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Original Articles

A pathway-based approach for identifying biomarkers of tumor progression to trastuzumab-resistant breast cancer

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ABSTRACT

Although trastuzumab is a successful targeted therapy for breast cancer patients with tumors expressing HER2 (ERBB2), many patients eventually progress to drug resistance. Here, we identified subpathways differentially expressed between trastuzumab-resistant vs. -sensitive breast cancer cells, in conjunction with additional transcriptomic preclinical and clinical gene datasets, to rigorously identify overexpressed, resistance-associated genes. From this approach, we identified 32 genes reproducibly upregulated in trastuzumab resistance. 25 genes were upregulated in drug-resistant JIMT-1 cells, which also downregulated HER2 protein by >80% in the presence of trastuzumab. 24 genes were downregulated in trastuzumab-sensitive SKBR3 cells. Trastuzumab sensitivity was restored by siRNA knockdown of these genes in the resistant cells, and overexpression of 5 of the 25 genes was found in at least one of five refractory HER2 + breast cancer. In summary, our rigorous computational approach, followed by experimental validation, significantly implicate *ATF4, CHEK2, ENAH, ICOSLG*, and *RAD51* as potential biomarkers of trastuzumab resistance. These results provide further proof-of-concept of our methodology for successfully identifying potential biomarkers and druggable signal pathways involved in tumor progression to drug resistance.

Introduction

Worldwide, breast cancer kills over 521,000 women annually, associated with over 1.7 million new cases [1]. Approximately 25% of cases express the human epidermal growth factor receptor-2 (HER2, ERBB2), a 185-kDa transmembrane tyrosine kinase. HER2 has no known ligand, but plays a major role in the progression of breast and other cancers [2]. A humanized monoclonal antibody against HER2, trastuzumab (Herceptin®), was approved for the treatment of HER2-positive breast cancer in 1998 [3]. The HERA (Herceptin Adjuvant) phase III trial showed that in early HER2-positive breast cancer patients, one-year trastuzumab therapy following surgery resulted in 32% greater disease-free survival [4]. However, an additional 1 year of adjuvant trastuzumab therapy showed no additional benefit [5]. While the therapy is initially effective against metastatic breast cancer, drug-resistant relapse is almost universal [6]. Due to the cost vs. benefit of trastuzumab, and the emerging study of other HER2-targeted therapies, drug-response-predictive biomarkers are strongly needed.

A number of studies have now reported various possible cellular/molecular mechanisms of trastuzumab resistance, largely from comparing sensitive vs. resistant breast cancer cell lines. Of these is overactivity of the mitogenic PI3K/Akt pathway. In particular,

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activating mutations in PI3K and loss of its antagonist, PTEN, have been identified in both preclinical [7,8] and clinical [9,10] studies of trastuzumab-resistant breast cancer. However, another study, examining both trastuzumab-treated and -untreated patients over three years, found no association of resistance with either of these PI3K pathway members [11]. Other reported resistance contributors include DARPP, HSP90, cytosolic sequestration of the cell cycle inhibitor CDKN1B ($p27^{KIP1}$), and downregulated tubulin III [3,12,13].

In addition to the above-described intracellular signal mediators, several cell surface transmembrane proteins and kinases have been implicated in trastuzumab resistance via either indirect or direct interaction with HER2. These include the hyaluronate receptor CD44, MUC4, c-MET, integrins α6β1 and α6β4, and HER2 heterodimerization with other EGFR family members [3]. As CD44 expression combined with low or absent CD24 has been reported to demarcate breast "cancer stem cells" [14] it has also been hypothesized that trastuzumab resistance may arise from a failure to eradicate tumor progenitors [3]. Analogously, the cancer stem cell-associated epithelial-to-mesenchymal transition has also been implicated in trastuzumab resistance [15].

In the current study, rather than examining HER2 signal effectors or pathways in isolation, we used a systems biology [16–18] approach, and applied our recently established subpathway identification [16] and network permutation method [17], which resulted in identification of 32 upregulated KEGG subpathway genes common to two preclinical and two clinical datasets. This discovery method allows the consideration of pathway "edges" as well as individual "nodes" [16]. The roles of these genes in trastuzumab resistance were experimentally validated by their upregulation in refractory HER2 + breast cancer cells and tumors. Based on these results, we believe our subpathway and network permutation approach is effective for identifying biomarker genes and pathways responsible for resistance to trastuzumab and other targeted, antineoplastic agents.

Materials and Methods

Trastuzumab-resistant network and its reproducibly upregulated, resistance-associated genes

To delineate a trastuzumab resistance network, we applied an association rule [16], based on gene expression and KEGG pathways, to identify all possible subsets of signaling pathways. This rule was applied to a preclinical dataset [GEO: GSE15043] composed of two BT474 drug-sensitive parent cells and eight BT474 daughter subclones selected for resistance by continuous growth in 0.2 and 1.0 μM trastuzumab [19]. The subsets, i.e., subpathways, included not only nodes but also edges such as activation or inhibition among the nodes [16]. The subpathways associated with drug resistance are represented by a network (Fig. 1), visualized using Cytoscape [20]. This network consisted of 4502 subpathways, with 916 gene entries. The statistical significance of the network was measured by "the mean correlation over all edges in the network", henceforth designated as "r" [17,21], to describe interactions between entities, rather than a single entity [22]. A correlation of an edge was calculated from the Pearson correlation coefficient of the expressions between two neighboring nodes. The r value was obtained from the mean of the correlations of all the edges. With the network topology conserved, we randomly permuted the node labels in the network to obtain the null distribution of the statistic r. The true mean correlation prior to permutation was set to r_0. We performed 5000 permutations, and the empirical p-value was calculated from $Pr(r > r_0)$.

Reproducible gene entries belonging to the network were identified using multiple trastuzumab-resistant preclinical ([GEO: GSE15376] [23] [ArrayExpress: E-TABM-157] [24]) and resistance-associated clinical datasets. We also compared Herceptin-nonresponsive and -responsive cells from the two preclinical datasets. The HER2-positive control cells, SKBR3 and BT474 are Herceptin-responsive, while two HER2-negative control cell lines, MCF7 and MDA-MB-231 are Herceptin-nonresponsive [25]. Additional clinical datasets were used to assess survival associated with gene expression profiles in HER2 + recurrent breast cancer from The Cancer Genome Atlas (TCGA) [26] dataset under the heading "Breast Invasive Carcinoma (TCGA, Nature 2012)" provided by cBioPortal [27], including 47 disease-free HER2 + patients vs. 7 recurred HER2 + patients, and the Netherlands Cancer Institute dataset contained 25 disease-free HER2 + patients vs. 24 recurrent HER2 + patients [28]. These four trastuzumab resistance-associated upregulated gene intersections (Fig. 2) resulted in the identification of 32 genes (Table 1) with their corresponding KEGG pathways listed in Appendix S1: Supplementary Table S1, including "Hallmarks of Cancer" in Appendix S1: Supplementary Table S2.

We performed overall survival analyses, based on 66 TCGA HER2 + breast cancer patients. The 66 patients were selected based on PAM50 classification that was annotated in the dataset TCGA_BRCA_exp_HiSeqV2-2014-05-02 (column PAM50Call_RNAseq in file clinical_data) from the UCSC Cancer Genomics Browser (https://genome-cancer.ucsc.edu) [29]. The expression subset of the 32 genes among the 66 patients was extracted from the dataset, and we divided the patients into two groups (high, low) in terms of the expression of each gene in the subset: gene expression greater than the median expression of the patient was denoted as "high;" otherwise it was denoted "low." Subsequently, we performed log-rank tests between the two groups for each gene. We also generated Kaplan–Meier curves for the gene *MMP9* as well as *PER2*, a gene of interest due to the recent emergence of circadian rhythm dysfunction as a risk factor for breast cancer [30].

Procurement and processing of tumor tissues

Patients consented to the use of the tissue specimens for research purposes, as approved by the Institutional Review Board of National Cancer Center, Republic of Korea (Ilsan, Korea). Histologic classification and tumor stage were reviewed by a pathologist in the Department of Pathology at the National Cancer Center. Included in our analysis were eight breast tumor samples. Clinical characteristics of 6 out of the 8 patients, including Herceptin sensitivity, are summarized in Table 2 (Appendix S1: Supplementary Fig. S1A and S1B).

Cell lines and reagents

We obtained human breast cancer cell lines SKBR3 and JIMT-1 cells from the American Type Culture Collection (ATCC, Manassas, VA, USA) and The German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). ATCC and DSMZ uses short tandem repeat profiling to authenticate all cell lines [31]. Reagents used on SKBR3 and JIMT-1 breast cancer cells, including a HER2 monoclonal antibody, were purchased from Abgent (San Diego, CA).

Cell culture

SKBR3 and JIMT-1 human breast cancer cell line studies were performed within six months of cell resuscitation. SKBR3 cells were grown in McCoy's 5A medium (Sigma-Aldrich, St. Louis, MO) and JIMT-1 cells were grown in DMEM (Hyclone, Logan, UT) with 10% FBS (Hyclone) at 37 °C in 5% CO_2. Cells (2.5×10^5) were seeded and grown under normoxic conditions to 70–80% confluency, and then incubated with or without 10 μg/ml trastuzumab [32] for up to 4 days, according to the required time study.

Western blotting

Cells grown with or without 10 μg/ml trastuzumab were harvested, washed twice in phosphate-buffered saline solution, lysed, and subjected to Western blot, as we previously described [33]. The blots were quantified using Image Lab software (Bio-Rad, Hercules, CA).

Real-time RT-PCR analysis

Total RNA was isolated from cell lysates using Isol-RNA Lysis Reagent (5PRIME, Hamburg, Germany) and processed with ReverTra Ace® qPCR RT Master Mix with gDNA Remover Kit (Toyobo, Osaka, Japan) to synthesize cDNA. Quantitative PCR was conducted using iQ™ SYBR® Green Supermix (Bio-Rad), according to the manufacturer's protocol, using a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad). Primer sequences were obtained from RTPrimerDB (http://rtprimerdb.org/), designed via GenScript Real-time PCR (TaqMan) Primer Design (https://www.genscript.com/ssl-bin/app/primer) or manually designed, as indicated in Appendix S1: Supplementary Table S3. Measurements of all 32 genes were initially analyzed. Twenty-five met the following quality standards: (1) No template control of primer sets must read a null (no signal detected) Ct value. (2) Resulting Ct value must be between 32 and 37. Normalization procedures and folds-change were carried out using β-actin, as we have previously reported [33] using the 2(-delta-delta C(T)) method as the internal reference [34]. P-values for tumor samples were calculated between each trastuzumab-resistant patient sample with each of the trastuzumab-responding patient sample. Trastuzumab-resistant patient samples with statistically significantly (i.e. $p < 0.05$ with all three trastuzumab-responding patients)