Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

Research-Based PAM50 Subtype Predictor Identifies Higher Responses and Improved Survival Outcomes in HER2-Positive Breast Cancer in the NOAH Study

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Abstract

Purpose: We report a retrospective exploratory analysis of the association of the research-based prediction analysis of microarray 50 (PAM50) subtype predictor with pathologic complete response (pCR) and event-free survival (EFS) in women enrolled in the NeOAdjuvant Herceptin (NOAH) trial.

Experimental Design: Gene expression profiling was performed using RNA from formalin-fixed paraffin-embedded core biopsies from 114 pretreated patients with HER2-positive ($HER2^+$) tumors randomized to receive neoadjuvant doxorubicin/paclitaxel (AT) followed by cyclophosphamide/methotrexate/fluorouracil (CMF), or the same regimen in combination with trastuzumab for one year. A control cohort of 42 patients with HER2-negative tumors treated with AT-CMF was also included. The PAM50 subtypes, the PAM50 proliferation score, and the PAM50 risk of relapse score based on subtype (RORS) and subtype and proliferation (RORP) were evaluated.

Results: HER2-enriched (HER2-E) tumors predominated within $HER2^+$ disease, although all PAM50 intrinsic subtypes were identified across the three cohorts. The OR for achieving pCR with trastuzumab-based chemotherapy for $HER2^+$/HER2-E and $HER2^+$/RORP-high were 5.117 ($P = 0.009$) and 8.469 ($P = 0.025$), respectively, compared with chemotherapy only. The pCR rates of $HER2^+$/HER2-E and $HER2^+$/RORP-high after trastuzumab-based chemotherapy were 52.9% and 75.0%, respectively. A statistically nonsignificant trend was observed for more pronounced survival benefit with trastuzumab in patients with $HER2^+$/HER2-E and $HER2^+$/RORP-high tumors compared with patients with $HER2^+$/non-HER2-E and $HER2^+$/non-RORP-high tumors, respectively.

Conclusions: As determined by EFS and pCR, patients with $HER2^+$/HER2-E tumors, or $HER2^+$/RORP-high tumors, benefit substantially from trastuzumab-based chemotherapy. The clinical value of this genomic test within $HER2^+$ disease warrants further investigation. *Clin Cancer Res; 20(2); 511–21.* ©*2014 AACR.*

Introduction

In the clinic, breast cancer is classified as hormone receptor (HR)-positive, $HER2^+$ and triple-negative breast cancer. This classification, based on the expression levels of estrogen receptor (ER), progesterone receptor (PR), and the HER2 receptor, currently dictates the choice of therapy (1). However, in the last decade, studies based on global gene expression analyses have shown a far more complex breast cancer portrait and have identified at least four intrinsic molecular breast cancer subtypes [luminal A, luminal B, HER2-enriched (HER-E), and basal like] and a normal breast-like group, which differ significantly in terms of incidence, prognosis, and response to therapies (6, 7). Among them, the HER2-E subtype is characterized by the high expression of HER2-regulated genes (e.g., *ERBB2*, *GRB7*, and *FGFR4*) and proliferation/cell-cycle–related genes (e.g., ribonucleotide reductase M2 and cell division cycle; ref. 6), together with lower expression of luminal-related genes (e.g., *ESR1*, *PGR*, and forkhead box A1) than the luminal A and B subtypes (1, 8). Thus, this molecular entity is likely to be driven by the EGFR/HER2 pathway (1, 2, 6). Importantly, the HER2-E and the other molecular subtypes are not fully

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-13-0239

Translational Relevance

HER2-positive ($HER2^+$) breast cancer is biologically heterogeneous (1, 2) and not all patients benefit from anti-HER2–based regimens to the same extent (3–5). Recent data from The Cancer Genome Atlas breast cancer project suggest that a subset of $HER2^+$ tumors with a prediction analysis of microarray 50 (PAM50) HER2-enriched (HER2-E) gene expression profile shows the highest activation of the HER2/EGFR signaling pathway, suggesting that they may benefit the most from anti-HER2 therapies (6). In this report, we provide clinical evidence suggesting that advanced $HER2^+$/HER2-E tumors, as well as $HER2^+$ tumors predicted to have a high baseline risk of relapse and proliferation status ($HER2^+$/RORP-high), benefit substantially from trastuzumab-based chemotherapy. Clinically applicable gene expression-based tests such as the PAM50 assay warrant further investigation in predicting response and survival during treatment with anti-HER2 agents.

concordant with subtypes based on pathology-based biomarkers such as ER, PR, and HER2 (1, 8).

In 2009, a clinically applicable gene expression-based test that provides an intrinsic subtype diagnosis was introduced (8). Known as prediction analysis of microarray 50 (PAM50), this genomic test measures the expression of 50 genes and has a 93% accuracy to identify the various intrinsic subtypes compared with subtype classifications based upon approximately 1,900 intrinsic genes (8). Since 2009, the PAM50 subtype predictor has shown to provide prognostic and predictive value across multiple cohorts of patients with breast cancer (8, 9–13). For example, two studies have shown that the PAM50 subtype predictor predicts pathologic complete response (pCR) after neoadjuvant anthracycline-taxane–based chemotherapy (8, 14).

In addition to identifying the various subtypes, the PAM50 predictor provides a proliferation score as well as a supervised risk predictor known as risk of relapse based on subtype only (RORS), which was optimized in a large cohort of patients with node-negative tumors who did not receive adjuvant systemic therapy (i.e., a pure prognostic population; ref. 8). The combined RORS and proliferation score, known as RORP, has recently been shown to outperform standard clinicopathologic variables in predicting outcome in a large ER^+ breast cancer cohort treated with endocrine therapy only (9).

The NeOAdjuvant Herceptin (NOAH) trial demonstrated that trastuzumab significantly improves pCR rates and 3-year event-free survival (EFS) in combination with neoadjuvant chemotherapy compared with neoadjuvant chemotherapy alone in patients with $HER2^+$ breast cancer (15). In this exploratory analysis, we evaluated the ability of the research-based PAM50 gene expression-based test in predicting pCR and EFS in women enrolled in the NOAH trial.

Materials and Methods

NOAH patient population

The details of the NOAH study population have been previously reported (15). Briefly, women with $HER2^+$ locally advanced or inflammatory breast cancer were treated with a neoadjuvant chemotherapy regimen consisting of doxorubicin (60 mg/m^2) and paclitaxel [150 mg/m^2, every 3 weeks (q3w)] × 3, followed by paclitaxel (175 mg/m2, q3w) × 4, followed by cyclophosphamide [600 mg/m^2, every 4 weeks (q4w)], methotrexate (40 mg/m^2, q4w), and fluorouracil (600 mg/m^2 q4w) on days 1 and 8 × 3, and either received 1 year of treatment with trastuzumab (arm 3; given as neoadjuvant and adjuvant treatment; $n = 117$) or no trastuzumab (arm 2; $n = 118$). HR-positive patients also received adjuvant endocrine therapy for 5 years. A parallel cohort of 99 patients with HER2-negative disease (arm 1) was included and treated with the same chemotherapy regimen (without trastuzumab). In the NOAH study, HER2 positivity was defined as 3+ overexpression in more than 10% of tumor cells by immunohistochemical (IHC) testing (Herceptest, Dako) or HER2 amplification ratio 2.0 or more by FISH (Pathvysion HER2 test, Abbott/Vysis) according to a central laboratory (Klinikum Kassell). HR status was defined by local assessment. The primary study endpoint was EFS.

TransNOAH patient selection

From the 334 enrolled patients in the NOAH trial, 247 formalin-fixed, paraffin-embedded (FFPE) core biopsies were prospectively collected before treatment, and 195 samples remained for RNA extraction and subsequent gene expression profiling analyses. In addition to tumor cells, information about infiltrating inflammatory cells, stroma cells, and normal epithelial cells from the pretreatment samples was recorded by a pathologist at a central laboratory.

Gene expression microarrays

FFPE tumor-derived RNA was amplified with the WT-Ovation FFPE System V2 (NuGEN). Biotin labeling of the cDNA was performed using the FL-Ovation cDNA Biotin Module V2 (NuGEN). Hybridization of labeled probes on the Affymetrix GeneChip Human Genome U133 Plus 2.0 Array were conducted using the GeneChip Hybridization, Wash, and Stain Kit (Affymetrix). Target preparation, hybridization of samples to microarrays, and washing of microarrays were conducted according to the respective manufacturers' manuals. For this workflow, a GeneChip Fluidics Station 450 and a GeneChip Scanner 3000 7G were used.

After extensive quality control, 156 samples met the quality criteria and were used for further gene expression analysis (Supplementary Fig. S1 and Supplementary Table S1). Similar distribution of the main clinicopathologic variables was observed in the subpopulation evaluated here compared with the entire population included in the NOAH trial (Supplementary Table S2). Raw data have been deposited in the Gene Expression Omnibus under the accession number GSE50948.

PAM50 gene expression analysis

The research-based PAM50 subtype predictor was applied using the publicly available algorithm (8). Only the probes with the highest interquartile range of expression were used for PAM50 subtyping (Supplementary Table S3). The expression of seven of the 50 *PAM50* genes was not included in the analysis (*CEP55*, *EXO1*, *GRB7*, *MIA*, *ORC6L*, *UBE2C*, and *UBE2T*) because they did not meet quality control standards. Of note, loss of genes can affect the robustness of the PAM50 subtyping (16, 17).

In addition to identifying the various intrinsic subtypes (luminal A, luminal B, HER2-E, basal like, and normal like), the PAM50 proliferation score, the PAM50 "RORS", and the "risk of relapse score based on subtype and proliferation" (RORP) were evaluated. The proliferation score is the mean expression of the 11-proliferation-related genes in the PAM50 assay (9). The RORS and RORPs were evaluated as continuous variables and as low-/medium-/high-risk groups using the previously reported cutoffs (8, 9).

Independent evaluation of the PAM50 subtype predictor

We evaluated research-based PAM50 data in three independent publicly available microarray and clinically annotated datasets (ISPY-1; ref. 12; MDACC; ref. 18; and XeNA; ref. 19) that include 91 patients with clinically $HER2^+$ tumors treated with neoadjuvant trastuzumab-based chemotherapy. From the ISPY-1 clinical trial ($n = 14$; ref. 12) and the MDACC dataset ($n = 50$; ref. 18), we selected clinically $HER2^+$ tumors treated with anthracycline/taxane-based chemotherapy and trastuzumab. From the XeNA clinical trial ($n = 27$; ref. 19), we selected all clinically $HER2^+$ tumors treated with four cycles of docetaxel-capecitabine and trastuzumab.

Statistical analysis

pCR was defined by the absence of residual invasive tumor in the breast and axillary lymph nodes (the presence of carcinoma *in situ* was allowed). Multivariate logistic regression analyses were used to evaluate the association of each signature with pCR. The following standard clinicopathologic variables were included in the multivariate models: age at diagnosis, tumor size, nodal status by clinical assessment, hormonal receptor status (when applicable), and histologic grade. ANOVA tests were used to evaluate the association of the percentage of inflammatory cells, invasive tumor cells, normal epithelial cells, and stromal cells across the predicted intrinsic subtypes. Student *t* test was used to compare the expression of individual genes or the correlation to each PAM50 centroid between the two response groups (pCR vs. residual disease). Cox models were used to test the independent associations of each signature with EFS.

Results

Clinicopathologic characteristics

The main clinicopathologic features of each arm of the NOAH microarray dataset (transNOAH) are shown in Table 1. No statistical significant differences in clinicopathologic features were observed between both $HER2^+$ arm, except for pCR rates, which were 44.44% (28/63) and 23.53% (12/51) with and without trastuzumab ($P = 0.033$), respectively. The observed pCR rates are similar to the pCR rates reported in both arms of the NOAH trial (15). Compared with both $HER2^+$ arms, the HER2-negative arm showed a higher percentage of ER^+ (59,5%) and PR-positive (PR^+, 40.5%) tumors, as well as a lower percentage of inflammatory breast cancer (12%). Finally, no significant differences in terms of pCR rates were observed between both chemotherapy-only arms (arm 1: 19.1% vs. arm 2: 23.5%).

PAM50 intrinsic subtypes versus clinicopathologic characteristics

Selected clinicopathologic features were evaluated across the PAM50 intrinsic subtypes (Table 2). Patients with basal-like tumors showed a lower mean age at diagnosis compared with patients with non-basal–like tumors (46.5 vs. 52.4 years, $P = 0.016$), whereas luminal A tumors showed a lower rate of histologic grade 3 compared with non-luminal A tumors (29.2% vs. 60.8%, $P = 0.028$). The concordance between the pathology-based subtypes [HR^+/HER2-negative (luminal A), $HR^+/HER2^+$ (luminal B), HR-negative/$HER2^+$ (HER2-E), HR-negative/HER2-negative (basal like)] and the PAM50 subtypes was moderate (87/129, 67.4%; multirater kappa statistic = 0.5), and comparable with previous reports (1, 8, 20).

The presence of various cell types in each tumor biopsy varied substantially across the PAM50 subtypes. For example, the highest and lowest mean percentages of inflammatory-related cells (e.g., lymphocytes) were identified in basal-like (13.4%) and luminal A tumors (6.5%), respectively, whereas the highest percentage of normal epithelial (4%) and stromal cells (30.7%), together with the lowest percentage of invasive tumor cells (48.1%), was identified in normal-like tumors. This is in agreement with the hypothesis that these tumors are referred to as normal like due to their high normal breast tissue content. Interestingly, these differences observed across the PAM50 subtypes were not well identified across the pathology-defined subtypes (Supplementary Table S4).

Response after chemotherapy only (arms 1 and 2)

The pCR rates of the PAM50 subtypes, the proliferation score, RORS and RORP groups after chemotherapy only (arms 1 and 2 combined) are shown in Table 3. Non-luminal A tumors (luminal B, HER2-E, basal-like, and normal-like tumors as one group) showed higher pCR rates than luminal A tumors [26.7% (20/75) vs. 0% (0/18); $P = 0.010$]. Interestingly, 33.3% (6/18) of luminal A tumors were found to be HR-negative by local pathology assessment. In terms of RORS and RORP, tumors predicted to have high-risk scores showed higher pCR rates than those tumors with intermediate or low scores (Table 3 and Supplementary Table S5). For example, the OR of the high-risk RORP group for achieving a pCR was 2.99 compared with

Table 1. Clinicopathologic characteristics of the TransNOAH dataset[a].

	HER2$^-$ disease		HER2$^+$ disease				
	Arm 1 (w/o T)		Arm 2 (w/o T)		Arm 3 (with T)		P^b
Number of patients	42	—	51	—	63	—	
Age (mean)	51.5	—	53.3	—	50.4	—	0.130
Histologic grade							
Grade 2	16	39.0%	21	41.2%	31	50.0%	0.463
Grade 3	25	61.0%	30	58.8%	31	50.0%	
ER Status							
ER$^+$	25	59.5%	12	23.5%	15	23.8%	0.852
ER$^-$	17	40.5%	39	76.5%	48	76.2%	
PR Status							
PR$^+$	17	40.5%	6	11.8%	12	19.0%	0.422
PR$^-$	25	59.5%	45	88.2%	51	81.0%	
Tumor stage							
T2	9	21.4%	7	13.7%	10	15.9%	0.950
T3-T4	33	78.6%	44	86.3%	53	84.1%	
Node							
N0	11	26.2%	8	15.7%	8	12.7%	
N1	10	23.8%	16	31.4%	28	44.4%	0.851[a]
N2	19	45.2%	25	49.0%	23	36.5%	
N3	2	4.8%	2	3.9%	4	6.3%	
Histology							
IBC	5	11.9%	14	27.5%	15	23.8%	0.820
Non-IBC	37	88.1%	37	72.5%	48	76.2%	
Treatment response							
pCR	8	19.1%	12	23.5%	28	44.4%	0.033
RD	34	80.9%	39	76.5%	35	55.6%	

Abbreviations: T, trastuzumab; w/o, without; IBC, invasive breast cancer; RD, residual disease.

[a]N0 and N1-3 have been compared.

[b]χ^2 or Fisher's exact test, except for age, which was compared using a Student *t* test.

the medium-/low-risk groups ($P = 0.043$; only adjusted for HR and HER2 status). Similar results were obtained with the PAM50 proliferation score.

Response after trastuzumab-based chemotherapy (arm 3)

HER2-E tumors showed increased pCR rates compared with non-HER2-E tumors (luminal A, luminal B, basal-like, and normal-like tumors as one group) following trastuzumab-based chemotherapy (arm 3; Table 3). There was no statistical difference in pCR rates between HER2-E compared with non-HER2-E tumors (52.9% vs. 34.5%, OR = 2.66, $P = 0.100$) but was significant when the highly chemotherapy-sensitive basal-like tumors were removed from the non-HER2-E group (53% vs. 29%, OR = 3.612, $P = 0.038$). However, HER2-E tumors were found to be associated with higher pCR rates compared with non-HER2-E tumors within HR-negative disease (62% vs. 31%, OR = 11.318, $P = 0.012$; data not shown). Consistent with the observed association between HER2-E profile and higher pCR rates, HER2$^+$ tumors that achieved a pCR after trastuzumab-based chemotherapy showed higher expression of the PAM50 HER2-E signature (as a continuous variable) than pretreated HER2$^+$ tumors that achieved residual disease (data not shown).

PAM50 RORP was also found associated with response to trastuzumab-based chemotherapy (Table 3 and Supplementary Table S5). The OR for achieving a pCR in the RORP high-risk group compared with the medium-/low-risk group was 5.85 ($P = 0.013$). The association of RORP with pCR after trastuzumab-based chemotherapy was found to be independent of clinicopathologic variables when evaluated as a continuous variable (Supplementary Table S5). Similar results were obtained using the proliferation score (Table 3). The OR for achieving a pCR in the proliferation score high group compared with the medium/low group was 4.19 ($P = 0.015$).

Prediction of response to trastuzumab (arm 3 vs. 2)

The response of the HER2-E and the non-HER2-E subtypes after neoadjuvant treatment with and without trastuzumab was evaluated by comparing response data between arms 3 and 2 (Table 4). For HER2-E tumors, the ORs for achieving a pCR with trastuzumab and chemotherapy

Table 2. Clinicopathologic characteristics of the PAM50 intrinsic molecular subtypes[a]

	Luminal A		Luminal B		HER2-E		Basal-like		Normal-like		
	N	%	*N*	%	*N*	%	*N*	%	N	%	p-value**
Number of patients	25	—	18	—	67	—	19	—	27	—	
Age (mean)	51.6	—	53.2	—	53.4	—	46.5	—	50.22	—	0.090
Histologic grade											
Grade 2	17	70.8	6	33.3	26	38.8	5	26.32	14	53.8	0.017
Grade 3	7	29.2	12	66.7	41	61.2	14	73.68	12	46.2	
Clinical IHC status											
$HR^+/HER2^-$	12	48.0	7	38.9	1	1.5	0	0	5	18.5	
$HR^+/HER2^+$	6	24.0	9	50.0	8	11.9	3	15.8	8	29.6	<0.001
$HR^-/HER2^+$	6	24.0	2	11.1	55	82.1	5	26.3	12	44.4	
$HR^-/HER2^-$	1	4.0	0	0.0	3	4.5	11	57.9	2	7.4	
Histology											
IBC	3	12.0	2	11.1	20	29.9	0	0.0	9	33.3	0.014
Non-IBC	22	88.0	16	88.9	47	70.1	19	100	18	66.7	
Cell-type histologic examination of (mean%)											
Inflammatory cells	—	6.5	—	8.7	—	10.4	—	13.4	—	12.0	0.009
Invasive tumor cells	—	61.0	—	65.8	—	64.2	—	57.9	—	48.1	<0.001
Normal epithelial cells	—	2.1	—	1.1	—	1.8	—	0.5	—	4.0	0.030
Stromal cells	—	25.1	—	20.5	—	18.5	—	19.5	—	30.7	<0.005

[a]IBC, invasive breast cancer.

[b]χ^2 or Fisher's exact test, except for age, which was compared using an ANOVA test.

Table 3. Response data of the PAM50 intrinsic subtypes and the RORS, RORP, and Proliferation groups across the three arms[a]

	$HER2^-$ disease					$HER2^+$ disease									
	Arm 1 (w/o T)					Arm2 (w/o T)					ARM3 (with T)				
	pCR	%	RD	%	*P*[b]	pCR	%	RD	%	*P*[b]	pCR	%	RD	%	*P*b
Subtype															
Luminal A	0	0.0	13	100.0		0	0.0	5	100.0		2	28.6	5	71.4	
Luminal B	2	28.5	5	71.4		3	42.9	4	57.1		2	50.0	2	50.0	
HER2-E	2	50.0	2	50.0	0.020	8	27.6	21	72.4	0.50	18	52.9	16	47.1	0.320
Basal-like	4	36.4	7	63.6		0	0.0	3	100.0		3	60.0	2	40.0	
Normal-like	0	0.0	7	100.0		1	14.3	6	85.7		3	23.1	10	76.9	
RORS															
Low	0	0.0	13	100.0		0	0.0	2	100.0		1	20.0	4	80.0	
Med	3	15.8	16	84.2	0.014	7	21.9	25	78.1	0.61	13	36.1	23	63.9	0.060
High	5	50.0	5	50.0		5	29.4	12	70.6		14	63.6	8	36.4	
Proliferation score															
Low	0	0	18	100.0		0	0.0	9	100.0		4	16.0	21	84.0	
Med	2	22.2	7	77.8	0.012	5	20.8	19	79.2	0.07	11	57.9	8	42.1	<0.01
High	6	40.0	9	60.0		7	38.9	11	61.1		13	68.4	6	31.6	
RORP															
Low	0	0.0	12	100.0		0	—	0	—		1	16.7	5	83.3	
Med	4	21.1	15	78.9	0.082	6	17.1	29	82.9	0.22	15	36.6	26	63.4	0.010
High	4	36.4	7	63.6		6	37.5	10	62.5		12	75.0	4	25.0	

[a]T, trastuzumab; w/o, without; RD, residual disease.

[b]χ^2 or Fisher's exact test.

Table 4. Prediction of response based on trastuzumab treatment within HR-defined and PAM50-defined groups

		Response				
		pCR	OR[a]	95% Low	95% High	$P_{interaction}$
ER/PR	HER2$^+$/HR-negative					
	No-trastuzumab ($n = 37$)	21.6%	5.799	1.799	18.703	
	Trastuzumab ($n = 43$)	51.2%				
	HER2$^+$/HR-positive					0.095
	No-trastuzumab ($n = 14$)	28.6%	1.061	0.213	5.29	
	Trastuzumab ($n = 20$)	30.0%				
Subtype	HER2$^+$/HER2-E					
	No-trastuzumab ($n = 29$)	27.6%	5.117	1.498	17.48	
	Trastuzumab ($n = 34$)	52.9%				
	HER2$^+$/nonHER2-E					0.527
	No-trastuzumab ($n = 22$)	18.2%	2.091	0.442	9.893	
	Trastuzumab ($n = 29$)	34.5%				
	HER2$^+$/HR$^-$/HER2-E					
	No-trastuzumab ($n = 28$)	25.0%	8.743	2.171	35.217	
	Trastuzumab ($n = 27$)	63.0%				
	HER2$^+$/HR$^-$/nonHER2-E					0.417
	No-trastuzumab ($n = 9$)	11.1%	2.364	0.111	50.486	
	Trastuzumab ($n = 16$)	31.3%				
ROR and/or Proliferation Score	HER2$^+$/RORS-high					
	No-trastuzumab ($n = 17$)	29.4%	8.109	1.624	40.497	
	Trastuzumab ($n = 22$)	63.6%				
	HER2$^+$/RORS-medium					0.433
	No-trastuzumab ($n = 32$)	21.9%	2.156	0.686	6.773	
	Trastuzumab ($n = 36$)	36.1%				
	HER2$^+$/RORS-low					
	No-trastuzumab ($n = 2$)	0.0%	—	—	—	
	Trastuzumab ($n = 5$)	20.0%				
	HER2$^+$/proliferation-high					
	No-trastuzumab ($n = 18$)	38.9%	4.064	0.909	18.182	
	Trastuzumab ($n = 19$)	68.4%				
	HER2$^+$/proliferation-medium					0.207
	No-trastuzumab ($n = 24$)	20.8%	10.036	1.836	54.87	
	Trastuzumab ($n = 19$)	57.9%				
	HER2$^+$/proliferation-low					
	No-trastuzumab ($n = 9$)	0.0%	—	—	—	
	Trastuzumab ($n = 25$)	16.0%				
	HER2$^+$/RORP-high					
	No-trastuzumab ($n = 16$)	37.5%	8.469	1.307	54.864	
	Trastuzumab ($n = 16$)	75.0%				
	HER2$^+$/RORP-medium					0.245
	No-trastuzumab ($n = 35$)	17.1%	4.037	1.17	13.92	
	Trastuzumab ($n = 41$)	36.6%				
	HER2$^+$/RORP-low					
	No-trastuzumab ($n = 0$)	—	—	—	—	
	Trastuzumab ($n = 6$)	16.7%				

[a]ORs provided in the table have been adjusted for the following clinicopathologic variables: age at diagnosis, tumor size, nodal status by clinical assessment, HR status (when applicable), and histologic grade. For the interaction test, RORS, proliferation and RORP have been evaluated as continuous variables.

compared with chemotherapy only were 5.117 in all patients and 8.743 within HR-negative disease. For non-HER2-E tumors, the ORs were 2.091 in all patients and 2.364 within HR-negative disease. However, the interaction between subtype and treatment for pCR was not significant ($P = 0.527$).

The response of the RORP-high and RORP-medium groups after treatment with and without trastuzumab (arm 3 vs. arm 2) was also evaluated (Table 4 and Supplementary Table S5). For the RORP-high group, the OR for achieving a pCR with trastuzumab compared with chemotherapy only was 8.469 in all patients (pCR rates of 75.0% vs. 37.5%) and 13.914 within HR-negative disease (78.6% vs. 30.8%). For the RORP-medium group, the ORs were 4.037 in all patients (pCR rates of 36.6% vs. 17.1%) and 5.792 within HR-negative disease (16.7% vs. 40.0%). Similar to subtype, the interaction between RORP and treatment for pCR was not significant ($P = 0.245$).

Prediction of event-free survival benefit with trastuzumab (arm 3 vs. 2)

The EFS outcomes of the HER2-E and non-HER2-E subtypes after treatment with and without trastuzumab were evaluated by comparing EFS data between arms 3 and 2 (Table 5 and Fig. 1A). First, a more pronounced trastuzumab benefit was observed in the HER2-E tumors (EFS hazard ratio = 0.430) compared with non-HER2-E tumors (EFS hazard ratio = 0.807), although no interaction was observed between HER2-E subtype and trastuzumab treatment for EFS ($P = 0.480$). Within HR-negative disease, patients with HER2-E tumors showed a better outcome than those with non-HER2-E tumors (Fig. 1B).

Second, we explored the ability of the RORP-high and -medium group categories to predict EFS benefit with trastuzumab (Fig. 1C and D and Supplementary Table S5). Consistent with the response data in the neoadjuvant setting, a more pronounced trastuzumab benefit was observed in the RORP-high tumors (EFS hazard ratio = 0.108 with an improvement in 3-year EFS rate from 66.7% to 93.8%) compared with RORP-medium tumors (EFS hazard ratio = 0.706), although no interaction was observed between RORP and trastuzumab treatment for EFS ($P = 0.244$). No differences in survival were observed within the RORP-medium group despite a 19.5% absolute increase in the pCR rate after the addition of trastuzumab to chemotherapy (Table 4). Similar results were observed when both RORP-high and -medium groups were evaluated within $HER2^+$/HR-negative disease (Fig. 1D).

Independent evaluation of the PAM50 subtype predictor

We evaluated the association of the HER2-E subtype, and RORP-high group, with pCR after trastuzumab-based chemotherapy in 91 patients with $HER2^+$ disease of the ISPY-1, XeNA, and MD Anderson Cancer Center (MDACC) datasets (Supplementary Tables S6 and S7). Concordant with our previous findings, HER2-E tumors showed increased pCR rates after trastuzumab-based chemotherapy compared with non-HER2-E tumors (66.0% vs. 26.8%; OR = 4.72, $P = 0.004$) after adjustment for ER status, tumor size, and study. Similarly, RORP-high tumors showed increased pCR rates compared with medium/low RORP tumors after trastuzumab-based chemotherapy (62.2% vs. 34.8%; OR = 2.90, $P = 0.026$).

Discussion

In this study, we evaluated the research-based PAM50 subtype predictor in a cohort of patients from the NOAH trial. Our results suggest that (i) HER2-E tumors show a high response rate after trastuzumab-based chemotherapy, (ii) within $HER2^+$ disease, HER2-E tumors achieve higher response rates after trastuzumab-based chemotherapy compared with chemotherapy only, (ii) PAM50-RORP identifies a cohort of $HER2^+$ tumors with a high likelihood of achieving a pCR and increased EFS after trastuzumab-based chemotherapy, (iv) the PAM50 intrinsic subtypes are not fully concordant with standard clinicopathologic variables such as ER, PR, and HER2, and PAM50 subtype information might help to better characterize the biology of a given tumor sample. To our knowledge, this is the first report to suggest that a clinically applicable gene expression-based assay might help identify a subset of patients with non-metastatic $HER2^+$ disease that benefit the most from the addition of trastuzumab to chemotherapy. However, it is important to note that we could not exclude a benefit of trastuzumab for patients whose tumors are either not HER2-E or not ROR-high.

One area of critical importance in breast cancer is the identification of biomarkers of response and/or resistance to anti-HER2 therapies. A number of biomarkers have been proposed (e.g., p95HER2; ref. 21; PTEN inactivation; refs. 22, 23; cyclin E1 amplification; ref. 24; PIK3CA mutations; refs. 25, 26; MYC amplification; ref. 27; and activation of insulin-like growth factor receptor; ref. 28), and the field is eagerly awaiting for the results of ongoing validation studies for these markers. For example, PIK3CA mutations, and PTEN deficiency determined by IHC, have not been found predictive of trastuzumab benefit in the FinHER and N9831 phase III adjuvant trials (23, 26), respectively. Interestingly, one of the main biomarkers of pCR to chemotherapy or anti-HER2–based therapies is HR-status. Data from NeoALTTO (29), GEPARQUINTO (30), and NeoSphere (31) clinical trials show that HR-negative tumors achieve higher pCR rates than HR^+ tumors after treatment with HER2-blocking therapies alone (31) or in combination with chemotherapy (29, 30). However, this finding alone does not exclude a potential benefit of trastuzumab in HR-positive tumors. In fact, trastuzumab-based therapy in the adjuvant setting has been shown to reduce the risk of recurrence across all clinical subgroups, although the magnitude of trastuzumab benefit in $HER2^+/HR^+$ disease seems lower than in $HER2^+$/HR-negative disease (3, 32). For example, in the NOAH trial, ER-negative and PR-negative tumors derived a larger EFS benefit from trastuzumab (EFS hazard ratio = 0.46) than ER-positive and/or PR^+ tumors (EFS hazard ratio = 0.87; ref. 15).

Table 5. Prediction of outcome based on trastuzumab treatment within HR-defined and PAM50-defined groups

		Outcome				
		3-year EFS	Hazard ratio[a]	95% Low	95% High	$P_{interaction}$
HR	$HER2^+$/HR-negative					
	No-trastuzumab ($n = 37$)	52.8%	0.413	0.188	0.911	
	Trastuzumab ($n = 43$)	72.3%				
	$HER2^+$/HR-positive					0.140
	No-Trastuzumab ($n = 14$)	61.5%	1.406	0.431	4.588	
	Trastuzumab ($n = 20$)	55.0%				
PAM50 Subtype	$HER2^+$/HER2-E					
	No-trastuzumab ($n = 29$)	60.7%	0.430	0.152	1.218	
	Trastuzumab ($n = 34$)	79.1%				
	$HER2^+$/nonHER2-E					0.480
	No-trastuzumab ($n = 22$)	47.6%	0.807	0.343	1.901	
	Trastuzumab ($n = 29$)	50.3%				
	$HER2^+/HR^-$/HER2-E					
	No-trastuzumab ($n = 28$)	59.3%	0.365	0.112	1.189	
	Trastuzumab ($n = 27$)	85.0%				
	$HER2^+/HR^-$/nonHER2-E					0.800
	No-trastuzumab ($n = 9$)	33.3%	0.402	0.112	1.441	
	Trastuzumab ($n = 16$)	48.9%				
PAM50 ROR and/or Proliferation score	$HER2^+$/RORS-high					
	No-trastuzumab ($n = 17$)	68.8%	0.500	0.118	2.119	
	Trastuzumab ($n = 22$)	77.3%				
	$HER2^+$/RORS-medium					0.630
	No-trastuzumab ($n = 32$)	51.6%	0.588	0.269	1.285	
	Trastuzumab ($n = 36$)	60.2%				
	$HER2^+$/proliferation-high					
	No-trastuzumab ($n = 18$)	58.8%	0.217	0.038	0.961	
	Trastuzumab ($n = 19$)	89.5%				
	$HER2^+$/proliferation-medium					0.990
	No-trastuzumab ($n = 24$)	65.2%	1.016	0.326	3.161	
	Trastuzumab ($n = 19$)	56.4%				
	$HER2^+$/RORP-high					
	No-trastuzumab ($n = 16$)	66.7%	0.108	0.011	1.063	
	Trastuzumab ($n = 16$)	93.8%				
	$HER2^+$/RORP-medium					0.244
	No-trastuzumab ($n = 35$)	50.0%	0.706	0.351	1.420	
	Trastuzumab ($n = 41$)	55.0%				

[a]Hazard ratios provided in the table have been adjusted for the following clinicopathologic variables: age at diagnosis, tumor size, nodal status by clinical assessment, HR status, and histologic grade. For the interaction test, RORS, Proliferation, and RORP have been evaluated as continuous variables. Outcome data of the RORS-low, Proliferation-low, and RORP-low groups are not shown due to the low numbers of patients (or lack of patients) in at least one of the treatment arms.

The 2013 St. Gallen Consensus Panel Recommendations for the systemic treatment of primary breast cancer are based on the identification of the intrinsic subtypes using surrogate pathologic-based definitions (33). Aware of the biologic heterogeneity within $HER2^+$ disease, $HER2^+$ disease has been divided into the luminal B category, if HR^+, and $HER2^+$ category, if HR-negative. However, although the majority of PAM50 luminal B and PAM50 HER2-E tumors are HR^+ and HR-negative, respectively, the discrepancy between pathologic-based definitions and gene expression-based subtype definitions is still considerable (34). Interestingly, up to 30% to 50% of HER2-E tumors are clinically HER2-negative (1, 6, 8), a category that includes tumors that are tested with IHC only, FISH only or both. Of note, both pathologic-based definitions of $HER2^+$ disease (i.e., IHC 3+ vs. FISH) are also discordant in 5% to 10% of

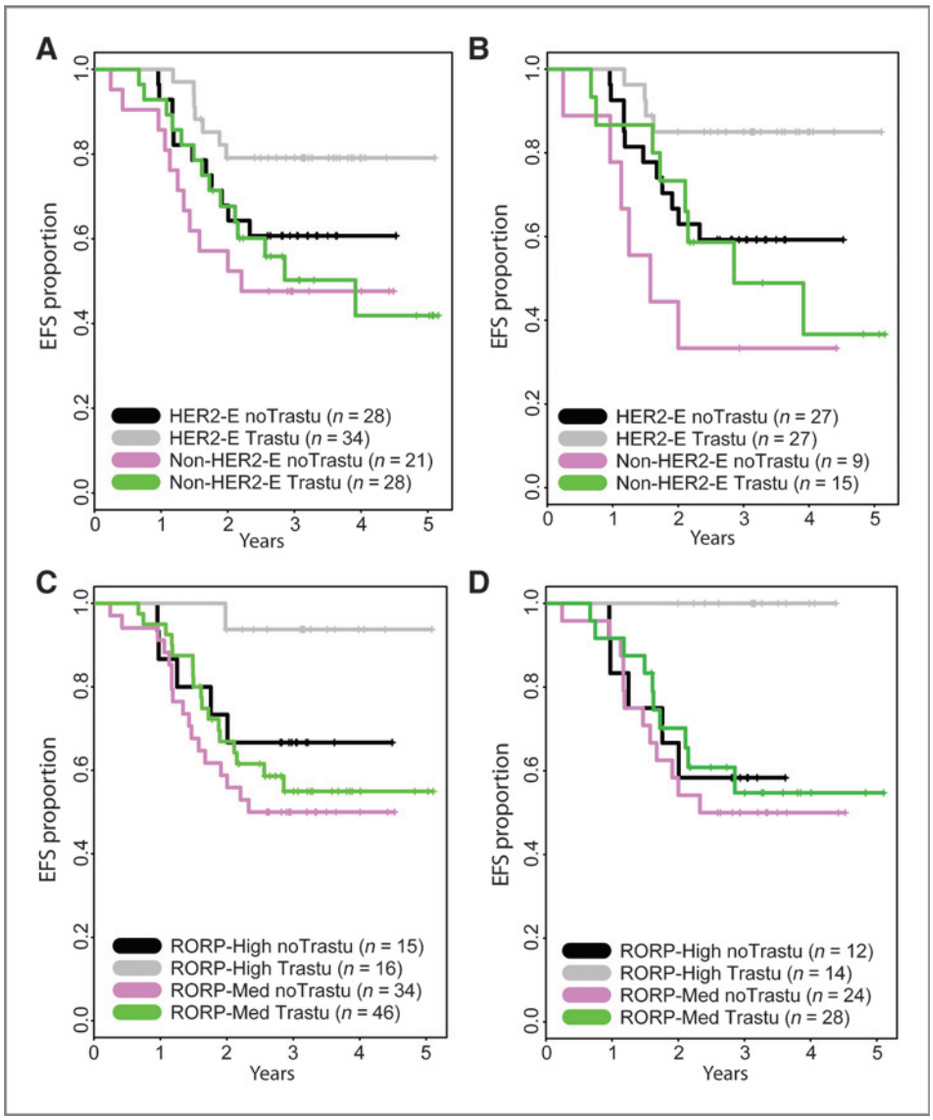

Figure 1. EFS of PAM50-defined groups with and without trastuzumab administration within patients with HER2$^+$ (A and C) and within patients with HER2$^+$/HR-negative (B and D). Outcome data of the RORS-low and RORP-low groups are not shown due to the low numbers of patients (or lack of patients) in at least one of the treatment arms.

the cases (35). In any case, future studies evaluating anti-HER2 therapies within HER2-negative/HER2-E tumors seem warranted.

Our data suggest that the identification of the HER2-E intrinsic molecular subtype, either alone or in combination with current pathologic HER2 testing, is likely to identify a more biologically homogenous group of tumors that are driven by the HER2 pathway and that might benefit the most from HER2 targeting therapies. Consistent with this hypothesis, phospho-proteomic analyses from The Cancer Genome Atlas project suggest that breast cancers that are both HER2$^+$ and HER2-E are uniquely characterized by high activation of EGFR/HER2 signaling (6).

Further studies are needed to address the clinical value of the PAM50 assay and other gene expression assays within HER2$^+$ disease. For example, the PAM50 subtype predictor could be tested retrospectively in large clinical trials that have evaluated trastuzumab in the adjuvant setting, and also in studies that have evaluated other anti-HER2 therapies such as lapatinib or pertuzumab, especially those that have evaluated the trastuzumab-lapatinib or trastuzumab-pertuzumab combinations. In fact, based on our results, the PAM50 subtype predictor might be able to identify a subset of patients with HER2$^+$ disease that are extremely sensitive to dual HER2 blockade and thus might not need chemotherapy. On the basis of the pCR rates of the trastuzumab and pertuzumab arm (without chemotherapy) observed in the NeoSphere clinical trial (31), this subpopulation might represent approximately 20% of the HER2$^+$ population.

Beyond the association of the PAM50 intrinsic molecular subtypes with response and outcome, additional information was observed from the PAM50 RORP. Indeed, HER2$^+$ tumors with high RORP scores, which are basically a subset of HER2$^+$/HER2-E tumors (Supplementary Table S8), are the ones to derive the largest benefit from

trastuzumab-based chemotherapy. This benefit was less in tumors with an intermediate baseline prognosis and/or proliferation status. A potential hypothesis could be that, in high-risk HER2$^+$ tumors, trastuzumab enhances the antitumor effects of chemotherapy. In medium-risk tumors however, trastuzumab might not be able to enhance the antitumor effects of chemotherapy because chemotherapy itself might not be as effective. These data are supported by the high chemotherapy benefit observed within the high risk of recurrence groups identified by the OncotypeDX (36) and MammaPrint (37) diagnostic assays.

Several caveats of our study require special consideration. First, the research-based PAM50 subtype predictor was applied to gene expression data obtained from a microarray platform (Affymetrix) that differs substantially from the original PAM50 microarray platform (Agilent) and, although proper normalization methods were applied, some of the subtype and/or ROR calls could have changed. However, the PAM50 predictor has already been applied successfully using gene expression data from Affymetrix platforms (8, 38, 39), and the clinicopathologic features of the subtypes identified in our study are consistent with previous studies (1, 8, 20). Second, it is of note that the input RNA in the TransNOAH study comes from FFPE tumor tissues, and this can affect the quality of the gene expression data. In fact, seven genes of the PAM50 assay were not evaluated because they did not meet our stringent quality control requirements, and loss of genes can affect the robustness of the PAM50 subtyping (16, 17). Finally, 53% of all tumor samples from the NOAH trial were not gene expression profiled and therefore some of our analyses have limited power to detect significant differences. Thus, for example, we cannot exclude the possibility that HER2$^+$/non-HER2-E tumors or RORP-medium groups derive survival benefit from the addition of trastuzumab to chemotherapy.

To conclude, the PAM50 subtype predictor provides additional and useful information of response to chemotherapy only and trastuzumab-based chemotherapy beyond that provided by standard pathologic markers. The clinical value of this genomic test within HER2$^+$ disease warrants further investigation.

Disclosure of Potential Conflicts of Interest

A. Prat is a consultant/advisory board member of Nanostring Technologies. G. Bianchini is a consultant/advisory board member of Roche. M. Thomas is employed as a biomarker experimental medicine leader in Roche. A. Belousov is head of Biostatistics BEDA in Roche Diagnostics GmbH. M.C.U. Cheang has ownership interest (including patents) in PAM50 classifier Patent. A. Koehler is global project manager in Roche Diagnostics GmbH, Germany, and has ownership interest (including patents) in Hoffmann-La Roche AG. P. Gómez is a consultant/advisory board member of BAYER. M. Byakhow is a consultant/advisory board member of Roche. L. Gianni is a consultant/advisory board member of Roche, GSK, and Novartis. J. Baselga is a consultant/advisory board member of Roche. No potential conflicts of interest were disclosed by the other authors.

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Grant Support

A. Prat was supported by a Career Catalyst Grant from the Susan G. Komen Foundation and by the "Beca Roche en Onco-Hematologia 2012."

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Received January 25, 2013; revised October 8, 2013; accepted October 9, 2013; published online January 17, 2014.

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