpoint (Figure 2.1a). The six treatment arms opened for enrollment to HER2+

patients between 2010 to mid 2016 in the I-SPY 2 TRIAL included 3 investigational agents that target the HER2-pathway – neratinib (N), pertuzumab/trastuzumab (P/H) and TDM-1/P, the (trastuzumab) control arm, and two additional investigational agents given in combination with trastuzumab – the AKT inhibitor MK2206 (MK2206/H) and the angiopoietin-1/-2 neutralizing peptibody AMG-386 (AMG386/H) (Figure 2.1b). The efficacy data for the neratinib arm (versus control) has been published by Park et. al.[12]; the other arms of the trial have all been presented as abstracts at recent meetings[12–14]. Pretreatment biopsies from patients were classified by BP molecular subtyping in addition to conventional receptors in the trial. The utility of BluePrint in predicting response to these HER2-targeted therapies, aside from H and P/H, is currently untested. The aim of the current study is to evaluate BP subtype as a predictor of response in the HER2+/HR+ population as a whole and within each of the HER2-targeted treatment arms, including P/H, in the I-SPY 2 HER2+/HR+ patients. In addition, we also performed differential gene expression analysis to assess gene and pathway-level differences between BP molecular subtypes within the HER2+/HR+ subset.

Results

BluePrint subtype classification within conventional receptor groups in I-SPY2 cohort

A total of 508 patients were enrolled and randomized across 6 treatment arms – neratinib, veliparib/carboplatin, AMG386, MK2206, pertuzumab, T-DM1/pertuzumab – in the I-SPY 2 trial between 2010 to mid 2016 (Figure 2.1b). There were 132 patients concurrently randomized to the control arm treated with standard chemotherapy, with the addition of trastuzumab for patients with HER2+ disease.

The BluePrint (BP) subtypes were determined for all 640 patients. Figure 2.2 shows the distribution of BP subtypes within the 4 conventional receptor groups. As expected, nearly all (n = 209/210) of the triple negative patients were classified as BP Basal-type. Also consistent with expectation, the HR+/HER2- patients are predominantly BP Luminal-type (n = 128/186; 69%) and the HER2+/HR- patients are predominantly BP HER2-type (n = 69/88; 78%); however both contain a sizeable minority of BP Basal-type cancers (Figure 2.2). Interestingly, although all HER2+/HR+ tumors (n=156) are HER2+ by FISH and/or IHC, 37% (n = 58/ 156) were classified as BP Luminal-type.

In this study, our analysis is focused on the triple-positive (HER2+/HR+) patients who received at least one HER2-targeted agent in the trial. Of these 156 HER2+/HR+ patients, 42 were randomized to the neratinib arm (N), 29 to the pertuzumab and trastuzumab arm (P/H), 35 to the T-DM1 and pertuzumab arm (T-DM1/P), 16 to the MK2206 and trastuzumab (MK2206/H) arm and 15 to the AMG386 and trastuzumab arm (AMG386/H) and (Figure 2.3).

There were 19 concurrently randomized HER2+/HR+ controls treated with trastuzumab (H) and standard chemotherapy (H+T → AC).

HER2+/HR+ BP Luminal-type is associated with lower pCR rates than HER2+/HR+ BP HER2-type patients

We limited our analysis to patients with BP Luminal-type and BP-HER2-type tumors (n=152) since the number of HER2+/HR+ BP Basal-type patients (n = 4) was too small for meaningful evaluation. In this population as a whole, combining all treatment arms, HER2+/HR+ BP HER2-type patients were more likely to achieve a pCR compared to BP Luminal-type patients (51% vs. 12%)(Figure 2.4a, left). This translates to an odds ratio of 7.50 between BP HER2-type and BP Luminal-type patients (Fisher Exact test p-value = 7.29E-07) (Figure 2.4a, left). A similar trend was observed within the subset of patients in the 4 treatment arms with experimental HER2-targeted therapies (N, P/H, T-DM1/P) and concurrent controls (H); HER2+/HR+ BP HER2-type patients had a higher pCR rate compared to the BP Luminal-type patients (57.7% vs 8.0%; OR = 15.4; p = 1.04E-08)(Figure 2.4a, right).

Although the number of patients in each individual treatment arm is small, we observed a higher pCR rate in HER2+/HR+ BP HER2-type compared to HER2+/HR+ BP Luminal-type patients in all arms with the exception of the MK2206/H treatment arm, as shown in the bar plot in Figure 2.4b.

Differential Gene Expression between HER2+/HR+ BP Luminal and BP HER2 subtypes

To further investigate the potential biological underpinnings of differential response between the two BP subtypes, we performed a differential gene expression analysis. Using a 5% FDR ($Q < 0.05$), our analysis marked 57 of the 75 BluePrint genes in our dataset as statistically different between the 2 BP subtypes in the I-SPY2 cohort (Figure 2.5). As expected, genes associated with ER signaling such as ESR1, PGR and FOXA1 were more highly expressed in HER2+/HR+ BP Luminal-type patients than BP HER2-type patients. Similarly, HR+/HER2+ BP HER2-type patients had higher expression levels of genes associated with ErbB2 signaling such as ERBB2 and GRB7 (Figure 2.5).

In our whole-genome analysis, 2052 genes were differentially expressed between HR+/HER2+ BP HER2-type and BP Luminal-type patients ($Q < 0.05$). Relative to BP Luminal-type patients, 945 genes were upregulated, and the other 1107 genes were expressed at lower levels in BP HER2-type patients. Pathway enrichment analysis of the differentially expressed genes using DAVID software showed significant enrichment in a broad array of biological processes (Table 2.1), including immune-related pathways, such as inflammatory response and regulation of T cell proliferation. Further analysis revealed that HR+/HER2+ BP HER2-type patients demonstrated higher expression levels of immune-related genes, such as CTLA4 and ITGB2, compared to BP Luminal-type patients.

Differences in HER2 amplification/ copy number between the BluePrint subtypes

The twenty most highly upregulated genes in each subtype are presented in Table 2.2. Notably, the top 8 most up-regulated genes in the BP HER2-type patients are found on the HER2

amplicon located on Chromosome (Chr) 17 q12[15], including ERBB2/ HER2. This led us to evaluate whether there is a difference in HER2 copy number amplification and/or protein expression between the two BP molecular subtypes. Of the 101 HER2+/HR+ patients with detailed HER2 IHC results available, a higher proportion of BP Luminal-type patients (25/42) had equivocal HER2 results i.e. IHC 2+ as compared to BP HER2-type patients (1/59) (Figure 2.6a). At the chromosome level, the median FISH HER2 to chromosome enumeration probe 17 (HER2/CEP17) signal ratios of the BP HER2-type patients is significantly higher than that of the BP Luminal-type patients (7.4 vs. 2.4, p = 5.449e-08) (Figure 2.6b).

Discussion

In this study, we evaluated BluePrint subtype as a predictor of response in the HER2+/HR+ population as a whole and within each of the HER2-targeted treatment arms, in the I-SPY 2 TRIAL. Our results suggest that HER2+/HR+ BP Luminal-type patients show lower response rates to HER2-targeted agents compared to HER2+/HR+ BP HER2-type patients, with the exception of MK2206/H. HER2+/HR+ BP HER2-type patients had a pronounced improvement in response rate with the addition of pertuzumab to trastuzumab (H: 20% vs. P/H: 76.5%). On the other hand, we did not observe a significant improvement in pCR rates (H: 7.7% vs. P/H: 8.3%) with the addition of pertuzumab to trastuzumab in HR+/HER2+ BP Luminal-type patients, unlike the findings from the NBRST study[10], in which the treatment benefit from dual therapy was observed in both HER2+/HR+ BP HER2-type and BP Luminal-type patients.

Relative to the HER2+/HR+ BP HER2-type patients, HER2+/HR+ BP Luminal-type patients were associated with lower HER2 protein expression levels (higher proportion with IHC 2+ staining) and correspondingly lower FISH HER2/CEP17 ratios, and higher estrogen receptor (ESR1) expression. Our results are consistent with the findings from the HERA trial[8] in which HER2+/HR+ breast cancer patients with lower FISH ratio (≥2 to <5), or with higher ESR1 expression, derived less benefit – shorter disease-free survival – from adjuvant trastuzumab. Collectively, these findings support the notion that a subset of HER2+/HR+ patients – defined by relatively lower HER2 expression/ amplification and higher ESR1 expression – that may be less likely to benefit from HER2-targeted therapies, and also highlight the potentially important role estrogen receptor pathway may play in mediating poor response to HER2-targeted therapies in these patients. The exact biological mechanisms underlying this observation are unclear, though

previous studies[16,17] suggest that estrogen receptor may mediate poor response to HER2-targeted therapies through activation of various receptor tyrosine kinases such as IFG1R[18,19], providing an escape mechanism to evade HER2 inhibition.

Pathway enrichment analysis revealed a higher expression of immune-related genes in the HER2+/HR+ BP HER2-type patients compared to BP Luminal-type patients; this suggests that immunologic activity in BP HER2-type tumors may in part account for higher pCR rates in response to neoadjuvant HER2-therapies observed in this subset of patients. Association between immune signals and response to HER2-targeted therapies has been recently observed in other neoadjuvant and adjuvant trials. In the NCCTG N9831 trial[20], patients with tumors that had increased expression of a subset of 14 immune-related genes were associated with increased relapse-free survival when treated with adjuvant trastuzumab. Accordingly, immune gene signatures significantly correlated with pCR in patients receiving neoadjuvant trastuzumab and/or lapatinib in the NeoALTTO[21] and CherLOB[22] studies. The potentially critical role the immune system plays in modulating response to HER2-targeted therapies is further reinforced by the findings in the NeoSphere trial[23], in which lower expression of immune genes like MHC1 – known to inhibit antibody-dependent cell-mediated cytotoxicity[24,25], an important mechanism of action of P/H – was linked with higher pCR to neoadjuvant P/H combination.

Comparing the BP subtypes to the research-based PAM50 subtype predictor (intrinsic subtypes)[26–28] revealed significant concordance between the two molecular subtype schemas in I-SPY 2 HER2+/HR+ patient subset (Chi-squared test p = 3.52E-11) (Table 2.3). However, there are differences between the BP Luminal-type and PAM50 Luminal A/B intrinsic subtypes. Though nearly all HER2+/HR+ BP Luminal-type tumors were classified as Luminal A or

Luminal B by PAM50, the converse is not true for the HER2+/HR+ Luminal A/B intrinsic subtypes; HER2+/HR+ intrinsic Luminal tumors (Luminal A and Luminal B as a group) were almost evenly distributed between BP Luminal-type and BP HER2-type.

The I-SPY2 TRIAL design[11] presents both opportunities and challenges for evidence-based biomarker testing. On one hand, the biomarker-rich nature of the trial provides an excellent resource to investigate the molecular correlates of response and resistance. On the other hand, as the I-SPY 2 TRIAL was designed to efficiently evaluate multiple novel regimens compared to a shared control arm for future Phase III testing, the sample sizes of each of the HER2-targeted treatment arms available for our study is relatively small. This may limit our ability to draw definitive conclusions; hence our findings would require further exploration and/or validation in future trials.

In summary, our analysis demonstrates the potential clinical utility of BluePrint molecular subtyping in helping to identify/ define a subset of HER2+/HR+ patients who are less likely to benefit from neoadjuvant HER2-targeted therapies. These HER2+/HR+ BP Luminal patients may potentially benefit from alternative therapeutic strategies such as the addition of neoadjuvant endocrine therapy. Moreover, given the important role of immune signaling in modulation of response to HER2-targeted therapies, HER2+/HR+ BP Luminal-type patients may also benefit from future clinical trials designed to evaluate therapeutic approaches that might enhance the immune activity within tumors and thereby sensitize it to HER2-targeted therapies; an example of such a strategy would be to attenuate immune-suppressive signaling pathways by targeting PD-1 receptor and/or its ligand PD-L1.

Translational Relevance

HER2-positive breast cancer is biologically heterogeneous and not all patients benefit from HER2-targeted therapies to the same extent. In this study, we provide clinical evidence suggesting that a subset of triple positive HER2+/HR+ breast cancer patients – characterized by lower HER2 protein expression levels and/or gene amplification and higher ESR1 expression – may derive less benefit from HER2-targeted therapies. We also demonstrated the potential clinical utility of BluePrint – a gene expression-based molecular subtype classification – in identifying this aforementioned subset of HER2+/HR+ breast cancer patients (with poor response to HER2-targeted therapies), and in providing additional and useful information of response to HER2-targeted therapies beyond that provided by standard pathologic markers. Further investigation of the clinical value of BluePrint in the predicting response to HER2-targeted therapies is warranted.

Methods

Patients

In the I-SPY 2 TRIAL (NCT01042379)[11], HER2+ patients were randomized to receive standard chemotherapy and trastuzumab (paclitaxel and trastuzumab followed by doxorubicin/ cyclophosphamide) or one of the HER2-targeted investigational agents in combination with standard chemotherapy (Figure 2.1a). The HER2-targeted investigational agent may be used in place of trastuzumab or in combination with trastuzumab. Patients enrolled in 5 of the investigational arms receiving HER2-targeted agents and concurrent control arms were included in this study. The 5 investigational arms included are: i) neratinib (N), ii) pertuzumab and trastuzumab (P/H), iii) T-DM1 and pertuzumab (T-DM1/P), iv) MK2206 and trastuzuamb (MK2206) and v) AMG386 and trastuzumab (AMG386). Core biopsy was performed during screening, prior to enrollment and randomization, to obtain tumor samples.

Assessments

I-SPY 2 patients' 16-gauge needle biopsies were received from participating hospitals at the I-SPY 2 UCSF laboratory. Frozen sections were then stained with hematoxylin and eosin before being reviewed by an experienced breast pathologist. To ensure sufficient tumor volume for microarray analysis, all samples included in this study showed a tumor cell percentage of at least 30%. Subsequently, expression-array profiling of the frozen tumor samples was performed at the centralized Agendia laboratory (Irvine, CA) on full-transcriptome expression arrays. RNA isolation, labeling and hybridization were performed as described previously[29]. RNA was hybridized to the custom-designed diagnostic chip, each containing oligonucleotide probes for the profiles in triplicate or more. Fluorescent intensities on scanned images were quantified and

normalized using Feature extraction software (Agilent Technologies, Santa Clara, USA). Full transcriptome expression microarray data was received by the I-SPY 2 Program Management Office and used for this study.

The 80-gene BluePrint molecular subtype assignments were derived from the pre-treatment biopsies. BP identifies functional molecular subtypes based on the expression levels of 80 genes that discriminate between 3 breast cancer subtypes: Luminal-type, HER2-type and Basal-type[9].

ER status was assessed on formalin-fixed paraffin-embedded tissue blocks by IHC. Pre-treatment tumor biopsies were classified as ER-positive when ≥1% invasive tumor cells showed definitive nuclear staining, irrespective of staining intensity. HER2 expression was evaluated by immunohistochemistry (IHC), and/ or fluorescent in-situ hybridization (FISH). Cases were considered HER2-positive if scored 3+ by IHC and/or amplified by FISH (dual probe HER2/CEP17 ratio >= 2 or <2 but with an average HER2 copy number >= 6 signals/cell) in accordance to the ASCO-CAP HER2 testing guidelines[3,4].

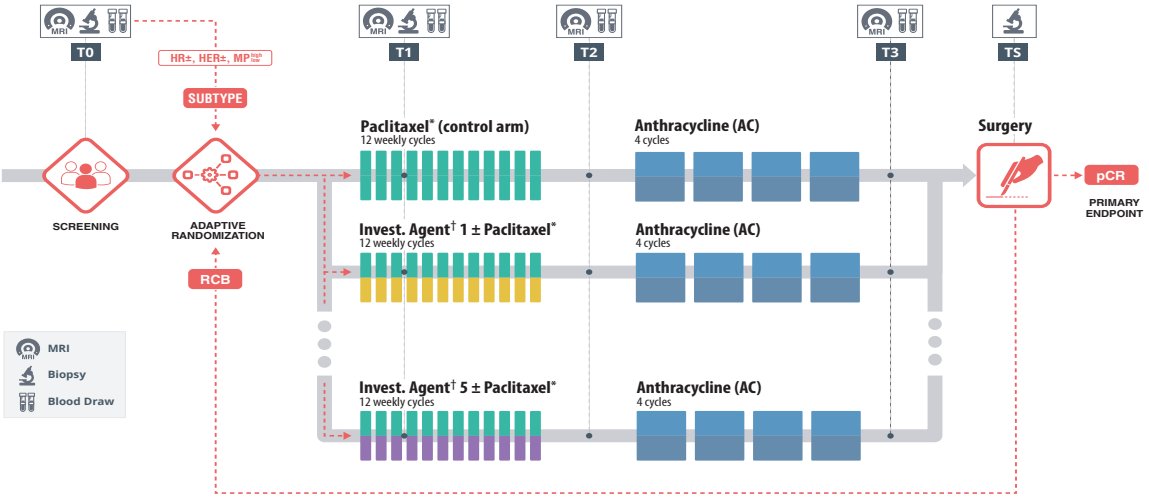
Statistical analysis

The R programming environment (version 3.3.3) was used to process raw data, perform statistical calculations, and perform differential expression analysis. We assessed the association between BP subtypes and response rate i.e. pCR using Fisher's exact test. For pre-processing of the gene expression data, Agilent gene expression arrays were quantile-normalized, log transformed, and median-centered. To identify genes expressed differentially between

HER2+/HR+ BP-HER2 and BP-Luminal tumor samples, we applied a Wilcoxon rank sum test and fitted a logistic model i.e. BP subtype ~ gene. Benjamini-Hochberg (BH) multiple testing correction was applied to the p-values to control for false discovery, and genes were identified as being significantly associated to BP subtype classification if BH $p<0.05$ from both tests. We then performed pathway enrichment analysis of significant genes using DAVID software tool (version 6.8)[30,31]. Our study is exploratory and does not adjust for multiplicities of other biomarkers in the trial outside this study.

Figure 2.1 | a) I-SPY 2 TRIAL (Investigation of Serial Studies to Predict Your Therapeutic Response with Imaging and Molecular Analysis 2) schematic. The I-SPY 2 TRIAL is a multi-center Phase 2 adaptive standing platform trial for women with high-risk clinical stage II or III breast cancer. The trial is designed to screen multiple experimental regimens in addition to standard neoadjuvant chemotherapy, with pathologic complete response (pCR) i.e. no invasive cancer left in the breast or lymph nodes as the primary endpoint. This trial is adaptive, in that a patient randomized to receive experimental treatment is assigned preferentially to the arm where her cancer subtype is most likely to respond. Subtype is defined by hormone receptor (HR) status, HER2 status and MammaPrint (MP) risk status of pretreatment biopsies (T0). The goal of I-SPY 2 is to identify (graduate) regimens with >85% predicted probability of succeeding in a 1:1 randomized 300-patient phase 3 trial where pCR is the endpoint, in the signatures defined by HR, HER2, and MP where the drug is most effective (graduates). (*) HER2-positive patients also receive trastuzumab. An investigational agent may be given in place of trastuzumab e.g. neratinib. AC, doxorubicin/cyclophosphamide; HER2, human epidermal growth factor receptor 2; MRI, magnetic resonance imaging. **b)** Timeline and graduating biomarker signatures of the six I-SPY 2 investigational arms and their concurrent controls between 2010 – mid 2016. HER2-positive patients were recruited in all the arms shown here, with the exception of the veliparib + carboplatin arm. The graduating biomarker signature(s) in which the investigational regimen graduated in is annotated on the right side of each regimen.

A



*HER2-positive participants also receive Trastuzumab. An investigational agent may be used instead of Trastuzumab.

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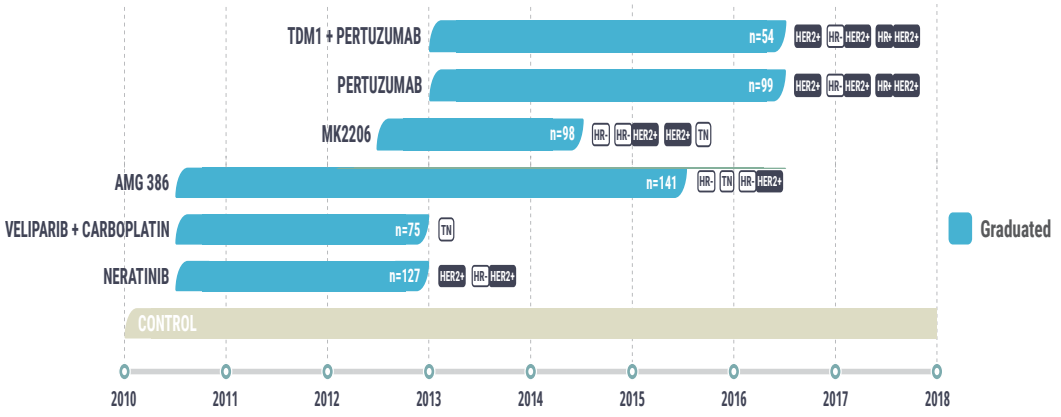

Figure 2.2 | Distribution of BluePrint molecular subtypes within all four conventional FISH/IHC receptor groups – HR+/HER2-, Triple Negative, HR+/HER2-, HER2+/HR+ – for 640 patients across 6 investigational arms (and concurrent controls) recruited to the I-SPY 2 TRIAL between 2010 to mid-2016.

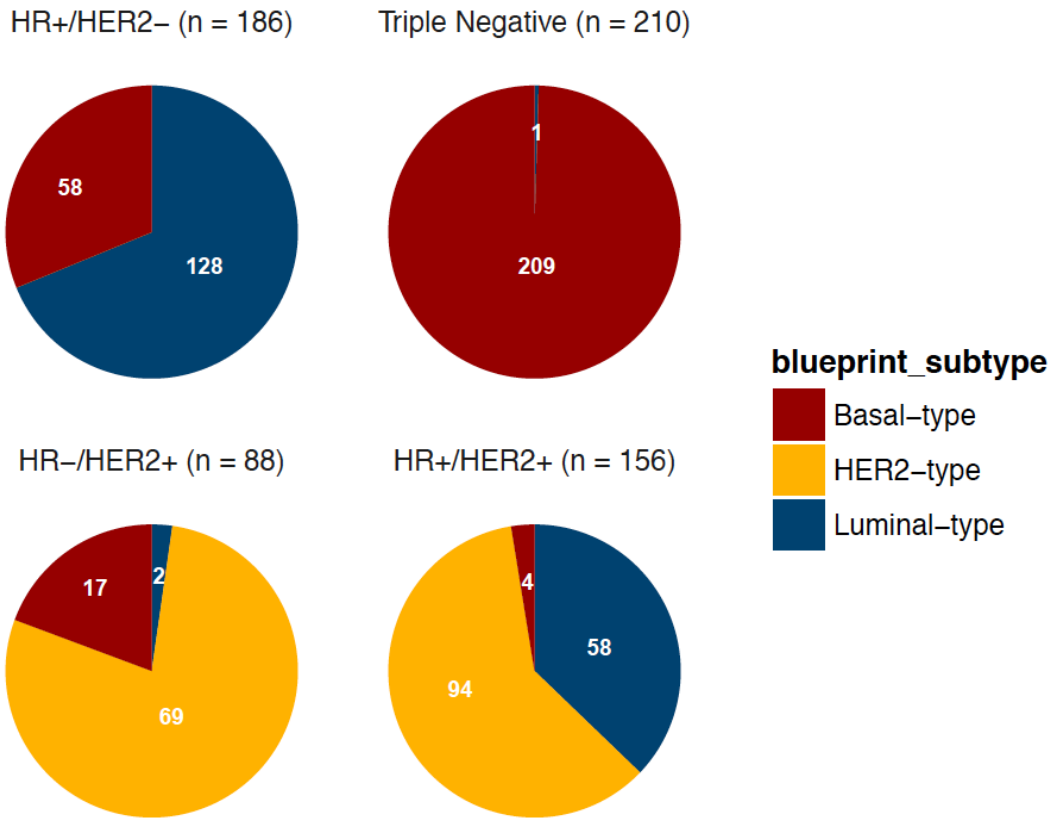

Figure 2.3 | CONSORT diagram that outlines the number of HER2+/HR+ patients in each treatment arm (receiving at least one HER2-targeted agent) in the I-SPY 2 TRIAL and their respective BluePrint subtype classifications. The four HER2+/HR+ patients classified as BP Basal-type were excluded from subsequent analysis. IHC, immunohistochemistry; FISH, fluorescent *in situ* hybridization; pCR, pathological complete response; BP, BluePrint.

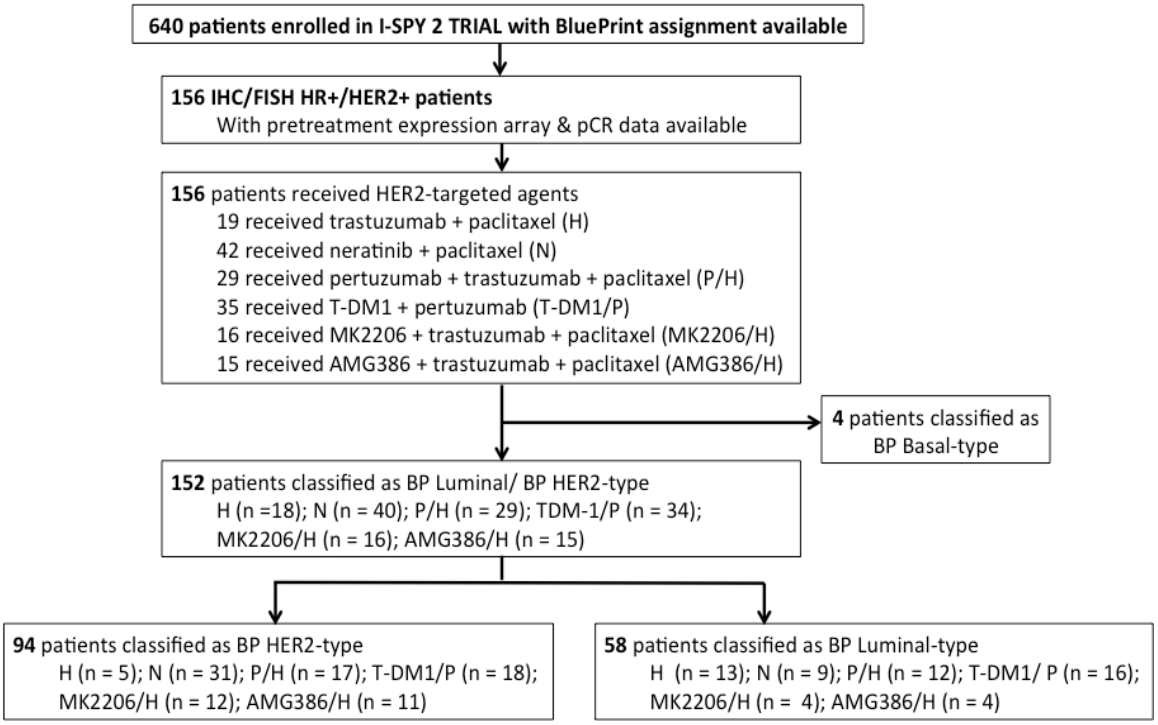

Figure 2.4 | HER2+/HR+ BP Luminal-type patients were associated with lower pathological complete response (pCR) rates, as compared to BP HER2-type patients. a) Mosaic plots of pCR distribution by BluePrint (BP) subtype across all 5 investigational treatment arms with HER2-targeted therapies (N, P/H, T-DM1/P, AMG386/H, MK2206/H) and concurrent controls (H) (left) and across 4 treatment arms with exclusively HER2-targeted therapies (H, N, P/H, T-DM1/P) (right). The odds ratio of pCR by subtype with Fisher's Exact test p-value is indicated below each of the mosaic plots. **b)** Bar plot of the pCR rates of HER2+/HR+ patients by treatment arm and BluePrint subtype. The number of patients in the H, N, MK2206/H and AMG386/H arms are too small for statistical assessment.* P<0.01 (Fisher's Exact test).BP, BluePrint; H, trastuzumab; N, neratinib; P, pertuzumab; T-DM1, trastuzumab-emtansine.

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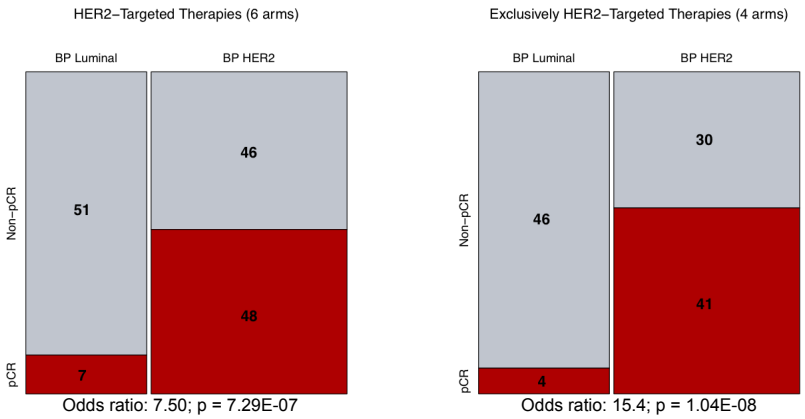

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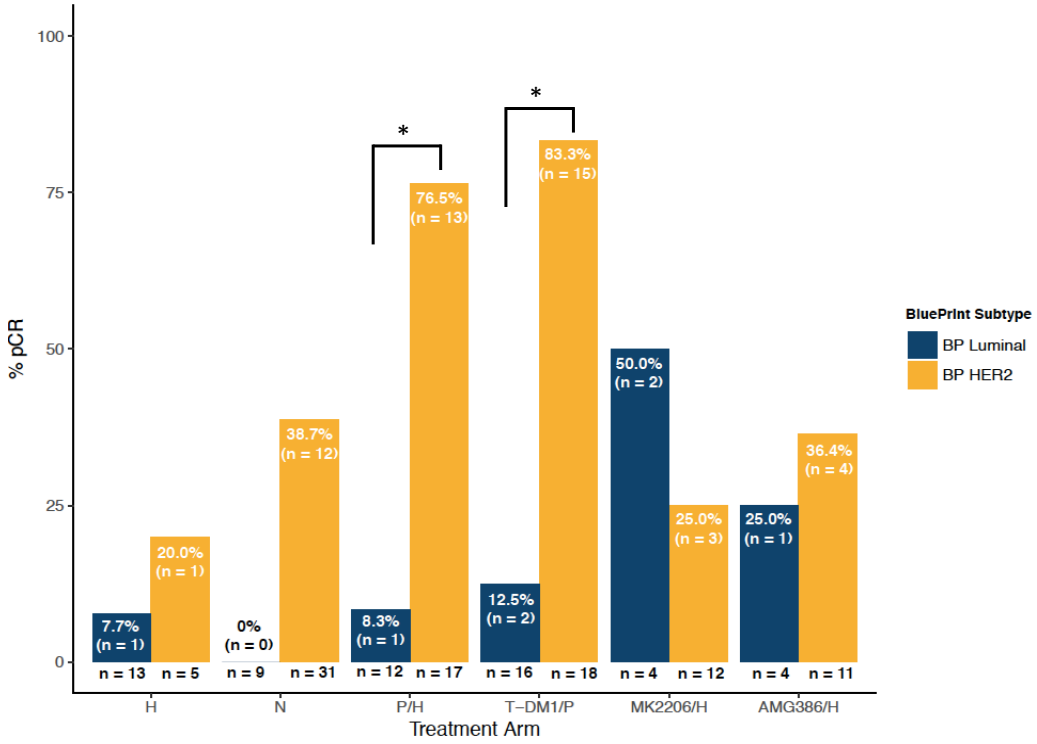

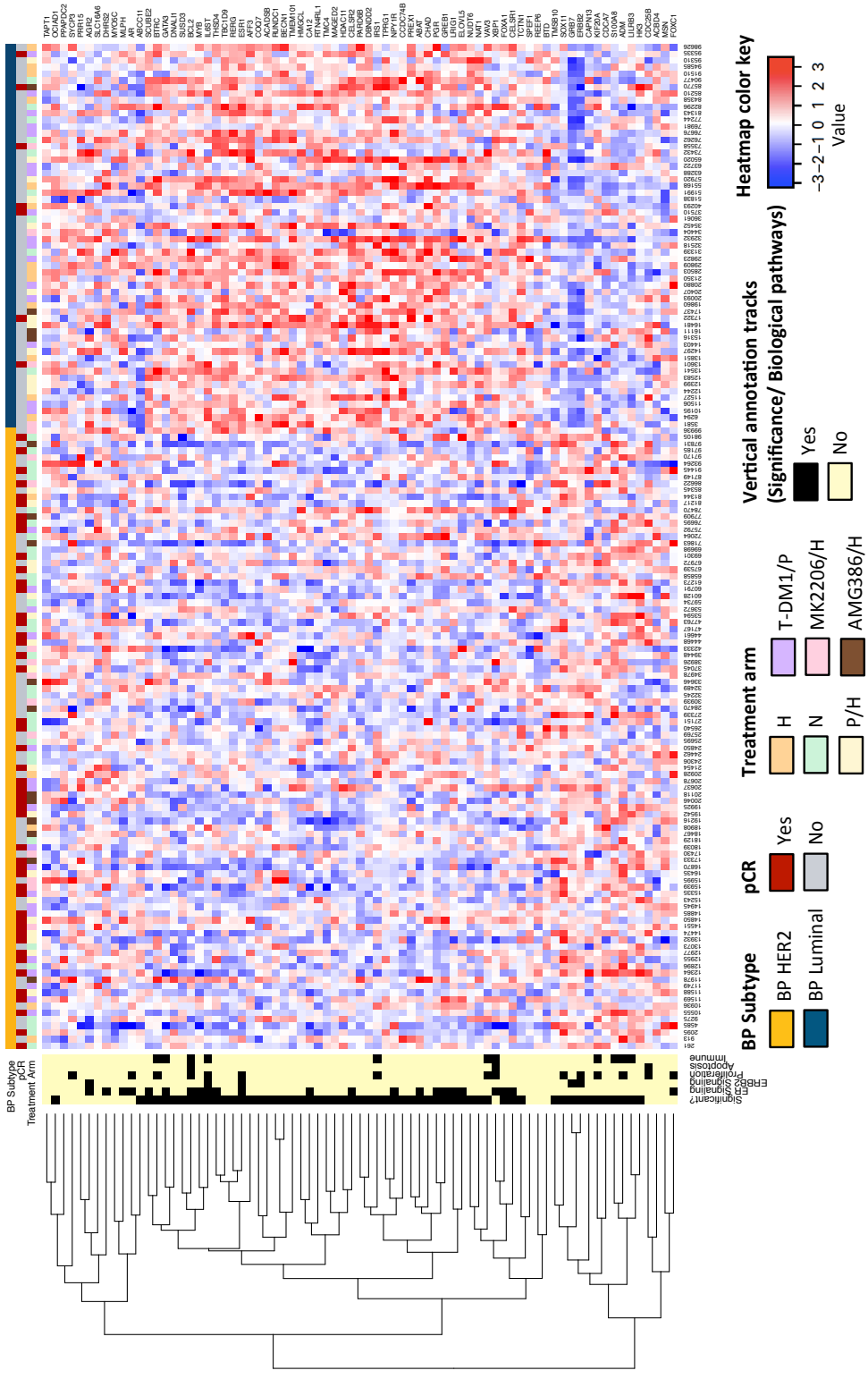

Figure 2.5 | Heatmap of gene expression levels of the 75 BluePrint genes in the 152 I-SPY 2 HER2+/HR+ patients included in this study. Patient data are arranged by BluePrint (BP) subtype along columns, and genes are hierarchically clustered along rows. Our analysis marked 57 of the 75 BluePrint genes as statistically different between the 2 BluePrint subtypes; these genes are marked by the black squares in the first column of the vertical annotation track on the left. The most prevalent biological pathways represented by these genes are included in the vertical annotation track (black: yes; cream: no). pCR, pathologic complete response; ER: estrogen receptor; ERBB, erythroblastic oncogene B.

Figure 2.6 | HER2 protein expression and/or copy number amplification differences between the two BluePrint (BP) molecular subtypes. **a)** Mosaic plot showing the distribution HER2 immunohistochemistry (IHC) staining results by BluePrint subtype. **b)** Boxplot showing the distribution of HER2 to chromosome enumeration probe 17 (HER2/CEP17) signal ratios by fluorescent in situ hybrization (FISH) within BP Luminal and BP HER2 subtypes. The color of the dots represent different HER2 IHC staining results. A blue dot represents HER2 IHC 3+; a green dot represents HER2 IHC 2+; and a red dot indicates that the HER2 IHC result was not reported. P<0.01 (unpaired t-test)

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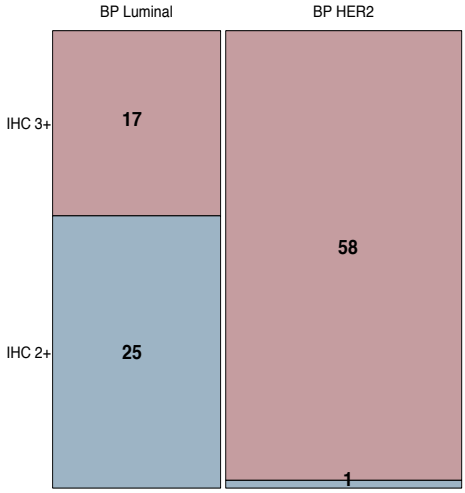

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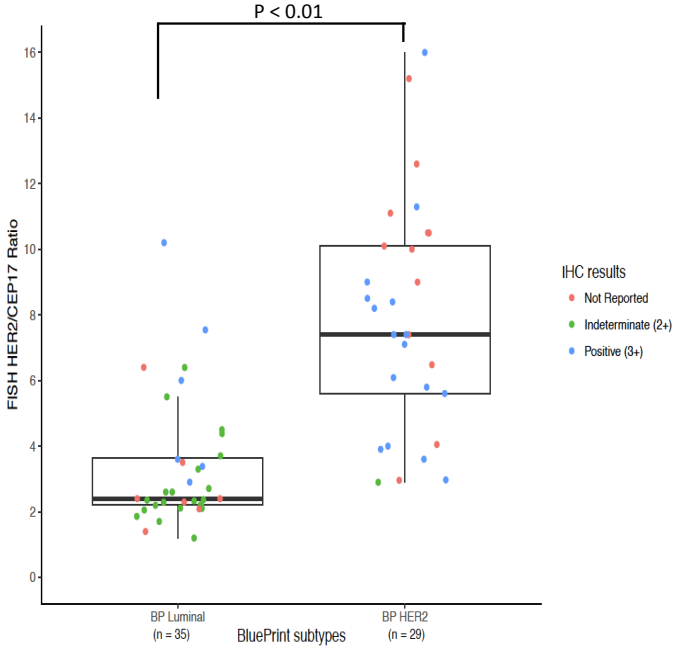

Table 2.1 | Biological processes/ pathways that showed significant enrichment based on DAVID functional enrichment analysis of the differentially expressed genes between BluePrint (BP) Luminal-type and BP HER2-type tumors. Immune-related pathways are highlighted in red.

Category	Term	p-value	Adjusted p-value
GOTERM_BP_DIRECT	negative regulation of T cell proliferation	3.60E-07	1.50E-03
GOTERM_BP_DIRECT	inflammatory response	7.30E-06	1.50E-02
KEGG_PATHWAY	Cytokine-cytokine receptor interaction	9.60E-05	2.70E-02
GOTERM_MF_DIRECT	protein binding	4.30E-04	4.30E-01
GOTERM_BP_DIRECT	positive regulation of inflammatory response	4.80E-04	4.90E-01
GOTERM_BP_DIRECT	neutrophil chemotaxis	5.30E-04	4.20E-01
GOTERM_BP_DIRECT	adaptive immune response	5.40E-04	3.70E-01
GOTERM_BP_DIRECT	positive regulation of gene expression	7.50E-04	4.10E-01
GOTERM_BP_DIRECT	regulation of G1/S transition of mitotic cell cycle	9.50E-04	4.30E-01
GOTERM_BP_DIRECT	response to lipopolysaccharide	1.10E-03	4.30E-01
GOTERM_BP_DIRECT	response to wounding	1.10E-03	3.90E-01
GOTERM_BP_DIRECT	negative regulation of interferon-gamma production	1.10E-03	3.60E-01
GOTERM_BP_DIRECT	xenobiotic catabolic process	1.10E-03	3.40E-01
GOTERM_BP_DIRECT	apoptotic signaling pathway	1.10E-03	3.20E-01
GOTERM_MF_DIRECT	chemokine activity	1.20E-03	5.40E-01
GOTERM_BP_DIRECT	chemotaxis	1.30E-03	3.50E-01

Table 2.2 | Top 20 up-regulated (indicated by the positive fold change) and down-regulated (indicated by the negative fold change) genes in BluePrint (BP) HER2-type tumors (relative to BP Luminal-type) in HER2+/HR+ patients. Log_2 fold change describes expression in BP HER2-type tumors relative to that in BP Luminal-type tumors.

Gene symbol	Gene Name	Fold Change	p-value	Adjusted p value
GRB7	Growth Factor Receptor Bound Protein 7	2.50	4.21354E-21	7.25571E-17
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2	1.53	1.10371E-19	9.50294E-16
MIEN1	Migration and Invasion Enhancer 1	2.04	1.53471E-17	8.80926E-14
TCAP	Titin-Cap	1.93	2.21837E-16	9.55006E-13
PGAP3	Post-GPI Attachment to Proteins 3	1.87	3.67058E-16	1.26415E-12
STARD3	StAR-Related Lipid Transfer Domain Containing 3	1.59	9.27484E-15	2.66188E-11
SOX11	SRY-Box 11	1.69	8.12486E-13	1.55456E-09
PNMT	Phenylethanolamine N-Methyltransferase	3.27	1.61532E-12	2.78157E-09
MPHOSPH6	M-Phase Phosphoprotein 6	0.94	9.63829E-11	9.76303E-08
TNFRSF21	TNF Receptor Superfamily Member 21	0.71	2.13716E-10	1.84102E-07
MFSD2A	Major Facilitator Superfamily Domain Containing 2A	0.57	4.45015E-10	3.33181E-07
C15orf39	Chromosome 15 Open Reading Frame 39	0.53	5.53158E-10	3.96891E-07
TGFBR1	Transforming Growth Factor Beta Receptor 1	0.49	1.53988E-09	9.82103E-07
KMO	Kynurenine 3-Monooxygenase	0.82	2.95765E-09	1.75623E-06
DPEP3	Dipeptidase 3	0.67	4.27331E-09	2.29957E-06
C2orf54	Chromosome 2 Open Reading Frame 54	1.74	4.79076E-09	2.42638E-06
LIMK2	LIM Domain Kinase 2	0.45	7.71216E-09	3.58928E-06
ADAM19	ADAM Metallopeptidase Domain 19	0.39	9.65345E-09	4.15581E-06
PSAT1	Phosphoserine Aminotransferase 1	0.96	1.00955E-08	4.24009E-06
ORMDL3	ORMDL Sphingolipid Biosynthesis Regulator 3	1.10	1.12871E-08	4.62773E-06
RERG	Ras Like Estrogen Regulated Growth Inhibitor	-1.49	1.00916E-13	2.48253E-10
TBC1D9	TBC1 Domain Family Member 9	-1.21	3.93883E-13	8.47833E-10
AL133644	Protein phosphatase 1J	-1.28	1.23594E-11	1.93481E-08
MAPT	Microtubule Associated Protein Tau	-1.68	1.73626E-11	2.29987E-08
PPM1J	Protein Phosphatase, Mg2+/Mn2+- dependent 1J	-1.16	1.69159E-11	2.29987E-08
ESR1	Estrogen Receptor 1	-1.08	3.72165E-11	4.57763E-08
CCDC74B	Coiled-Coil Domain Containing 74B	-1.56	5.38966E-11	6.18733E-08
ZSWIM5	Zinc Finger SWIM-Type Containing 5	-0.74	6.27664E-11	6.75523E-08
DBNDD2	Dysbindin Domain Containing 2	-1.01	1.41998E-10	1.35844E-07
AGBL2	ATP/ GTP Binding Protein Like 2	-0.49	2.24514E-10	1.84102E-07
PARD6B	Par-6 Family Cell Polarity Regulator Beta	-1.32	2.1905E-10	1.84102E-07
KIAA1324L	KIAA1324 Like	-0.79	2.53895E-10	1.98731E-07
NXNL2	Nucleoredoxin Like 2	-0.69	8.31753E-10	5.72911E-07
SLC39A6	Solute Carrier Family 39 Member 6	-1.37	1.40153E-09	9.28246E-07
SPR	Sepiapterin Reductase	-0.74	2.57399E-09	1.58301E-06
PDZK1	PDZ Domain Containing 1	-1.95	3.24377E-09	1.86192E-06
SLC19A2	Solute Carrier Family 19 Member 2	-1.06	3.76702E-09	2.09252E-06
MZT2B	Mitotic Spindle Organizing Protein 2B	-0.52	4.68039E-09	2.42638E-06
EIF3J-AS1	EIF3J Antisense RNA 1 (Head-to-Head)	-0.42	5.24803E-09	2.58203E-06
PCP2	Purkinje Cell Protein 2	-0.95	7.20778E-09	3.44772E-06

Table 2.3 | Concordance between BluePrint molecular subtypes and PAM50 intrinsic subtype classifications of 155 I-SPY 2 HER2+/HR+ patients.

HER2+/HR+ subset (n= 155)		BluePrint subtypes		
		BP Basal-type	**BP HER2-type**	**BP Luminal-type**
Intrinsic subtypes	**Basal-like**	1	1	0
	HER2-enriched	0	40	1
	Luminal A	2	22	31
	Luminal B	0	31	26

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Chapter 3:

Acquired resistance to neratinib in HER2-positive breast cancer

Introduction

Overexpression and/or amplification of human epidermal growth factor receptor (HER2 occurs in approximately 15-20% of breast cancers and clinically defines the HER2-positive breast cancer subtype[1]. HER2-positive (HER2+) breast cancer is associated with high proliferation rates and, prior to the advent of HER2-targeted therapies, a poor prognosis[2]. Several anti-HER2 agents, such as trastuzumab[3], lapatinib[4] and pertuzumab[5], have been developed to target the HER2 oncogene through different mechanisms of action and have significantly improved the outcomes of HER2+ breast cancer patients.

Neratinib is an irreversible, pan-HER tyrosine kinase inhibitor that blocks HER2 and EGFR phosphorylation and downstream signal transduction through the PI3K/AKT and RAS/RAF/MEK/ERK pathways[6]. Neratinib was most recently approved by the FDA for extended adjuvant treatment of patients with early stage HER2+ breast cancer after adjuvant trastuzumab[7–9]; the role of neratinib in the neoadjuvant[10] and metastatic[11–14] disease setting is still currently under clinical investigation. Despite major advances made in the treatment of HER2+ metastatic breast cancer, complete, durable responses to HER2-targeted therapies like neratinib are rare, and patients invariably succumb to the emergence of drug-resistant disease (acquired resistance). Furthermore, 40-50% of HER2+ patients fail to respond initially to neratinib and exhibit intrinsic resistance[11–13]. Identifying molecular events that limit the

response, as well as biomarkers that predict which patients will benefit to neratinib, are essential for enhancing clinical outcomes.

Preclinical[15–18] and clinical[19,20] studies have implicated somatic mutations in HER2 and HER3 as predictive of neratinib response or resistance; however, the prevalence of these mutations is low (<5% for HER2; <1% for HER3)[21] and most of the studies were conducted in the context of HER2 non-amplified breast cancer, thus the findings may not fully explain variability in neratinib response observed in HER2+ breast cancer patients.

Recent biomarker analyses[22] from the I-SPY 2 TRIAL[23] revealed that activation of some HER family phosphoproteins predict response to neratinib. The I-SPY 2 TRIAL is a phase II, adaptive neoadjuvant therapy trial in which the primary goal is to determine the predictive probabilities of phase III trial success for various targeted therapeutics[23]. Neratinib was available to patients with all tumor subtypes in the trial, and it graduated in the HER2-positive signature[10] (Figure 3.1a). The biomarker study evaluated the levels of 18 phosphoproteins/proteins – comprising neratinib drug targets (EGFR, HER2) and downstream effector molecules – in pre-treatment biopsies by reverse phase protein array (RPPA) as specific biomarkers of neratinib response[22]. Six of the 18 HER family phosphoproteins/proteins markers were associated with neratinib response; of which, EGFR Y1173 appeared to add predictive value to the clinical HER2 status in identifying patients who specifically respond to neratinib (relative to trastuzumab-treated controls)[22]. However, these HER family biomarkers may not be perfectly predictive and do not provide insights into the mechanisms driving resistance in patients who did

not respond to neratinib; better biomarkers are clearly needed to predict response and optimize therapeutic decisions.

Given the potential of HER family phosphoproteins in predicting neratinib response in the clinic, we sought to assess the predictive capability of a larger repertoire of HER family phosphoproteins and evaluate the dynamics of these phosphoproteins/protein levels in response to different concentrations of neratinib in an *in vitro* setting – using 4 HER2-positive breast cancer cell lines and their neratinib-resistant derivatives. The goals of this study were to validate the existing HER family phoshoprotein biomarkers, identify additional biomarkers of response, and elucidate mechanisms of resistance to neratinib. To this end, we interrogated and compared the activation state of 104 key signaling phosphoproteins/ proteins from prosurvival, mitogenic, apoptotic, and growth regulatory pathways (Table 3.1) between 4 neratinib-sensitive HER2+ breast cancer cell lines and their drug resistant derivatives using RPPA.

Results

Development of *in vitro* models of acquired resistance to neratinib

We developed multiple *in vitro* models of acquired resistance to neratinib by continuously exposing 4 neratinib-sensitive cell lines – AU565, BT474, HCC1954 and SK-BR-3 – to neratinib (Figure 3.1b). 8 neratinib-resistant derivatives/ sub-lines – AU565R1, AU565R2, AU565R3, AU565R4, BT474R1, SK-BR-3R1, HCC1954R1 and HCC1954R2 – were derived independently. The resistant cell lines were viable at higher concentrations of neratinib, as indicated by the right shift in cell viability curves of the resistant cell lines relative to the their respective parental cell lines; the concentration of neratinib required to cause 50% growth inhibition i.e. GI_{50} of the resistant cell line derivatives is between 20 – 340-fold higher than of the parental cell lines.

Activation of HER family signaling proteins is associated with neratinib sensitivity

We evaluated the baseline activation state of 104 key signaling phosphoproteins/ proteins from prosurvival, mitogenic, apoptotic, and growth regulatory pathways (Table 3.1) as predictors of neratinib sensitivity using RPPA data from the 4 parental cell lines and their 8 resistant derivatives. Unsupervised clustering of the 104 key signaling proteins/ phosphoproteins revealed that baseline phosphoprotein/ protein levels clustered primarily by cell line of origin, regardless of neratinib sensitivity (Figure 3.2a). Upon median-centering the baseline phosphoprotein/ protein levels of cell lines with a common parental cell line, unsupervised clustering analysis revealed 2 clusters, with a predominance of sensitive parental cell lines in one of the clusters (Figure 3.2b); notably, this cluster was characterized by higher baseline HER family

phosphoprotein signaling activation (EGFR Y1173, EGFR Y1068, HER2 Y1248, HER2 Y877, HER3 Y1289).

Using logistic regression (in a model adjusting for cell line of origin and applying multiple testing correction, see *Methods*), 13 of the 104 phosphoproteins/ proteins tested were significantly associated with neratinib sensitivity (Table 3.2). Of these 13 phosphoproteins/ proteins, almost half were HER family signaling proteins – EGFR Y1173, total HER2, HER2 Y1248, EGFR Y1068, ERBB2 Y877, total EGFR and HER3 Y1289; among which, 4 of these HER2 family signaling proteins were also associated with neratinib response in the I-SPY2 TRIAL. Having the same set of phosphoprotein/ protein endpoints appear as markers associated with neratinib sensitivity in both HER2-positive breast cancer cell lines and tumor samples from patients increase our confidence that the selected cell lines are clinically relevant models. The other seven phosphoproteins/ proteins that were associated with neratinib sensitivity represent a multitude of pathways; these include AKT/ mTOR signaling (p70 S6K T389 and T412), JAK/STAT signaling (STAT5 Y694), proliferation (p27 T187) and other receptor tyrosine kinase signaling pathways (total MET and IRS1 S612).

Differential changes in RPPA endpoints with increasing concentrations of neratinib

The 4 parental HER2-positive cell lines and the 8 neratinib-resistant derivatives were treated with 5 different concentrations of neratinib ranging from 2nM to 100nM for an hour (Figure 3.3). Protein lysates were then collected for RPPA analysis to evaluate the changes in the aforementioned 104 protein endpoints in response to increasing concentrations of neratinib. As expected, activation of HER family phosphoproteins (EGFR Y1068, EGFR Y1173, ERBB2

Y1248) – drug targets of neratinib – were negatively correlated with increasing drug concentrations in parental cell lines (indicated by the blue rows / boxes in Figure 3.4; adjusted $p < 0.05$). Activation of downstream effectors of HER family – AKT T308, AKT S473 and ERK 1/2 T202/ Y204 – was also downregulated with an increase in neratinib concentration. Interestingly, most of the resistant cell line derivatives showed a similar concentration-dependent reduction in activation of HER family phosphoproteins and the downstream effector proteins.

Comparative analysis revealed distinct differences in neratinib-induced phosphoprotein activation between resistant cell lines from their parental cell lines, and also between resistant cell lines derived from the same parental cell line. For example, AU565R1 exhibited a significant concentration-dependent up-regulation in phospho-MEK1/2 S217/S221 while AU565R2 showed a concentration-dependent down-regulation of the same endpoint (Figure 3.4). These results suggest that each of the *in vitro* models of neratinib resistance may have distinctive underlying mechanisms driving neratinib resistance.

Inhibition of PI3K, AKT or MEK was insufficient to restore neratinib sensitivity

HER2 homodimers/ heterodimers are known to signal via downstream PI3K/ AKT and MAPK pathways to drive cell proliferation in HER2+ breast tumors[24,25] (Figure 3.5a). Our data indicated that while neratinib was able to down-regulate PI3K/AKT and MAPK pathways in both parental and resistant HER2+ cell lines, it was insufficient to fully abrogate signaling via these downstream pathways in the resistant HER2+ cell lines even at higher concentrations of neratinib. AU565 neratinib-resistant derivatives (AU565R1-R4) exhibit continual signaling via PI3K/AKT and MAPK pathways even when treated at high neratinib concentrations (50nM/

100nM) as shown by the higher levels of activation of AKT S473 and ERK T202/Y204 compared to AU565 parental cell line at these concentrations (Figure 3.5b). We postulated that elimination of residual PI3K/ AKT and/ or MAPK signaling by treatment with a combination of neratinib and inhibitors targeting either of these pathways might rescue neratinib sensitivity in the resistant HER2+ cell lines.

Parental AU565 cell lines exhibited significant cell growth inhibition only when treated at higher doses of PI3K inhibitor BKM120, AKT inhibitor MK2206 or MEK inhibitor trametinib alone; conversely, parental AU565 was highly sensitive to neratinib i.e. >80% cell growth inhibition at neratinib 10nM (Figure 3.5c). Though the addition of BKM120, MK2206 or trametinib enhanced the response to neratinib in AU565 resistant derivatives, with concurrent treatment with neratinib and each of the 3 agents eliciting greater growth suppression than either monotherapy, none of the combinations were able to fully restore neratinib sensitivity in the resistant cell lines; most of the combination treatments only achieved <50% growth inhibition in the AU565 resistant derivatives (Figure 3.5c). In addition, the treatment combinations comprising neratinib and either one of the three kinase inhibitors (BKM120, MK2206 or trametinib) were not uniformly effective across all the resistant cell lines, inhibiting growth at varying degrees; for instance, the combination of AKT inhibitor MK2206 and neratinib was able to achieve 75% growth inhibition in AU565R3 but only achieved <50% growth inhibition in the 3 other resistant lines (AU565R1, R2 and R4). These data further support our hypothesis that HER2-positive breast cancer cell lines acquire resistance to neratinib via diverse mechanisms, thus exhibiting differential dependence on PI3K/AKT and/or MAPK signaling.

BET family bromodomain inhibition re-sensitizes resistant cells to neratinib

In a recent study by Stuhmiller T.J. et. al.[26], the authors demonstrated that BET family bromodomain inhibition is able to overcome resistance to lapatinib – another EGFR/HER2 tyrosine kinase inhibitor – in HER2-positive breast cancer cell lines. Resistance to lapatinib was mediated through kinome reprogramming, leading to transcriptional up-regulation of multiple alternative kinases (such as ERBB3, DDR1, IGF1R and MET) capable of bypassing HER2-directed signaling. Inhibition of BET family bromodomains – epigenetic factors that link acetylated transcription factors and histones to the activation of RNA polymerase II – prevents the lapatinib-induced kinome reprogramming response by suppressing the transcription of the kinases implicated in drug resistance.

Given that neratinib share a similar mechanism of action as lapatinib, we hypothesized that HER2-positive breast cancer cells might develop acquired resistance to neratinib in a similar fashion through adaptive heterogeneous kinome reprogramming, leading to up-regulation of multiple receptor and/or intracellular tyrosine kinases that bypass HER2 inhibition to sustain HER2-positive cell proliferation (Figure 3.6a). To test this hypothesis, we treated the AU565 neratinib-resistant cell lines (AU565R1 – R4) with JQ1, an inhibitor of BET family bromodomains that has previously been shown to suppress lapatinib-induced kinome reprogramming at a transcriptional level[26]. Treatment with JQ1 alone decreased viability of the AU565 neratinib-resistant cell lines only at a higher drug concentration (300nM; $p<0.05$ across AU565R1 – R4) but when used in combination with neratinib resulted in a significant concentration–dependent reduction in viability across all the resistant cell lines ($p < 0.001$ for neratinib + JQ1 300nM combination across AU565R1 – R4) (Figure 3.6b). Concurrent treatment

with neratinib and JQ1 elicited greater reduction in cell viability than either monotherapy. Our data supports the proposal of adaptive kinome reprogramming as a potential mechanism of neratinib resistance in HER2-positive breast cancer, and that the combination of neratinib and BET bromodomain inhibitor appears to be a promising therapeutic strategy to overcome such resistance.

Discussion

In this study, we evaluated the baseline activation state of 104 key signaling phosphoproteins/ proteins as predictors of neratinib response using RPPA data from HER2-positive breast cancer cell lines with differential neratinib sensitivity. The use of a quantitative proteomics platform like RPPA to study signal transduction permits a comprehensive strategy to characterize protein networks and pathways that influence drug response.

Our data revealed that higher baseline activation of HER family signaling phosphoproteins (EGFR/ HER2/ HER3) is associated with neratinib sensitivity in HER2-positive breast cancer cell lines. This is consistent with clinical data from the I-SPY2 TRIAL, in which phosphorylation levels of HER2 and EGFR in pre-treatment biopsies correlate with pathological complete response in HER2-positive breast cancer patients treated with neratinib in a neoadjuvant setting[22]. Of the thirteen RPPA endpoints that associated with neratinib sensitivity in our *in vitro* study, four of the endpoints – EGFR Y1173, EGFR Y1068, HER2 Y1248, total HER2 – were also identified as potential biomarkers of neratinib response in the I-SPY2 TRIAL[22]; this helped to reinforce the clinical relevance of the breast cancer cell line models. Previous published studies[27–32] have also highlighted the potentially important role(s) phosphorylated EGFR/HER2 levels play as prognostic and/or predictive markers of drug response in breast cancer and other tumor types in providing additional information compared to total EGFR/HER2 protein levels. Our findings also demonstrate the need to evaluate the activation of the all HER family receptors (EGFR, HER2, HER3) in its entirety, not just HER2 alone, as potential markers of neratinib response, especially given that HER2 requires homodimerization or heterodimerization with one of the other HER family members – in

particular HER3 – to activate downstream signaling, in turn driving cell proliferation in HER2-positive breast cancer[24].

Inhibition of downstream PI3K/AKT or MAPK signaling pathways in combination with neratinib increased growth inhibition across the HER2-positive (acquired) resistant cell line models to varying degrees; however, none of the combinations was sufficient to fully restore sensitivity to neratinib in the resistant cell lines. Although some preclinical studies[33–35] have demonstrated the potential benefit of targeting PI3K/AKT or MAPK pathway to overcome resistance to trastuzumab and lapatinib in HER2-positive breast cancer in some cases, this was not the case in our study. One possible explanation could be that inhibition of either one of the downstream pathways could result in compensatory activation in the other pathway; this is supported by a study by Serra et. al.[36] in which the inhibition of PI3K/AKT/mTOR pathway by PI3K inhibitor in HER2-positive breast cancer models resulted in a compensatory activation of the ERK signaling pathway, reducing the efficacy of PI3K inhibitors. Simultaneous inhibition of PI3K/AKT and MAPK pathways may be necessary to overcome acquired resistance to neratinib in HER2-positive breast cancer though such a combination may be potentially challenging given that these PI3K/AKT inhibitors and MEK inhibitors may have overlapping toxicities such as skin rash.

The neratinib-resistant cell line models used in our study exhibited differential phosphorylation/ activation of phosphoproteins/ proteins in response to increasing concentrations of neratinib treatment (Figure 3.3b). This suggests that the cell lines may have acquired resistance to neratinib through diverse mechanisms, which poses a challenge in identifying a

therapeutic strategy that will be able to overcome resistance across all cell lines. Notably, the resistant cell lines showed a similar concentration-dependent reduction in activation of HER family phosphoproteins when treated with neratinib. This indicates that neratinib was still able to bind and engage its drug targets (EGFR and HER2) in the resistant cell lines, and drug resistance was unlikely mediated through mutation of the cysteine residue[37] critical for covalent binding of neratinib to its drug target. This is unlike ibrutinib, another irreversible tyrosine kinase inhibitor targeting Bruton's tyrosine kinase, which also binds covalently to its drug target like neratinib; the main mechanism through which patients with chronic lymphocytic leukemia develop acquired resistance to ibrutinib is through a cysteine to serine mutation (C481S) at the site covalently bound by ibrutinib[38,39].

Our findings indicate that combining neratinib with BET bromodomain inhibition might prove beneficial to overcome or prevent resistance to neratinib in HER2-positive breast cancer. Several preclinical studies[26,40–42] have investigated and demonstrated the promise and synergistic effect of combining BET bromodomain inhibitors and targeted therapies to overcome acquired resistance to targeted therapies like lapatinib and vemurafenib. BET bromodomain inhibiton as a therapeutic strategy has the advantage of being able to suppress the transcription of multiple kinases that may be implicated in drug resistance[40,43], especially given the plasticity and resiliency of cancer signaling networks and the ability of tumor cells to activate alternative compensatory pathways when one pathway is blocked to sustain cell proliferation and survival, and given the non-feasibility of using multiple kinase inhibitors or other drugs (>2-3) to counter resistance mechanisms due to cumulative toxicity. On the other hand, the broad-acting nature of BET bromodomain inhibitor may result in undesirable off-target effects from inhibition of

transcription of genes that are important for functions beyond driving drug resistance; further mechanistic and clinical studies on BET bromodomain inhibition, especially in the context of combination therapy, is warranted.

Translational relevance

Despite major advances made in the treatment of HER2-positive metastatic breast cancer, complete, durable responses to HER2-targeted therapies like neratinib are rare, and patients invariably succumb to the emergence of drug-resistant disease (acquired resistance). Furthermore, 40-50% of HER2+ patients fail to respond initially to neratinib and exhibit intrinsic resistance. Identifying molecular events that limit the response, as well as biomarkers that predict which patients will benefit to neratinib, are essential for enhancing clinical outcomes. In this study, we identified 13 phosphoproteins/ proteins that are associated with neratinib sensitivity in HER2-positive breast cancer cell line models. Among which, 4 of the phosphoproteins/proteins are associated with neratinib response in the clinical setting[22]; further investigation is required to assess the clinical relevance of the other 9 protein markers in predicting patient response to neratinib. The study also demonstrated in HER2-positive breast cancer cell line models that acquired resistance to neratinib could potentially be mediated through adaptive kinome reprogramming, and that the combination of neratinib and BET bromodomain inhibitor appears to be a promising therapeutic strategy to overcome such resistance. Further studies are needed to understand the underlying mechanisms and critical players mediating neratinib-induced kinome reprogramming.

Methods

Compounds

Stocks of neratinib, BKM120, MK2206, trametinib and JQ1 (Selleck Chemicals, Houston, TX) were prepared with DMSO.

Cell culture

AU565, BT474, SK-BR-3 and HCC1954 HER2-positive breast cancer cell lines were obtained from ATCC and were routinely cultured at 37°C in a humidified 5.0% CO_2 atmosphere in RPMI-1640 medium (AU565, BT474, HCC1954) or McCoy's 5a Medium Modified (SK-BR-3) (UCSF Cell Culture Core Facility), supplemented with 10% heat-inactivated fetal bovine serum and penicillin-streptomycin 100ug/ml (UCSF Cell Culture Core Facility). Neratinib-resistant derivatives of the 4 cell lines (AU565R1, AU565R2, AU565R3, AU565R4, BT474R1, SK-BR-3R1, HCC1954R1 and HCC1954R2) were derived independently. Cells were treated with gradually increasing concentrations of neratinib. When cells began proliferating at normal rates, drug doses were doubled until a concentration of 120nM of neratinib, the peak plasma concentration of neratinib attainable in patients in Phase I clinical studies[44] was reached. Fresh drug was added every 72-96hr. Resistant cell lines were maintained continuously in the presence of neratinib.

Cell growth assay

For the growth assays, each of the cell lines and their resistant derivatives were plated in 96-well plates at 5000 cells/ well and were exposed to neratinib at concentrations ranging from 0.0192nM to 7.5uM (5-fold dilutions) the next day. At 120h after drug addition, cells were lysed

with CellTiter-Glo Luminescent Cell Viability Assay reagent (Promega, Madison, WI) and luminescence was measured using a BioTek plate reader (BioTek Instruments Inc., Winooski, VT) according to the manufacturer's instructions. All experimental points were set up as sextuplicate biological replicates. Data are presented as percentage of viable cells compared with control cells (vehicle treatment).

Reverse Phase Protein Microarray

Cells were lysed with extraction buffer composed of Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA) and 1% Halt protease and phosphatase cocktail (ThermoFisher Scientific, Waltham, MA) at a concentration of approximately 4000 – 5000 cells per 1uL of extraction buffer. 2.5% beta-mercaptoethanol (BME) was added to the protein lysates. Samples were heated at 100°C for 5 minutes and brought to room temperature, briefly centrifuged and stored at -20°C until printing.

Cell lysates were printed in triplicate spots (approx. 10 nL per spot) onto nitrocellulose coated slides (Grace Biolabs, Bend, OR) using an Aushon 2470 Arrayer (Aushon Biosystems, Billerica, MA). Standard curves of control cell lysates were also included for quality assurance purposes[45]. 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 use[46], and are listed in Table 3.1. 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[47]. Significant concordance between RPPA data and

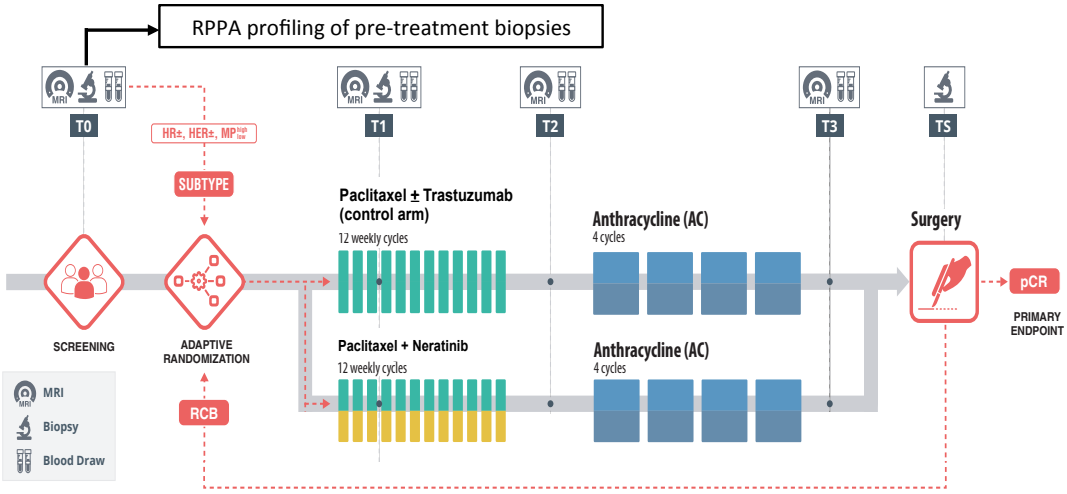
western blotting have been previously shown[48–50].Each slide was probed with one primary antibody targeting the protein of interest. Biotinylated goat anti-rabbit IgG (H+L)(1:7500, Vector Laboratories Inc., Burlingame, CA) and rabbit anti-mouse IgG (1:10, Dako Cytomation, Carpinteria, CA) were used as secondary antibodies. Signal amplification was performed using a tyramide-based avidin/ biotin amplification system (DakoCytomation, Carpinteria, CA) followed by streptavidin-conjgated IRDye 680 (LI-COR, Lincoln, NE) for visualization. Total protein was measured using Sypro Ruby protein blot staining per manufacturer's instructions (Molecular Probes, Eugene, OR). Images were acquired using a Tecan PowerScanner (Tecan, Mannedorf, Switzerland) and analyzed with MicroVigene software version 5.1.0.0 (Vigenetech, Carlisle, MA)[51]. The final results represent negative control-subtracted and total protein normalized relative intensity values for each endpoint within a given cell line sample.

Statistical Analysis of RPPA data

We assessed the association of protein endpoints at baseline with neratinib sensitivity of the cell lines treated as a dichotomous variable (sensitive=1; resistant=0) using the Wilxocon rank sum test and logistic regression. Analysis was also performed in a model adjusting for cell line of origin as a categorical variable i.e. neratinib sensitivity ~ protein endpoint at baseline + cell line of origin. Benjamini-Hochberg (BH) multiple testing correction was applied to the p-values to control for false discovery, and protein endpoints were identified as being significantly associated to neratinib sensitivity if BH $p < 0.05$. We also assessed the relationship of the protein/ phosphoprotein levels in response to treatment with increasing concentrations of neratinib by Pearson correlation. Correlation coefficient was considered significant if BH $p < 0.05$.

Figure 3.1 | a) I-SPY 2 TRIAL schematic for patients in the control and neratinib treatment arms. After screening, patients with HER2-positive and HER2-negative breast cancer were eligible to undergo adaptive randomization to receive neratinib and paclitaxel, followed by doxorubicin/cyclophosphamide (AC). Patients randomized to the control group received paclitaxel ± trastuzumab (depending on HER2 status), followed by AC. Pre-treatment biopsy specimens from patients in both neratinib and control arms were analyzed for HER family signaling protein activation by reverse phase protein array (RPPA)[22]. b) Schematic of the experimental workflow to develop neratinib-resistant HER2-positive breast cancer cell line models. Parental cell lines were treated with increasing concentrations of neratinib over time to establish acquired resistant cell line derivatives. Acquired resistant cell line models (colored curves) are able to survive under higher concentrations of neratinib as demonstrated by a right shift in the cell viability curves (neratinib GI_{50}) relative to parental cell line (black curve). The neratinib GI_{50} profiles of the AU565 parental cell line and its resistant derivatives are shown here as an example; results were normalized to those obtained with DMSO. RPPA was then performed on the protein extracts of both parental and resistant derivative cell lines to identify candidate markers of drug response/ resistance.

A



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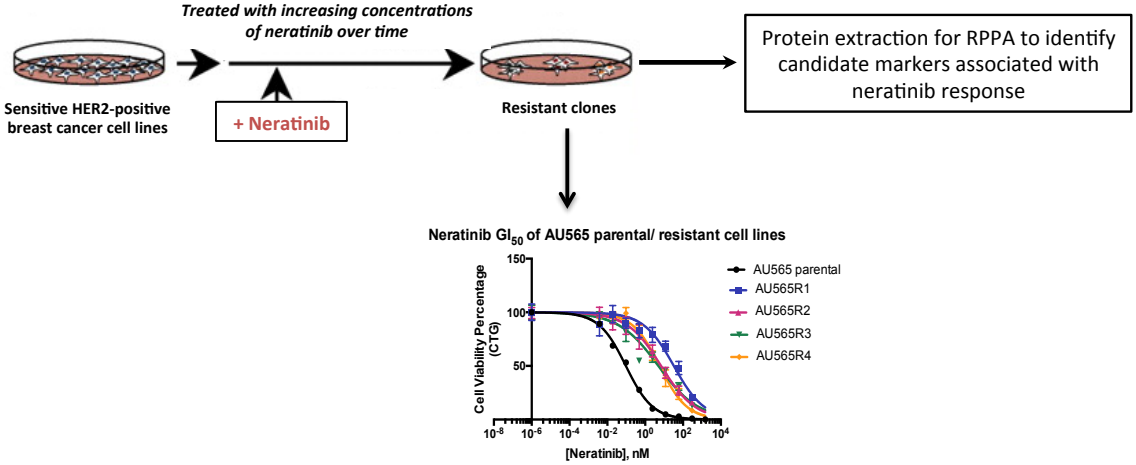
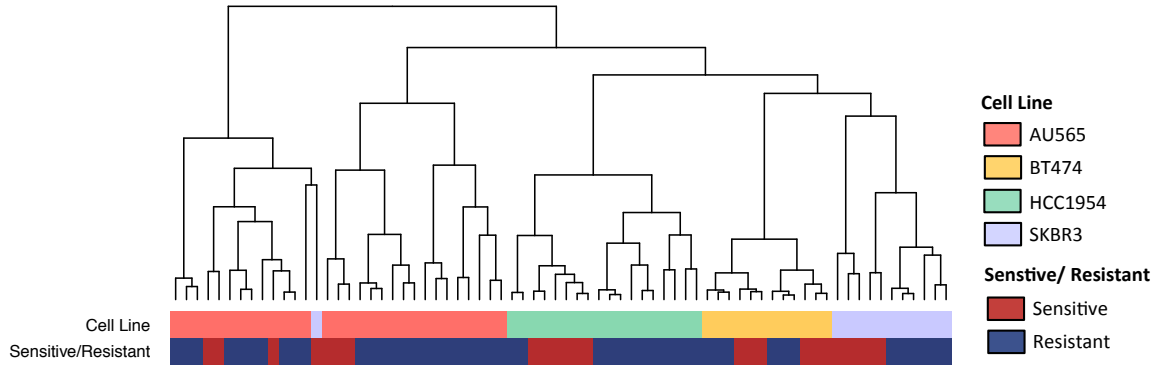


Figure 3.2 | Unsupervised hierarchical clustering of the baseline activation of 104 proteins/ phosphoproteins of the 4 parental HER2-positive breast cancer cell lines and their resistant derivatives. a) Cell lines are hierarchically clustered along columns. Hierarchical clustering, when performed without median-centering within cell lines, reveals clusters primarily driven by cell line of origin. Individual cell lines – AU565, BT474, HCC1954, SKBR3 – are denoted by 4 different colors (pink, yellow, green, purple respectively) on the first row of the annotation track. Neratinib sensitivity of the cell lines are indicated on the second row of the annotation: red represents neratinib sensitivity (parental cell lines); blue represents neratinib resistance (resistant derivative). b) Heatmap of the protein (activation) levels of the 104 phosphoproteins/ proteins in the 4 parental HER2-positive breast cancer cell lines and their resistant derivatives. Each column represents a cell line, and each row represents a RPPA protein endpoint (phosphoprotein/ protein). Hierarchical clustering of cell lines, when performed with median-centering within individual cell lines, reveals 2 main clusters. Most of the sensitive cell lines (indicated by red bars on the sensitive/resistance horizontal annotation strip) are found within the cluster on the right. Using logistic regression (in a model adjusting for cell line of origin and applying multiple testing correction, see *Methods*), 13 of the 104 phosphoproteins/ proteins tested were significantly associated with neratinib sensitivity (adjusted $P < 0.05$); these phosphoproteins/ proteins are marked by the squares on the vertical annotation track (black: yes; cream: no)

A



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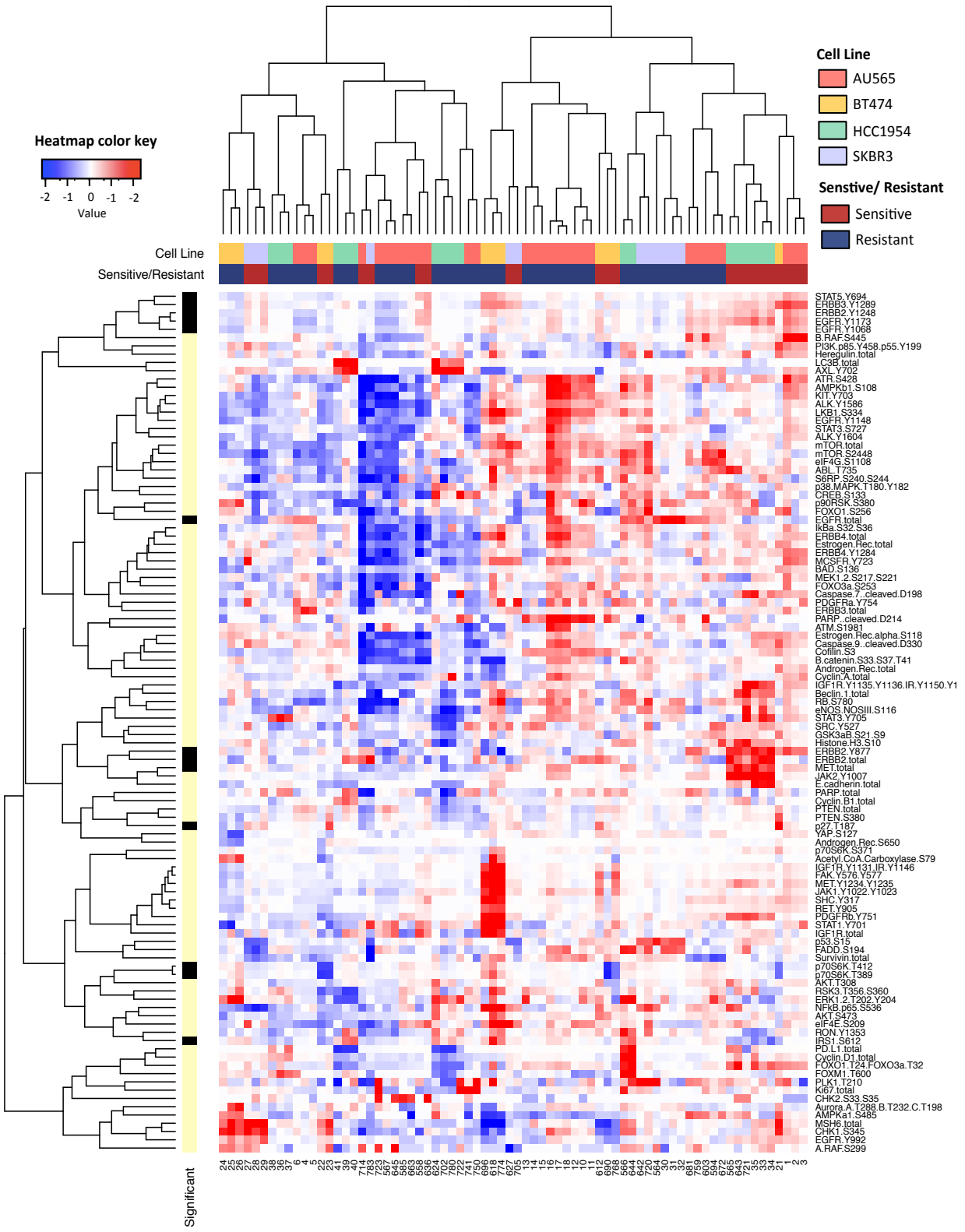
Heatmap color key
-2 -1 0 -1 -2
Value
Cell Line
AU565
BT474
HCC1954
SKBR3
Senstive/ Resistant
Sensitive
Resistant
Cell Line
Sensitive/Resistant
Significant
STAT5.Y694
ERBB3.Y1289
ERBB2.Y1248
EGFR.Y1173
EGFR.Y1068
B.RAF.S445
PI3K.p85.Y458.p55.Y199
Heregulin.total
LC3B.total
AXL.Y702
ATR.S428
AMPKb1.S108
KIT.Y703
ALK.Y1586
LKB1.S334
EGFR.Y1148
STAT3.S727
ALK.Y1604
mTOR.total
mTOR.S2448
eIF4G.S1108
ABL.T735
S6RP.S240.S244
p38.MAPK.T180.Y182
CREB.S133
p90RSK.S380
FOXO1.S256
EGFR.total
IkBa.S32.S36
ERBB4.total
Estrogen.Rec.total
ERBB4.Y1284
MCSFR.Y723
BAD.S136
MEK1.2.S217.S221
FOXO3a.S253
Caspase.7..cleaved.D198
PDGFRa.Y754
ERBB3.total
PARP..cleaved.D214
ATM.S1981
Estrogen.Rec.alpha.S118
Caspase.9..cleaved.D330
Cofilin.S3
B.catenin.S33.S37.T41
Androgen.Rec.total
Cyclin.A.total
IGF1R.Y1135.Y1136.IR.Y1150.Y1
Beclin.1.total
RB.S780
eNOS.NOSIII.S116
STAT3.Y705
SRC.Y527
GSK3aB.S21.S9
Histone.H3.S10
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ERBB2.total
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JAK2.Y1007
E.cadherin.total
PARP.total
Cyclin.B1.total
PTEN.total
PTEN.S380
p27.T187
YAP.S127
Androgen.Rec.S650
p70S6K.S371
Acetyl.CoA.Carboxylase.S79
IGF1R.Y1131.IR.Y1146
FAK.Y576.Y577
MET.Y1234.Y1235
JAK1.Y1022.Y1023
SHC.Y317
RET.Y905
PDGFRb.Y751
STAT1.Y701
IGF1R.total
p53.S15
FADD.S194
Survivin.total
p70S6K.T412
p70S6K.T389
AKT.T308
RSK3.T356.S360
ERK1.2.T202.Y204
NFkB.p65.S536
AKT.S473
eIF4E.S209
RON.Y1353
IRS1.S612
PD.L1.total
Cyclin.D1.total
FOXO1.T24.FOXO3a.T32
FOXM1.T600
PLK1.T210
Ki67.total
CHK2.S33.S35
Aurora.A.T288.B.T232.C.T198
AMPKa1.S485
MSH6.total
CHK1.S345
EGFR.Y992
A.RAF.S299

Figure 3.3 | Schematic of the experimental workflow to obtain drug-treated cell lysates for reverse phase protein array analysis. HER2-positive cell lines and their respective neratinib-resistant clones were treated at 5 difference concentrations of neratinib for 1 hour. Protein extraction was then performed for RPPA analysis.

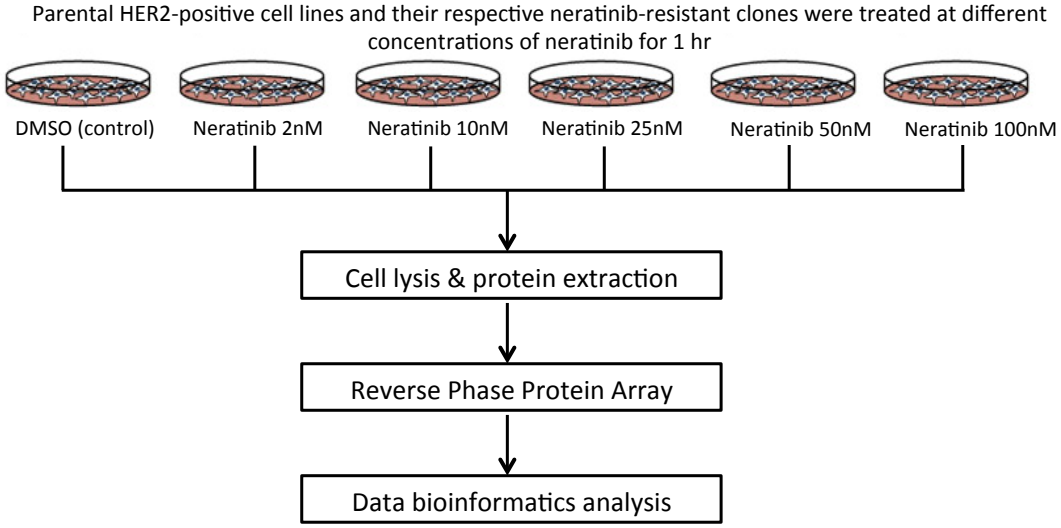

Figure 3.4 | Heatmap showing the relationship and strength of association between the phosphoprotein/ protein levels and concentration of neratinib. Each column represents a cell line, and each row represents a RPPA protein endpoint. Cell lines are arranged first by cell line of origin, then by neratinib sensitivity status (sensitive/ resistant), along columns, and phosphoproteins/ proteins are hierarchically clustered along rows. A blue square indicates significant negative correlation between the drug concentration and the level of the phosphoprotein/ protein (e.g. EGFR Y1173, on bottom right), whereas a red square indicates a significant positive correlation (e.g. ALK Y1586, on top right)(BH corrected; adjusted $p < 0.05$). The intensity of the color is equal to the log of the BH adjusted correlation p-value; darker colors indicate more highly correlated endpoints. (transformed data shown = sign(Pearson coefficient)*(-log10(BH p))). BH, Benjamini-Hochberg (to control for false discovery rate).

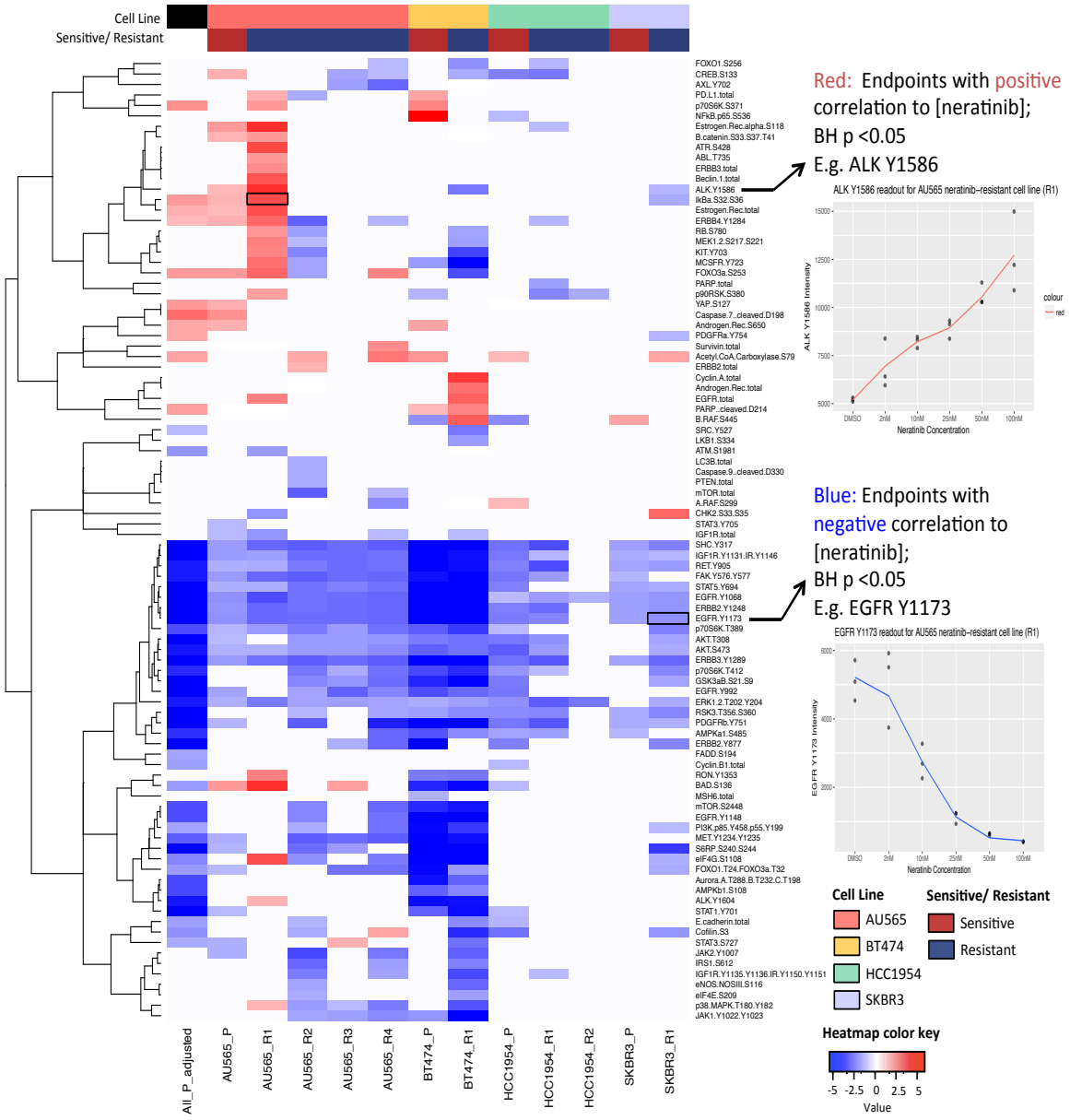

Figure 3.5 | Inhibition of PI3K, AKT or MEK was insufficient to restore neratinib sensitivity. a) Selected inhibitors target different effectors within the PI3K/AKT and Ras/Raf/MEK/ERK effector pathways downstream of HER2. b) Plots of AKT S473 and ERK T202/Y204 intensities (by reverse phase protein array) for AU565 parental cell line and its resistant derivatives (AU565R1-R4) when treated with varying concentrations of neratinib for 1 hr. Residual AKT S473 and ERK T202/Y204 signaling in resistant cell lines at higher neratinib concentrations (50nM/ 100nM). c) Viability of AU565 parental cells and resistant derivatives (R1 – R4) treated with DMSO or the indicated drug treatments for 120 h; results were normalized to those obtained with DMSO. * $P < 0.01$. Data are representative of three independent experiments (b-d); error bars b-d), s.d.

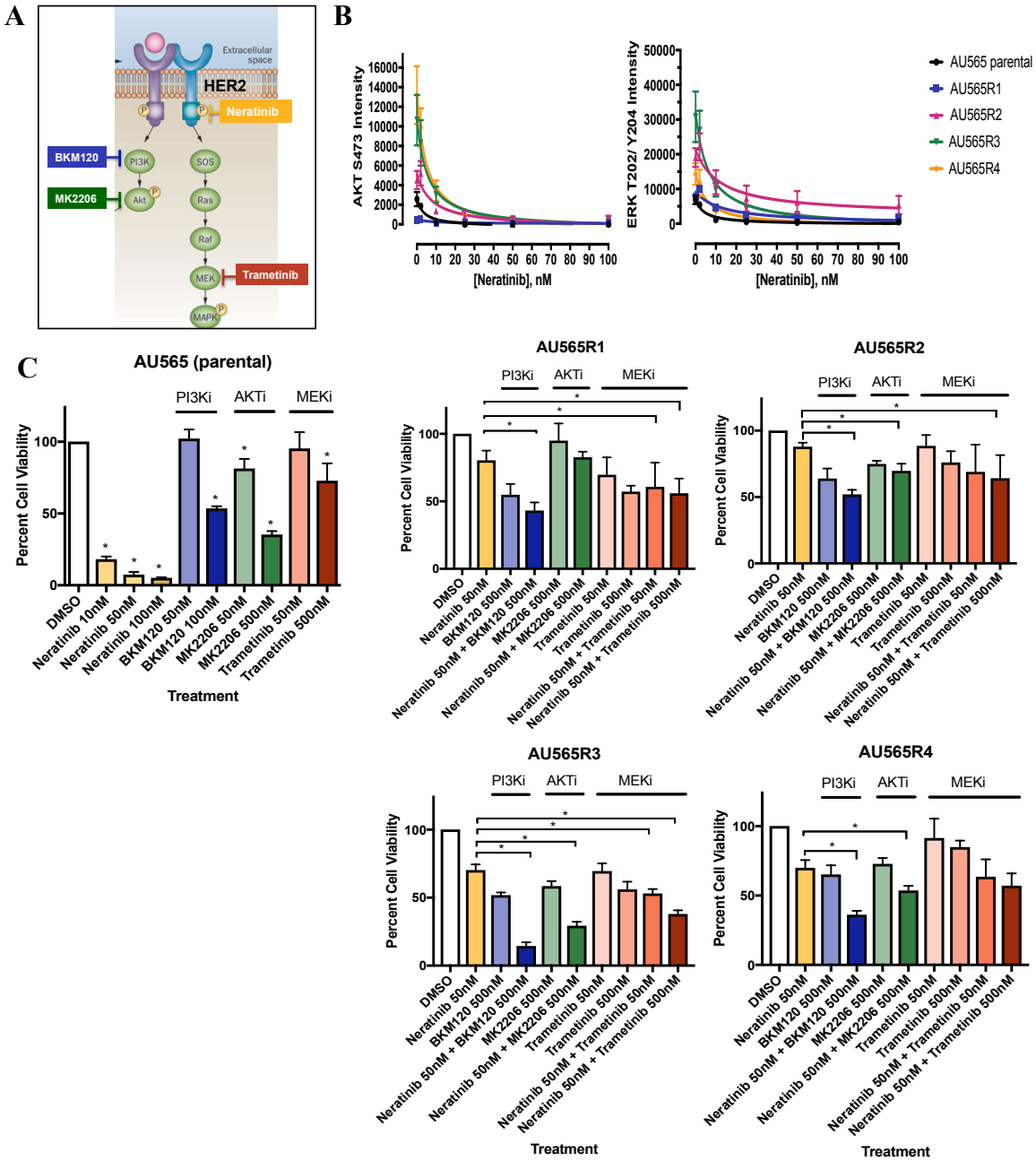

Figure 3.6 | a) Proposed mechanism by which HER2-positive breast cancer cell lines develop acquired resistance to neratinib. Neratinib induces transcriptional up-regulation of multiple alternative kinases capable of bypassing ERBB2-directed signaling. We hypothesize that by inhibiting the BET family of bromodomain-containing acetylation readers using JQ1 (a potent inhibitor of the BET family of bromodomain proteins), the neratinib-induced kinome adaptation can be prevented at an epigenetic level (image adapted from Stuhmiller T.J. et. al. *Cell Reports*. (2015)[52]) b) Viability of AU565 resistant cell lines (AU565R1-R4) treated with DMSO, JQ1, neratinib or combination of JQ1 and neratinib at the indicated concentrations for 120 h; results were normalized to those obtained with DMSO. Data are representative of three independent experiments. * $P < 0.05$; error bars, s.d.

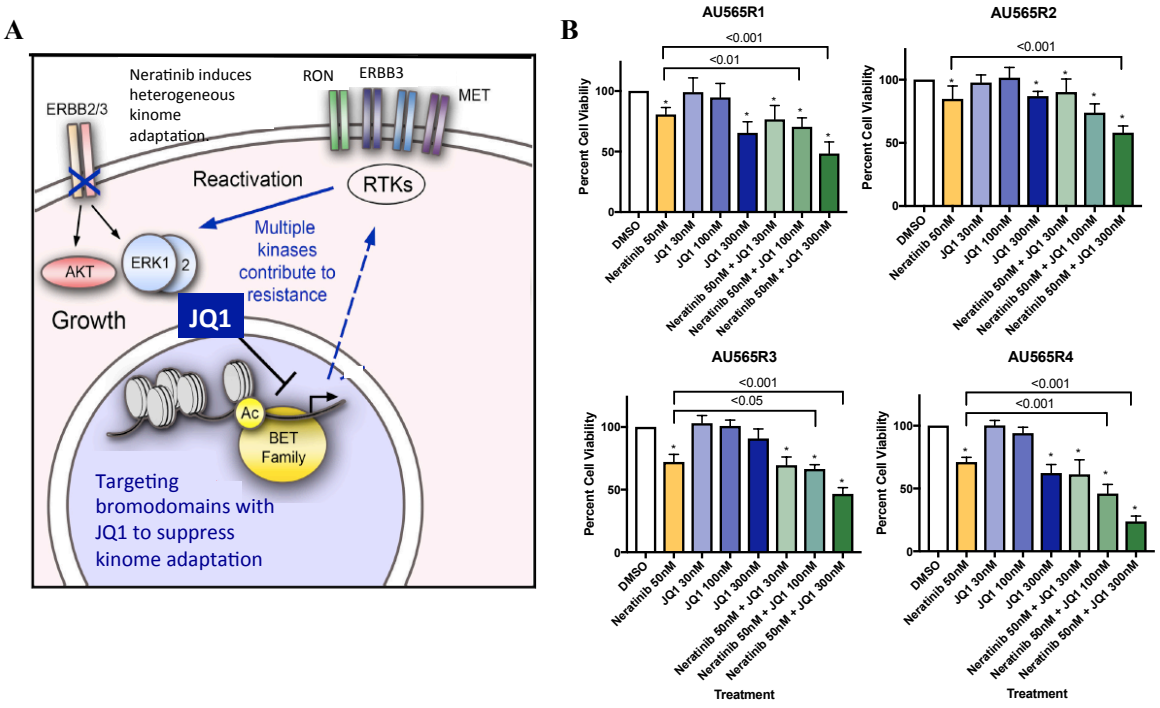

Table 3.1 | 104 phosphoproteins/ protein endpoints included in reverse phase protein array (RPPA) from different biological pathways commonly dysregulated in breast cancer.

HER family signaling pathway	MAPK signaling pathway	Hormonal receptors	JAK/ STAT signaling
EGFR total	A-RAF S299	Androgen Rec S650	JAK1 Y1022/Y1023
EGFR Y1068	B-RAF S445	Androgen Rec total	JAK2 Y1007
EGFR Y1148	ERK1/2 T202/Y204	Estrogen Rec alpha S118	STAT1 Y701
EGFR Y1173	MEK1/2 S217/S221	Estrogen Rec total	STAT3 S727
EGFR Y992	p90RSK S380		STAT3 Y705
ERBB2 total	RSK3 T356/S360		STAT5 Y694
ERBB2 Y1248	**Cell Proliferation**	**DNA Damage/ Repair**	**Other RTKs**
ERBB2 Y877	Cyclin A total	ATM S1981	AXL Y702
ERBB3 total	Cyclin B1 total	ATR S428	KIT Y703
ERBB3 Y1289	Cyclin D1 total	CHK1 S345	MET total
ERBB4 total	Histone H3 S10	CHK2 S33/S35	MET Y1234/Y1235
ERBB4 Y1284	Ki67 total	MSH6 total	PDGFRb Y751
Heregulin total	p27 T187	p53 S15	RET Y905
SHC Y317	Rb S780	PLK1 T210	RON Y1353
PI3K/AKT/mTOR signaling pathway	**Apoptosis**	**Cancer Metabolism**	**Other kinases**
AKT S473	BAD S136	Acetyl CoA Carboxylase S79	ABL T735
AKT T308	Caspase 7, cleaved D198	AMPKa1 S485	ALK Y1586
FOXO1 S256	Caspase 9, cleaved D330	AMPKb1 S108	ALK Y1604
FOXO1 T24/FOXO3a T32	FADD S194	IGF1R total	Aurora A T288/B T232/C T198
FOXO3a S253	PARP total	IGF1R Y1131/IR Y1146	SRC Y527
GSK3aB S21/S9	PARP, cleaved D214	IGF1R Y1135/Y1136-IR Y1150/Y1151	
mTOR S2448	Survivin total	IRS1 S612	
mTOR total		LKB1 S334	
p70S6K S371	**Autophagy**	**Metastasis**	**Transcription regulators**
p70S6K T389	Beclin 1 total	Cofilin S3	CREB S133
p70S6K T412	LC3B total	FAK Y576/Y577	YAP S127
PI3K p85 Y458-p55 Y199	**Inflammation**	FOXM1 T600	
eIF4E S209	IkBa S32/S36		
eIF4G S1108	MCSFR Y723	**Epithelial to Mesenchymal Transition**	**Others**
PTEN S380	NFkB p65 S536	E-cadherin total	eNOS/NOSIII S116
PTEN total	p38 MAPK T180/Y182	B-catenin S33/S37/T41	PD-L1 total
S6RP S240/S244	PDGFRa Y754		

Table 3.2 | 13 phosphoproteins/ proteins endpoints (of the 104 endpoints tested) associated with neratinib sensitivity at baseline (adjusted $P < 0.05$ in a logistic regression model adjusted for cell line of origin and applying multiple testing correction; see Methods). Of these 13 phosphoproteins/ proteins, 4 of them were associated with pathological complete response (pCR) in the I-SPY 2 TRIAL[22].

Protein endpoints	p-value	Adjusted p value (BH)	Tested as qualifying biomarker in I-SPY 2	Associated with pCR in I-SPY 2
ERBB2 Y877	4.84E-05	0.00315		
ERBB2 Y1248	6.40E-05	0.00315	Yes	Yes
EGFR Y1173	9.07E-05	0.00315	Yes	Yes
EGFR total	0.000601	0.0156	Yes	
p70S6K T412	0.000952	0.0167		
ERBB3 Y1289	0.00107	0.0167	Yes	
STAT5 Y694	0.00113	0.0167		
MET total	0.00153	0.0198		
IRS1 S612	0.00194	0.0212		
p70S6K T389	0.00204	0.0212		
EGFR Y1068	0.00237	0.0224	Yes	Yes
ERBB2 total	0.00372	0.0322	Yes	Yes
p27 T187	0.00577	0.0461		

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