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NSABP B-41, a Randomized Neoadjuvant Trial: Genes and Signatures Associated with Pathologic Complete Response

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Abstract

Purpose—In NSABP B-41, pathologic complete response (pCR) was associated with prolonged survival among women with HER2-positive operable breast cancer treated with neoadjuvant chemotherapy and lapatinib, trastuzumab, or the combination. We used a large human breast cancer gene expression panel to select candidate prognostic biomarkers for pCR among women treated with trastuzumab in NSABP B-41.

Patients and Methods—Eligible patients had a baseline pre-adjuvant treatment core biopsy sample, known pCR status, and no withdrawal of consent. We analyzed extracted RNA using the human nCounter® Breast Cancer 360™ gene expression panel. Gene counts were normalized to

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housekeeping genes and transformed into logarithmic scale with base two. To screen for candidate genes and meta-gene signatures prognostic of pCR, we used univariate logistic regression. Variable selection was done by multivariable logistic regression with lasso regularization.

Results—Analyses of data from 130 patients revealed that a composite of gene expression from 19 genes and one gene signature appeared to predict pCR in women with HER2-positive early-stage breast cancer undergoing neoadjuvant chemotherapy with trastuzumab-containing regimens. The identified genes are involved in important pathways such as epithelial-mesenchymal transition, adhesion and migration, estrogen receptor signaling, DNA damage and repair, apoptosis, and proliferation. The AUC from a 10-fold cross validation on predicting pCR, with these 20 genomic markers in a logistic regression model, was 0.73.

Conclusions—The expression level of *ERBB2, ESR1*, and few other genomic markers was highly predictive of pCR after trastuzumab-containing regimens. These findings need to be validated and calibrated in future studies.

Keywords

Neoadjuvant; breast cancer; genomic; biomarker; trastuzumab

Introduction

Neoadjuvant treatment in patients with HER2-positive breast cancer has been an excellent model for studying highly effective HER2-targeted therapy. Several studies have shown that tumors that are HER2-enriched (HER2E), as assessed by Prediction Analysis of Microarray 50 (PAM50) intrinsic subtypes, are associated with a higher pathologic complete response (pCR) rate (1–6). One of these studies has correlated this finding with a trend for increased event-free survival (EFS) (2). An analysis of baseline tumor samples from two clinical trials found that combined analysis of HER2E subtype and *ERBB2* mRNA into a single assay identified tumors with high responsiveness to HER2-targeted therapy (7).

The Cancer Genome Atlas (TCGA) molecular analyses of primary breast cancer tumors (8) have provided the foundation for numerous attempts to define other genes and gene signatures that are prognostic for pCR. In the Cancer and Leukemia Group B (CALGB) 40601 study, multivariable models revealed an association between pCR and *ESR1* and *ERBB2* gene expression, p53 *(TP53)* mutation signature, and immunoglobulin G signature, but no association with intrinsic subtype signature and clinical levels of estrogen receptor (ER) or HER2 (1). In all treatment arms of the NeoALTTO study, high *ERBB2*/HER2 expression and low *ESR1* expression were significantly associated with higher pCR rates; whereas only in the combination arm, high expression of immune gene signatures was positively associated with pCR and high expression of the stroma gene signatures was negatively associated with pCR (4). In a subsequent genomic analysis of samples from CALGB 40601, tumor genetics (mutations, DNA copy number alterations), tumor mRNA subtype (HER-E, luminal), and immune microenvironment (B-cell features) were independent predictors of pCR in patients treated with regimens containing trastuzumab and paclitaxel (9).

The National Surgical Adjuvant Breast and Bowel Project (NSABP) B-41 open-label, phase 3 randomized neoadjuvant trial compared trastuzumab, lapatinib and the combination in HER2-positive, operable breast cancer patients treated with concurrent standard neoadjuvant chemotherapy (10). From 2007 to 2011, 529 patients were enrolled. The results showed no statistically significant differences in pCR rate and EFS, but patients whose tumors achieved a pCR had a better outcome than those who did not (10, 11). Similar to other investigators, we previously reported that patients with HER2E tumors achieved a higher pCR rate, especially in the trastuzumab-containing arms of NASBP B-41 (12).

In the present, more detailed evaluation of the NSABP B-41 trastuzumab-containing arms, we have used a large human breast cancer gene expression panel to find other candidate biomarkers prognostic of pCR in women with early-stage HER2-positive, operable breast cancer treated with neoadjuvant chemotherapy combined with single or dual HER2-targeted therapy. The primary objectives of the present analysis were to determine the prognostic utility of these genomic signatures for pCR and their predictive ability for treatment benefit from the addition of lapatinib to trastuzumab in this patient population. A secondary objective was to determine the utility of genes and gene signatures in predicting EFS. This report adheres to REMARK criteria (Supplementary Table S1) (13).

Materials and Methods

Study design and patients

The NSABP B-41 study design and eligibility criteria have been previously reported (10). All patients received neoadjuvant chemotherapy with four cycles of standard doxorubicin and cyclophosphamide (AC) followed by four cycles of weekly paclitaxel. Concurrent with the weekly paclitaxel, patients were randomized to receive either weekly trastuzumab, daily lapatinib, or both lapatinib and trastuzumab until surgery. The study's primary endpoint was pCR in the breast and lymph (nodes, defined as absence of any invasive component in the resected breast specimen and absence of cancer in all resected lymph nodes after neoadjuvant therapy, (ypT0/Tis ypN0) (14). One secondary endpoint was EFS, defined as time from randomization to the first occurrence of local, regional, or distant recurrence, contralateral breast cancer, second primary cancer, or death from any cause. Also, a comparison was undertaken between pre- and post-treatment biopsy paired samples in patients treated with trastuzumab with or without lapatinib. During the B-41 trial, tumor specimens were collected and processed at local sites. Patients eligible for this correlative science study had to have a baseline core biopsy sample, known pCR status and no withdrawal of consent. A study protocol with objectives and statistical analysis plan for this correlative science project was submitted to the NSABP Foundation for the permission to use these tissue samples prior to further sample process and assay. At entry to the B-41 trial, all participants signed informed consent and allowed their tissue samples to be used in the future for the purpose of the study, including the development of molecular predictors of pCR.

RNA isolation

For each patient, the NSABP Department of Pathology produced serial 10 μM sections from selected formalin-fixed paraffin-embedded (FFPE) tumor samples and sent anonymized, matched unstained slides as well as hematoxylin and eosin (H&E) stained slides to the Genomics and Epigenomics Shared Resource (GESR) at Georgetown University Medical Center (GUMC). At the GUMC Histopathology and Tissue Shared Resource (HTSR), the samples underwent pathological examination to confirm diagnosis and identify malignant tissue. Using the matching H&E slides as templates, tumor-containing areas were macrodissected from the unstained slides and processed for RNA isolation.

After deparaffinization, the macrodissected tissues were processed using the Roche High Pure FFPET RNA Isolation Kit (Roche Molecular Systems, Pleasanton, CA). The concentration of the extracted RNA was estimated by ultraviolet-visible spectrophotometry (NanoDrop 1000 spectrophotometer; Thermo Fisher Scientific, Waltham, MA) to ensure sample purity (optical density 260/280 nm ratio 1.7–2.5). We assessed RNA quality using the Agilent RNA 6000 Nano Kit with the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) and the degree of RNA integrity with the Agilent 2100 Expert Software, as previously described (12).

Gene expression profiling

From each sample, 100 ng of RNA was hybridized to the human nCounter® Breast Cancer 360™ (BC360) gene expression panel (NanoString Technologies, Seattle, WA), and processed on the nCounter® SPRINT Profiler (NanoString Technologies, Seattle, WA) according to manufacturer protocols. The BC360 panel includes 776 individual genes, including 18 housekeeping genes for normalization, and 40 gene signatures associated with breast cancer signaling pathways and biological processes (15–18). Our analyses of BC360 data focus primarily on the 758 unique, non-housekeeping genes, and the 40 gene signatures. The nCounter® SPRINT Profiler system generates simultaneous, multiplexed digital measurements (i.e., reporter-code-counts) of the relative abundance of mRNA transcripts using sequence-specific probes; a reporter probe tagged with a target-specific, four-color, six-position fluorescent barcode; and a capture probe to immobilize the complex for data collection (19, 20). The reporter-code-count files for each patient sample were forwarded to NanoString Technologies (Seattle, WA) for analysis. The raw count data were log2-transformed and normalized to housekeeping genes. These data were used to calculate the PAM50 subtype calls and BC360 single and meta-gene signature scores for each sample using proprietary algorithms.

Statistical analysis

To determine if the included patient samples were representative, we used the Pearson's Chi-square test to compare treatment and stratification factors (i.e., age, clinical nodal status, clinical tumor size, estrogen receptor (ER) and progesterone receptor status (PR) between the included and excluded patient populations. The primary analyses focused on patient samples from the trastuzumab-containing regimens because the lapatinib-alone arm had a lower pCR rate than the trastuzumab-containing arms (10). Also, the outcomes from the

trastuzumab-containing arms would be more relevant to inform the present clinical management of patients with early stage HER2-positive breast cancer.

In the primary analyses, we initially screened candidate gene signatures and individual genes that were prognostic of pCR among patients treated with trastuzumab-based regimens. The BC360 analysis includes 34 carefully curated meta-gene signatures and select single genes. For each of the BC360 meta-gene signatures or single gene expression other than the PAM50 output of basal-like, HER2-enriched, luminal A, and luminal B subtype scores, we used univariate logistic regression model to obtain the *P* value and odds ratio (OR) associated with pCR. These signatures were subsequently ranked based on the *P* values. We then applied the Benjamini-Hochberg procedure to identify candidate signatures for pCR with the false discovery rate (FDR) controlled at 0.1 (21). We used the Holm's step-down procedure to identify gene signatures statistically significantly prognostic for pCR with a familywise error rate (FWER) controlled at 0.05 (22). Similarly, we obtained a list of candidate genes among the 758 genes on the BC360 panel with FDR controlled at 0.1. We entered the selected genes and gene signatures as predictors in a multivariate logistic regression model and applied the lasso regularization to select a final multivariate model for the prognosis of pCR. To assess the performance of this multivariate model, we used the area under the receiver operating characteristic (ROC) curve from 10-fold cross validation.

We compared the prognostic utility of the following conventional clinical factors: treatment (trastuzumab plus lapatinib plus chemotherapy, trastuzumab plus chemotherapy), ER (positive, negative), clinical nodal status (positive, negative), clinical tumor size (in cm), and tumor grade (well, moderate, poor) to the prognostic utility of known genes and gene signatures: *ESR1*, *ERBB2*, and Tumor Inflammation Signature (TIS). We then combined significant clinical factors and genomic markers in a multivariate logistic regression and evaluated whether clinical factors or genomic markers supplement each other in the prognosis of pCR among trastuzumab-treated patients. To compare these models, we used the likelihood ratio test between the nested model and a sub-model, and the Akaike information criterion (AIC) between non-nested models (23). Logistic regression models with treatment, individual gene expression, and their interaction as covariates were used to screen for potential predictive genetic markers for the benefit of the addition of lapatinib to trastuzumab among patients treated with trastuzumab-containing regimens.

In the wheel plots that describe expression profiles of each sample, signature scores were mapped to the empirical distribution of the calibrated breast invasive cohort data in TCGA using quantile normalization. Paired *t*-test was used to compare scores from pre- and post-treatment.

Results

A total of 219 tissue samples from 202 patients enrolled in NSABP B-41 were available, including 194 baseline samples from core biopsy prior to neoadjuvant therapy and 25 samples from surgical specimens after neoadjuvant therapy (21 from breast and four from lymph node material). All analyses were based on data from the 194 patients with core biopsy breast tissue samples prior to neoadjuvant therapy. Their median follow-up time was

5.2 years. As shown in Fig. 1, 69 patients had been randomized to neoadjuvant chemotherapy plus trastuzumab, 64 to chemotherapy plus lapatinib, and 61 to chemotherapy plus trastuzumab and lapatinib. Supplementary Fig. S1 shows the heatmap of mean expression levels of the BC360 single gene and meta-gene signatures across the 194 samples. Comparison of treatment and stratification factors between the patients included and not included in the analyses revealed no statistically significant differences, with P values of the Pearson's Chi-square test ranging from 0.1 to 0.87 (Supplementary Table S2).

Selection of biomarkers for pCR

In exploratory analyses of the gene signatures measured by BC360, we compared the mean gene expression differences between patients who did or did not achieve pCR following treatment with trastuzumab-containing (Fig. 2A) or lapatinib-only (Fig. 2B) regimens. Among patients randomized to trastuzumab-containing regimens, TIS, p53, hypoxia, and basal-like meta-gene signatures, and *IDO1* single gene were overexpressed in the pCR group; mast cell abundance, luminal B, ER signaling meta-gene signatures, and *B7-H3 (CD276), ESR1* single genes were overexpressed in the non-pCR group. When analyzing all 194 patients there were significant differences between patients with or without pCR in the features where the confidence intervals did not include 0. (Supplementary Table S3). For example, on average, the expression level of ESR1 was lower in patients with pCR than that in patients who did not achieve pCR while the opposite was the case for *IDO1* and *ERBB2* single genes or cytotoxic cell and TIS meta-gene signatures. There was no interaction between pCR status and treatment when comparing trastuzumab-containing arms versus the lapatinib only arm in all genes or signatures except *ERBB* (Unadjusted p=0.025, Supplementary Table S4).

In the primary analyses, we focused on data from 130 patients on trastuzumab-containing regimens. Applying the Benjamini-Hochberg procedure on results from univariate logistic regression models on the prognosis of pCR, we identified 10 candidate gene signatures out of 34 evaluated with FDR controlled at 0.1 (Table 1). *ERBB2* and *IDO1* were the top candidates with adjusted P values < 0.05. Among these, cytotoxic cells, cytotoxicity, and TIS signatures as well as *IDO1, PD1 (PDCD1)*, and *TIGIT* single genes were highly correlated, with coefficients ranging from 0.55 to 0.93 (data not shown). The correlation coefficient between *IDO1* and TIS was 0.91. Using the Benjamini-Hochberg procedure, we also identified 38 genes among the 758 individual, non-housekeeping genes on the BC360 panel, with FDR controlled at 0.1 (Supplementary Table S5). Among these 38 genes, *ERBB2*, *IDO1*, *ESR1* and *PGR* were also among the selected genes we identified in the primary analyses. In total, we found 44 individual genes or gene signatures whose expression levels were potentially prognostic for pCR.

With these 44 biomarkers as prognostic factors, we performed multivariate logistic regression models and applied lasso regularization for model selection to narrow the list to 19 individual genes and one gene signature (Table 2). These selected genes are involved in important pathways such as epithelial-mesenchymal transition (*HEMK1*, *GRB7*, *ERBB2*, *TMPRSS4*), adhesion and migration (*ITGB6*, *COL27A1*, *NRCAM*), ER signaling (*ELOVL2*, *IFT140*, *MAPT*), DNA damage and repair (*NPEPPS*, *PRKDC*), apoptosis

(*BCL2*), and proliferation (*TFDP1*). The AUC from a 10-fold cross validation on predicting pCR, with these 20 genetic markers in a logistic regression model, was 0.73 (Supplementary Fig. S2).

Similar univariate analyses were also performed to screen for potential prognostic markers for pCR among patients who were on the lapatinib-only regimen. With FDR controlled at 0.1, none of the gene signatures or genes were selected as candidate prognostic markers. The odds ratios, their 95% confidence intervals and unadjusted p-values for the top five meta-gene signatures and top 10 genes are presented in Supplementary Table S6. Only IFT140, ZNF205 and TCEAL1 appeared in the list of top genes for trastuzumab-containing regimens in Supplementary Table S5.

Clinical and genetic predictors of pCR

Table 3 summarizes the results from four multivariate logistics regression models for predicting pCR. In model 1, which investigated the predictive value of clinical factors (ER status, clinical nodal status, clinical tumor size, tumor grade), only ER status was a statistically significant predictor of pCR (OR = 0.46, 95% CI = 0.22–0.99; $P = 0.05$). In model 2, which tested the prognostic value of two known genes (*ESR1, ERBB2)* and a signature (TIS), both *ERBB2* (OR = 1.74, 95% CI = 1.27–2.38; $P < 0.001$) and TIS (OR = 1.75, 95% CI = 1.18–2.59; $P = 0.006$) were strong predictors, and *ESR1* (OR = 0.87, 95% CI = 0.73–1.02, $P = 0.09$) was a marginal predictor. In model 3, which tested the combined predictive value of ER and the three genes in model 2, there was no evidence that ER status ($P = 0.24$) provided any independent information in predicting pCR beyond the three genetic markers. *ESR1* was differentially expressed across ER status with respective mean and standard deviations of −5.0 and 0.88 among patients with ER-negative tumors, and −1.1 and 1.79 among patients with ER-positive tumors. In model 4, HER2 subtype (vs other subtypes) replaced *ERBB2* in model 2. Model 4 is a better fitted model than model 2 in predicting pCR because the AIC for model 4 is 146.8, which is smaller than that for model 2, 157.4.

In order to illustrate how the results of model 2 could be used in clinical practice, we selected expression levels of the three genetic markers over their dynamic ranges: *ESR1* (from −5, −2, 1), *ERBB2* (from 1, 3, 5), and TIS (from 5.5, 7, 8.5). Fig. 3 shows the predicted chance of pCR for patients with various gene expression profiles for HER2-positive breast cancer treated with neoadjuvant chemotherapy plus a trastuzumab-containing regimen.

Predictive utility of pCR with genetic markers

In the parent NSABP B-41 study, patients randomized to trastuzumab plus lapatinib had a higher pCR rate than those randomized to trastuzumab-alone (60.2% vs. 49.4%, $P = 0.056$) (10). In the present analysis, we used a logistic regression model for predicting pCR with treatment (trastuzumab plus lapatinib vs. trastuzumab), individual genes or signatures, and their interaction, to screen for potential predictive markers for benefit from the addition of lapatinib among the 758 genes and 34 signatures on the BC360 panel. We were not able to identify any candidate predictive markers with FDR controlled at 0.1 (data not shown). The top three genes on the list were *CCL21* (unadjusted $P = 0.007$), *PRLR1 (PRLR)* (unadjusted

$P = 0.015$), and *PTGDS* (unadjusted $P = 0.03$); the top three BC360 single or meta-gene signatures were p53 meta-gene signature (unadjusted p=0.08), *PD1* single gene expression (unadjusted p=0.13) and apoptosis meta-gene signature (unadjusted p=0.15). TIS did not predict treatment benefit in pCR from the additional lapatinib (unadjusted p=0.19).

ERBB2 gene expression was a statistically significant predictor of pCR among the 130 patients treated with trastuzumab-containing regimen. To study the prognostic utility of *ERBB2* in patients on the lapatinib-only arm, we used a logistic regression model with Pspline for a flexible characterization of the dose-response relationship. The model showed that *ERBB2* was not prognostic of pCR in patients on lapatinib-only regimen ($P = 1$).

Prognosis of EFS

The total number of EFS events were 33 (9 in trastuzumab-alone, 12 in lapatinib-alone and 12 in the combination arm.) Using data from the 130 patients treated with trastuzumab-containing regimens, we used univariate Cox proportional hazards models to study the prognostic utility of individual genes on EFS. None of the 758 individual genes identified using the BC360 panel were promising when FDR was controlled at 0.1. The volcano plot (Supplementary Fig. S3) shows the strength of the relationships between the candidate genes and EFS, along with their unadjusted *P* values.

Paired samples

For 14 of 194 patients, we had gene expression data from paired core biopsy and resected tissue samples. In an analysis of paired samples from the nine patients treated with trastuzumab-containing regimens, the expression levels in pre- and post-treatment samples were strongly correlated for 26% of the 758 non-housekeeping genes on the BC360 panel, with Pearson correlation coefficient > 0.8. Paired t-test identified differential expression levels among many genes; the box plots in Supplementary Fig. S4 show the differences for the top 10 differentially expressed genes. The wheel plots of selected gene signatures in pre- and post-treatment paired samples for two representative patients who did not achieve a pCR are shown in Supplementary Fig. S5. The patterns of signatures are similar in pre- and post-pairs, as expected.

Discussion

In the present exploratory analysis of the NSABP B-41 trastuzumab-containing arms, we used a large human breast cancer gene expression panel to define candidate biomarkers prognostic for pCR in women with early-stage HER2-positive operable breast cancer treated with neoadjuvant chemotherapy combined with single or dual HER2 targeted therapy. We found that TIS, p53, hypoxia, and basal-like signatures and *IDO1* single gene expression were overexpressed in the pCR group; mast cell abundance, luminal B, ER signaling signatures and *B7-H3, ESR1* single gene expression were overexpressed in the non-pCR group. After a variable selection procedure to control the correlation among candidate genomic markers, the combination of 19 genes and one gene signature predicted pCR in women with HER2-positive early-stage breast cancer treated with regimens containing trastuzumab alone or trastuzumab and lapatinib. These markers included pathways such as

epithelial-mesenchymal transition (*HEMK1*, *GRB7*, *ERBB2*, *TMPRSS4*), adhesion and migration (*ITGB6*, *COL27A1*, *NRCAM*), ER signaling (*ELOVL2*, *ESR1*, *IFT140*, *MAPT*), DNA damage and repair (*NPEPPS*, *PRKDC*), apoptosis (*BCL2*), and proliferation (*TFDP1*).

Furthermore, our data show that *ESR1* expression is a more powerful predictor of pCR than the clinical ER status. *ERBB2*, whose amplification defines HER2-positivity, is a natural and established quantitative predictor of pCR in patients undergoing targeted therapy for HER2-positive disease (1, 4). TIS is an 18-gene signature for the pathways associated with a suppressed adaptive immune response (24–26). In our analyses, TIS was overexpressed in patients who had a pCR while undergoing treatment with trastuzumab-containing regimens. Based on a multivariate logistic regression model with *ESR1*, *ERBB2*, and TIS, we could potentially predict the pCR for patients with HER2-positive breast cancer across a large variety of genomic profiles. The predictive pCR values and their confidence intervals could be used by patients and treating physicians to help decide if trastuzumab-containing systemic therapies would be an appropriate option with the estimated chance for achieving pCR.

Although pCR is not an established surrogate marker for long-term clinical outcomes, it is well known that patients who achieve pCR have a better prognosis than those without pCR (27–29). Using data from 11,955 patients with breast cancer treated with neoadjuvant therapies in 12 international trials, Cortazar, et al., showed that the HR for EFS between patients with pCR and without pCR was 0.49 (95% CI 0.33–0.71) in the hormone-receptor–positive, HER2-negative subgroup and 0.39 (95% CI 0.31–0.50) in the HER2-positive subgroup (30). In the NeoALLTO study, the 3-year EFS was 86% for patients with pCR and 72% for patients without pCR (HR = 0.38, 95% CI 0.22–0.63) (31). Therefore, it is important to identify prognostic markers of pCR for patients with HER2-positive tumors being treated with neoadjuvant trastuzumab-containing regimens. Among the conventional clinical factors, only ER status has been found to be associated with pCR in clinical trials (10, 11, 27, 28, 32, 33). The development of genomic markers for pCR has become an urgent need in the pursuit of personalized medicine in clinical practice.

Unlike the NSABP B-41 study (10), in the NeoALTTO study neoadjuvant therapy consisted solely of paclitaxel and HER2 targeted therapy (trastuzumab, lapatinib, or the combination) and then anthracycline-containing therapy after surgery (34). The addition of anthracyclines prior to surgery in the NSABP B-41 study might explain some of the differences in the findings between the two studies. Our current study support an RNA sequencing analysis of samples from the NeoALTTO study which showed that *ESR1* mRNA levels were more predictive of pCR than ER protein levels measured by immunohistochemistry (4). Di Cosimo and colleagues (35) profiled RNA from 226 pretreatment tumor biopsies from the NeoALTTO study to evaluate a trastuzumab risk (TRAR) prediction model based on 41 genes associated with early relapse (36). These authors reported that patients benefiting the most from trastuzumab treatment had tumors with higher levels of *CD8* immune cells, higher *ERBB2* expression, and lower *ESR1* expression (35). Confirming their findings, our analysis of NSABP B-41 samples identified *ERBB2*, *ESR1,* and TIS (which includes the *CD8* gene) as a powerful panel of signatures that are ready for implementation in management of patients with HER2-positive breast cancer.

In our study, *ERBB2* gene expression was highly predictive of pCR for trastuzumab-containing regimens but not for lapatinib-only regimens. This differs from other studies which do show benefit from lapatinib to trastuzumab either with endocrine therapy in the HER2-E group (6, 37) or NeoALTTO where the immune signature was significantly associated with pCR in the combination group (4). We did not find any genes or gene signatures to be predictive of EFS possibly because of the small sample size and limited number of events in our study. Future analyses are planned to combine data sets from several similar neoadjuvant trials to predict both benefit of adding lapatinib to trastuzumab and long-term outcomes.

The immune system has been shown to play a prognostic role in HER-positive breast cancer (38) and to modulate response to targeted HER2 therapies (39). The data presented here add to these studies and also identify two signatures that could reach significance as potential biomarkers in a larger dataset. We found the overexpression of *IDO1* to be associated with patients who achieved a pCR and TIS to be a predictor of pCR in patients treated with trastuzumab-containing regimens in NSABP B-41. *IDO1* is an immunoregulatory enzyme involved in immunosuppression (40) after an inflammatory response and has been shown to be present in breast cancer (41) in both the HER2 positive tumor and immune cells. However, *IDO1* gene expression, induced by IFN, is a general marker of inflammation and good response (17, 25). GeoMx™ Digital Spatial Profiling (42) is a novel technology that can more deeply explore immune interactions using quantitation of protein targets in space. The immune biomarkers distinguished with the gene expression panel along with additional immune biomarkers for responsiveness to targeted therapies in patients with HER2-positive breast cancer could be evaluated more deeply with spatial profiling (42, 43).

The serial monitoring of circulating tumor DNA (ctDNA) is another novel approach to elucidate biomarkers that predict sensitivity or resistance to neoadjuvant treatment. Recently, McDonald and colleagues tested ctDNA targeted digital sequencing (TARDIS) using personalized patient-specific mutations in the neoadjuvant setting to evaluate residual disease (44). This study found that patients with a pCR had lower levels of ctDNA compared with patients who did not have a pCR. Combining these different assay approaches to biomarker discovery could provide innovative tools to personalize therapy for women with HER2-positive breast cancer.

This biomarker study was prospectively designed to investigate the prognostic genomic markers from the NanoString Technologies nCounter® Breast Cancer 360™ Panel using FFPE tumor samples from patients on a randomized clinical trial. Participants of this biomarker study were similar to the others in patient characteristics. Quality RNA and associated annotations were preserved in these tissue samples. The panel of 776 genes under investigation was expertly curated in that the panel covered 23 key breast cancer pathways and processes. Statistical analyses followed standard approaches on adjusting for multiple testing and variable selection. However, our sample size is limited to 194 overall and 130 for analyses on trastuzumab-related questions. Interactions among genomic markers in predicting pCR could not be fully studied. Recently developed statistical methods on identifying individualized treatment rules based on biomarkers have yet to be explored (45). Bulk gene expression was a strong approach to evaluate many different areas of biology with

a small sample, however, some heterogeneity of disease may not be fully captured. Other methods of detection of residual disease or predictors of outcome such as ctDNA or digital spatial profiling combined with somatic genomic data will be important to further validate the studies.

The results of this study not only confirmed previous findings on the prognostic utility of *ERBB2* and *ESR1* gene expression, but also provided an additional list of 17 genes and one signature that jointly predicted pCR in HER2-positive patients treated with trastuzumab-containing regimens. It was illustrated how the prognostic power of these markers could be unleashed using a model based on *ERBB2, ESR1* and TIS. Our results need to be further calibrated in other existing and future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Translational Relevance

The development of biomarkers for pathologic complete response (pCR) has become an urgent need in the pursuit of personalized medicine in clinical practice. The NSABP B-41 clinical trial compared trastuzumab, lapatinib, and the combination given with concurrent chemotherapy until surgery for HER2-positive, operable breast cancer. Using expression data of the NanoString Technologies nCounter® Breast Cancer 360™ panel from available tissue samples, we screened potential prognostic biomarkers for pCR among women treated with trastuzumab in NSABP B-41. We found that the combination of 19 genes and one meta-gene signature predicted pCR in women with HER2-positive early-stage breast cancer treated with regimens containing trastuzumab alone or trastuzumab and lapatinib. Upon further validation, our data would help patients and treating physicians to decide if trastuzumab-containing systemic therapies would be an appropriate treatment option.

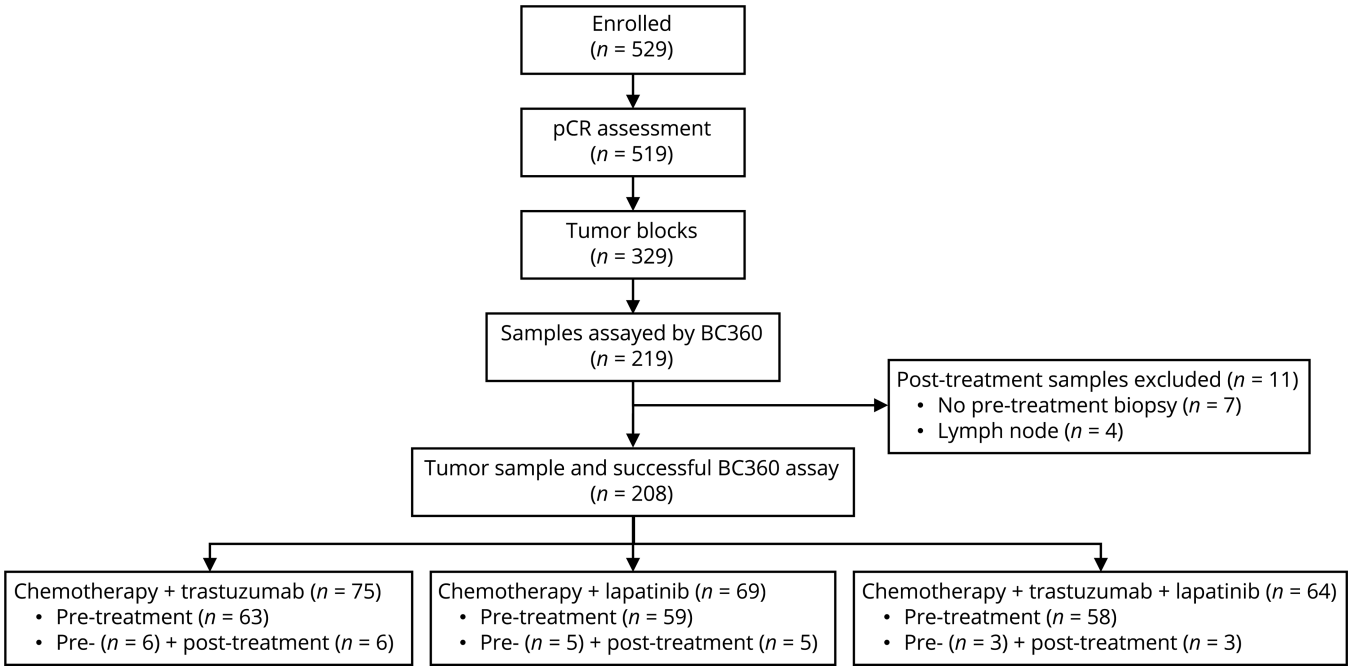

Figure 1.
NSABP B-41 patient sample flowchart

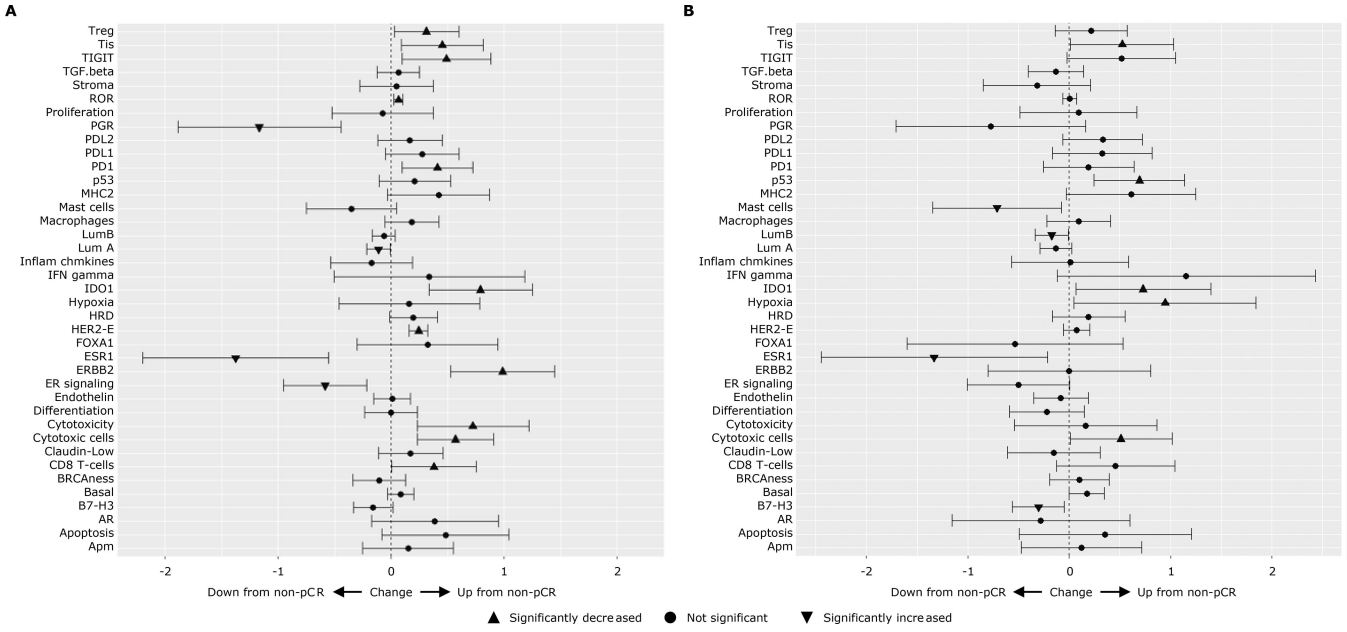

Figure 2.

Differences in mean gene expression and 95% confidence intervals between patients with and without pCR. (A) patients treated with trastuzumab or trastuzumab + lapatinib. (B) patients treated with lapatinib alone

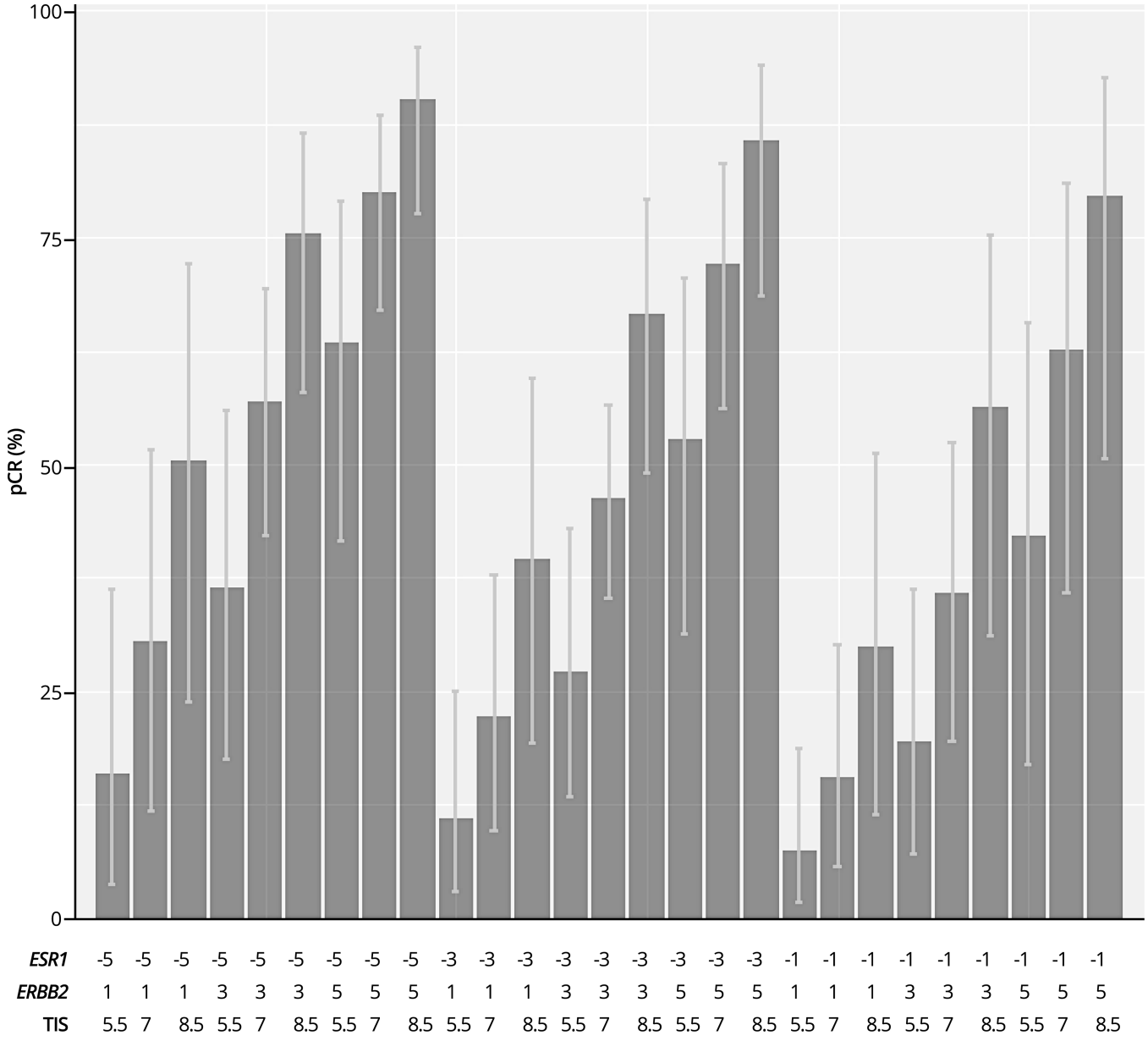

Figure 3.

Predicted chance of pCR and its confidence interval across various combinations in expression levels of ESR1, ERBB2 and TIS in patients on trastuzumab-containing regimens (n = 130)

Table 1.

Selected 10 gene signatures in prediction of pCR from univariate analysis on 34 meta-gene signatures (shown in bold) or single gene expression (shown in italics) from the BC360 panel among patients on trastuzumab-containing regimens (n = 130)

Signatures	OR (95% CI)	*P*	Adjusted *P*
ERBB2	1.73 (1.30, 2.31)	0.00016	0.005
IDO1	1.58 (1.19, 2.09)	0.0014	0.05
ESR1	0.79 (0.68, 0.91)	0.0018	0.06
Cytotoxic cells	1.83 (1.25, 2.68)	0.0019	0.06
PGR	0.76 (0.64, 0.91)	0.0027	0.08
ER signaling	0.6 (0.43, 0.84)	0.0029	0.08
Cytotoxicity	1.43 (1.11, 1.85)	0.006	0.17
PD1	1.67 (1.12, 2.51)	0.013	0.35
TIS	1.53 (1.08, 2.17)	0.016	0.42
TIGIT	1.47 (1.07, 2.03)	0.017	0.43

Table 2.

The selected list of 19 genes and one gene signature from the multivariate logistic regression model to predict pCR in patients on trastuzumab-containing regimens after lasso regularization (n = 130)

Pathways	Selected Genes or Gene Signature
Epithelial-mesenchymal transition	*HEMK1, GRB7, ERBB2, TMPRSS4*
Adhesion and migration	*ITGB6, COL27A1, NRCAM*
JAK-STAT	*SOCS2*
Hedgehog	*LRP2*
ER signaling	*ELOVL2, IFT140, MAPT*
DNA damage and repair	*NPEPPS, PRKDC*
MAPK	*DUSP6, PRKCB*
Apoptosis	*BCL2*
Proliferation	*TFDP1*
Multiple pathway	*MYCN*
Cytotoxicity pathway	*GZMA, GZMB, GZMH, PRF1, GNLY*

Table 3.

Multivariate logistic regression models for predicting pCR with clinical factors, genetic markers and combination of clinical factors and genetic markers (n = 130)

Variables	OR (95% CI)	*P*
Model 1: Prediction with clinical factors		
Trastuzumab + lapatinib (vs. trastuzumab-alone)	1.0 (0.47, 2.10)	0.99
ER (positive vs negative)	0.46 (0.22, 0.99)	0.05
Node (positive vs negative)	0.60 (0.29, 1.27)	0.18
Clinical tumor size (cm)	1.09 (0.92, 1.29)	0.34
Grade (moderate vs well)	0.83 (0.28, 2.50)	0.74
Grade (poor vs well)	0.75 (0.28, 2.03)	0.57
Model 2: Prediction with 3 genetic markers		
ESR1	0.87 (0.73, 1.02)	0.09
ERBB2	1.74 (1.27, 2.38)	<0.001
TIS	1.75 (1.18, 2.59)	0.006
Model 3: Prediction with ER and 3 genetic markers		
ER (positive vs. negative)	2.33 (0.57, 9.5)	0.24
ESR1	0.75 (0.55, 1.01)	0.06
ERBB2	1.71 (1.24, 2.35)	0.001
TIS	1.79 (1.20, 2.67)	0.004
Model 4: Prediction with HER2 subtype and 2 other genetic markers		
HER2 subtype (vs Other subtypes)	10.99 (3.72, 32.45)	<0.001
ESR1	0.87 (0.73, 1.04)	0.12
TIS	1.54 (1.04, 2.27)	0.03