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ORIGINAL REPORT

Intrinsic Subtypes, *PIK3CA* Mutation, and the Degree of Benefit From Adjuvant Trastuzumab in the NSABP B-31 Trial

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See accompanying editorial on page 1318 and articles on pages 1334 and 1407

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K.L.P.-G. and N.S. contributed equally in design and conduct of the study and analyses of the data.

Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

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ABSTRACT

Purpose
Considerable molecular heterogeneity exists among human epidermal growth factor receptor 2 (HER2) –positive breast cancer regarding gene expression and mutation profiling. Evidence from preclinical, clinical neoadjuvant, and metastatic clinical trials suggested that *PIK3CA* mutational status and PAM50 intrinsic subtype of a tumor were markers of response to anti-HER2 therapies. We evaluated the predictive value of these two biomarkers in the adjuvant setting using archived tumor blocks from National Surgical Adjuvant Breast and Bowel Project (NSABP) trial B-31.

Patients and Methods
Expression data for 49 genes using the nCounter platform were used to generate PAM50 intrinsic subtypes for 1,578 archived tumor blocks from patients in the B-31 trial. Six *PIK3CA* hotspot mutations were examined by mass spectrometry of the primer extension products in a randomly selected subset (n = 671). We examined the heterogeneity of trastuzumab treatment effect across different subsets defined by each marker using Cox regression and disease-free survival as the end point.

Results
Seven hundred forty-one (47.0%) of 1,578 tumors were classified as HER2-enriched (HER2E) subtype, and 166 (24.7%) of 671 tumors had *PIK3CA* mutations. Hazard ratios (HRs) for trastuzumab in HER2E and other subtypes were 0.44 (95% CI, 0.34 to 0.58; $P < .001$) and 0.47 (95% CI, 0.35 to 0.62; $P < .001$), respectively (interaction $P = .67$). HRs for trastuzumab in *PIK3CA* wild-type and mutated tumors were 0.51 (95% CI, 0.37 to 0.71; $P < .001$) and 0.44 (95% CI, 0.24 to 0.82; $P = .009$), respectively (interaction $P = .64$).

Conclusion
Unlike results seen in the metastatic and neoadjuvant clinical trials, *PIK3CA* and PAM50 intrinsic subtypes were not predictive biomarkers for adjuvant trastuzumab in NSABP B-31. These data suggest that results from the metastatic and neoadjuvant setting may not be always applicable to the adjuvant setting.

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INTRODUCTION

Randomized clinical trials have demonstrated the clinical efficacy of trastuzumab added to chemotherapy in treatment of human epidermal growth factor receptor 2 (HER2) –positive stage II and III breast cancer with approximately 50% reduction in recurrence rate.[1]

Considerable molecular heterogeneity exists among HER2-positive breast cancer in regard to gene expression and mutation spectrum, and tumor response to trastuzumab may also be heterogeneous accordingly. Using an unsupervised approach not based on trastuzumab response data, the Cancer Genome Atlas data demonstrated that only approximately 50% of clinically HER2-positive breast cancers were classified as the HER2-enriched (HER2E) intrinsic subtype by the PAM50 algorithm, with the rest classified largely as luminal subtypes.[2,3] In the neoadjuvant therapy setting (Cancer and Leukemia

Group B 40601 trial), 80% of HER2E tumors had pathologic complete response (pCR) on neoadjuvant chemotherapy plus HER2-targeted therapies, whereas the pCR rate was 25% to 32% in the other subtypes, which was similar to the pCR rate in the chemotherapy arm.[4] Thus, the PAM50 intrinsic subtype may identify a much larger subset (approximately 50%) of patients with HER2-positive breast cancer who may not benefit from adjuvant trastuzumab.[4] This is in contrast to our recently reported 8-gene (*ESR1*, *CA12*, *GATA3*, *IGFR1*, *NAT1*, *ERBB2*, *GRB7*, *C17orf37*) predictive signature developed from a supervised approach using clinical data from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-31 trial, which classified only 10% of HER2-positive patients as a no-benefit group.[5]

Another proposed marker of trastuzumab response is *PIK3CA* mutational status. In vitro data suggested that *PIK3CA* mutations present in approximately 25% of HER2-positive tumors were a marker of resistance to trastuzumab.[6] Activating mutations in the *PIK3CA* gene and loss of PTEN expression have been proposed as the most important markers of PI3K pathway activation that lead to intrinsic resistance to trastuzumab.[7] However, whereas several clinical studies in the metastatic or neoadjuvant setting demonstrated such an association,[8,9] other studies failed to replicate the association.[10] A recent meta-analysis of published studies failed to confirm the interaction between *PIK3CA* mutation or PTEN loss and resistance to trastuzumab.[9] Furthermore, retrospective analyses of the Finland Herceptin (FINHER)[10] and North Central Cancer Treatment Group (NCCTG) N9831 trials,[11] two randomized clinical trials of adjuvant trastuzumab, found no association of *PIK3CA* mutation (FINHER) or PTEN loss (N9831) with trastuzumab resistance. To test their worth as predictive markers for adjuvant trastuzumab, we have assayed PAM50 intrinsic subtypes and *PIK3CA* mutations using archived tumor blocks from the NSABP B-31 trial, which tested the worth of adding 1 year of trastuzumab to adjuvant chemotherapy in the treatment of HER2-positive stage III breast cancer.[1]

PATIENTS AND METHODS

Study Design and Patients

The general design of this study and description of available materials are presented in Figure 1. Among patients who participated in the B-31 trial (N = 2,130), 1,878 patients signed informed consent to permit use of banked tissue and had clinical follow-up data, available tumor blocks, and data on estrogen receptor (ER) status and number of positive nodes. The discovery cohort (n = 800) was selected by random sampling stratified by treatment and tested to ensure that it had a similar benefit from trastuzumab as did the entire cohort.

As previously reported, nCounter (nanoString Technologies, Seattle, WA) gene expression profiling for 462 genes was performed on 1,579 cases, and the data will be available through the Database of Genotypes and Phenotypes of the National Institutes of Health.[5] (However, one patient

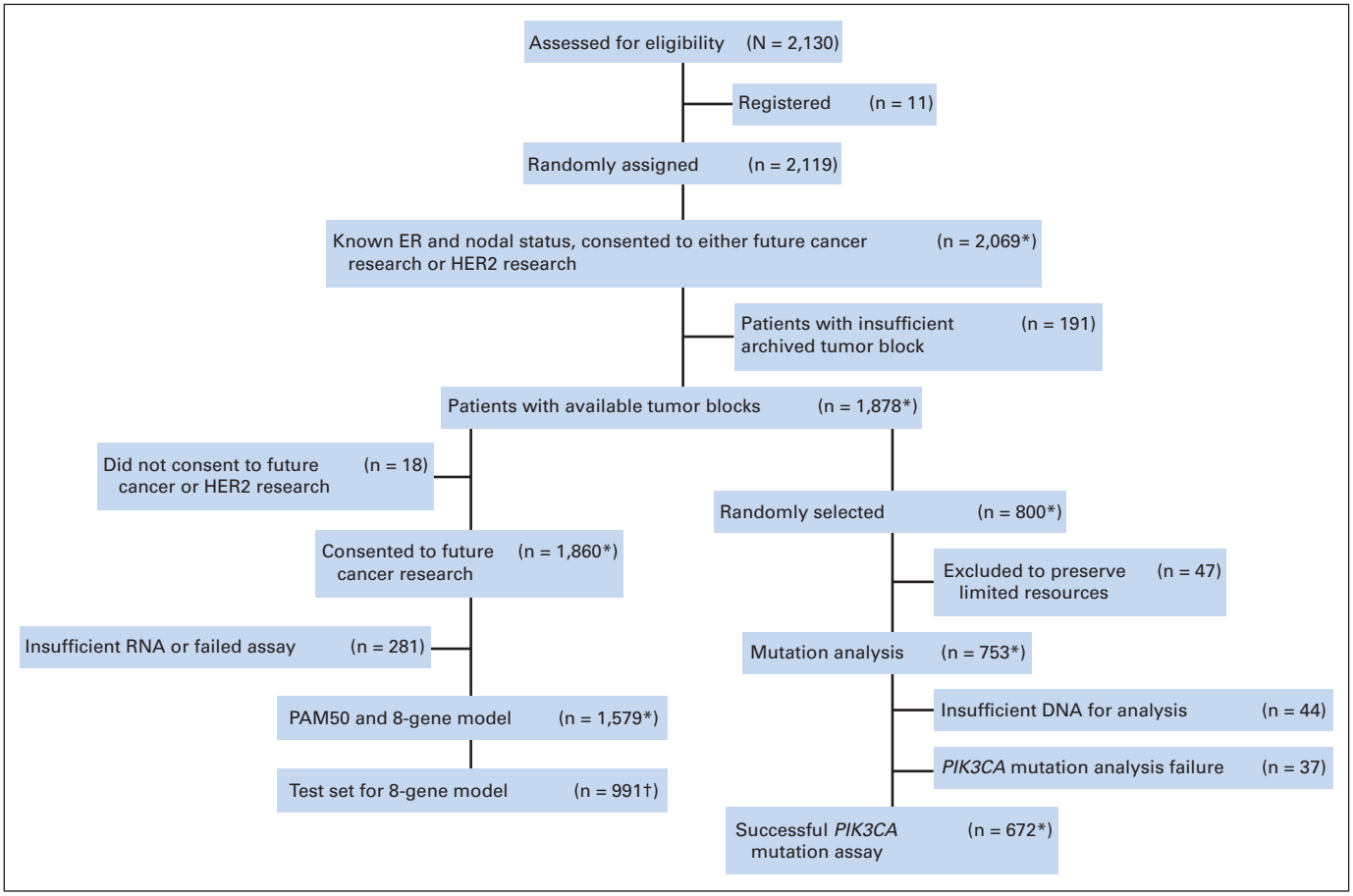

Fig 1. CONSORT diagram for National Surgical Adjuvant Breast and Bowel Project B-31 trial. (*) One patient retracted consent in 2012 after the work of Pogue-Geile et al[5] had been completed. (†) Used to test 8-gene model. ER, estrogen receptor; HER2, human epidermal growth factor receptor 2.

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recently has retracted consent after the work on Pogue-Geile et al[5] had been completed). Among 1,878 cases, 800 were randomly selected and used for evaluation of *PIK3CA*. (One was excluded because of removal of consent.) Of these, 47 with thin blocks were excluded to preserve precious resources, leaving 753 for mutation analyses. The clinical and pathologic characteristics of the study cohort were similar to the original B-31 trial cohort (Data Supplement).

PAM50 Intrinsic Subtype Classification. The nCounter platform-based gene expression profiling of archived tumor blocks from NSABP B-31 has been previously published.[5] Among 462 genes examined using the nCounter platform, 49 from the PAM50 gene list were represented. One gene, *CCNE1*, was unintentionally excluded from the code set, but this exclusion had little impact on the results. The misclassification rate within the Parker et al[3] data set of 200 cases was only 0.5% when the 49 genes were used (Data Supplement).

To identify intrinsic subtype, we first normalized B-31 nCounter gene expression data to remove technical platform differences and patient population differences between B-31 (who were mainly HER2 positive) and the original PAM50 development cohort published by Parker et al[3] (which included all breast cancer). Details and R code used for normalization are included in the Data Supplement. Based on the published PAM50 algorithm, we then identified the following intrinsic subtypes: luminal A, luminal B, HER2E, basal like, and normal like.

PIK3CA *Mutation*

Two 5-μm sections were cut from formalin-fixed, paraffin-embedded blocks, tumor areas with 80% or more tumor cellularity were macrodissected by comparison to matching hematoxylin and eosin–stained slides, and DNA was isolated using the Mag-Bind FFPE DNA kit (Omega Bio-Tek, Norcross, GA). DNA was then subjected to mutation detection using TypePlex chemistry and the Mass-Array platform (Sequenom, San Diego, CA), as described previously.[12] In brief, the method involves polymerase chain reaction amplification of the target region followed by a single base extension reaction and resolution of alleles by matrix-assisted laser desorption ionization–time of flight mass spectrometry. In this study, we evaluated the following amino acid changes at six hotspots in *PIK3CA*: N345K, C420R, E542K, E545K, H1047L, and H1047R. These hotspots cover 92% of *PIK3CA* mutations (1,918 of 2,075 mutations) reported in the Catalogue of Somatic Mutations in Cancer database[13] and have been functionally validated as strongly activating mutations.[14,15] Additional methodologic details are included in the Data Supplement.

Statistical Analysis

Follow-up information was included up to October 2010. Patients from the control arm who crossed over to receive trastuzumab were censored at the time of crossover. Among 1,578 patients who were profiled with nCounter assays, 265 of the 796 patients who were on the doxorubicin and cyclophosphamide followed by paclitaxel arm crossed over to receive trastuzumab. For 671 patients who were successfully profiled for *PIK3CA* mutational status, 112 of the 341 patients who were on the doxorubicin and cyclophosphamide followed by paclitaxel arm crossed over to receive trastuzumab. The definition of the primary end point for this analysis, disease-free survival (DFS), has been previously described.[1] All experiments were performed blinded to clinical data before data anonymization by the NSABP Biostatistical Center.

For analysis of each marker's association with time to event, we first compared DFS rates among subgroups using the Kaplan-Meier method. Cox proportional hazard models were then used to assess the associations of each marker with the end point and the marker-treatment interaction simultaneously controlling for treatment, nodal status (one to three, four to nine, or $\geq$ 10 positive nodes), ER status (negative or positive), age ($<$ or $\geq$ 60 years), and tumor size ($\leq$ 2, 2 to 5, or $>$ 5 cm). All reported *P* values are two-sided. All statistical analyses were performed in R (http://www.r-project.org).

We calculated the power of observing a treatment-group interaction for both PAM50 (HER2E *v* other subtypes) and *PIK3CA* (mutants *v* wild type [WT]). For PAM50, with the current sample size (n = 1,578), the proportion of benefit versus nonbenefit, with an α level of .05, the hazard ratio (HR) between HER2E subtype versus other subtypes must be less than 0.5 to have 80% power. Similarly, for analysis of *PIK3CA* mutational status, to have 80% power, the HR between mutation versus WT must be greater than 2.9 (details shown in the Data Supplement).

RESULTS

All PAM50 Intrinsic Subtypes Receive Benefit From Trastuzumab in B-31

Before examining potential treatment interaction between intrinsic subtypes and trastuzumab, it was important to ensure that our research PAM50 assay resulted in accurate intrinsic subtype classification. The association between PAM50 intrinsic subtypes and clinical ER and HER2 status is shown in Table 1. The distribution of clinical ER and HER2 status among subtypes was similar to that seen in other studies; 741 tumors (47.0%) were classified as HER2E subtype, 103 (6.5%) as basal-like, 435 (27.6%) as luminal A, 223 (14.1%) as luminal B, and 76 (4.8%) as normal-like subtypes.[16,17] As expected, 516 (74.9%) of 689 of the ER-negative/HER2-positive tumors were classified as the HER2E subtype, whereas 97 (91.5%) of 106 of the ER-positive/HER2-negative tumors were classified as luminal subtypes. To further verify that our research PAM50 assay was able to correctly subtype B-31 tumors, we checked expression levels of *ESR1* and *ERBB2* mRNAs according to assigned subtypes. As expected, HER2

Table 1. Distribution of PAM50 Intrinsic Subtypes With Clinical ER and HER2 Status in NSABP B-31

	No. of Patients				
ER and HER2 Status	Basal Like	HER2 Enriched	Luminal A	Luminal B	Normal Like
ER negative/HER2 negative	29	5	3	2	1
ER negative/HER2 positive	64	516	44	10	55
ER negative/HER2 NA	2	4	1	0	1
ER positive/HER2 negative	3	3	66	31	3
ER positive/HER2 positive	4	213	318	179	14
ER positive/HER2 NA	1	0	3	1	2
Total (N = 1,578)	103	741	435	223	76
%	6.5	47.0	27.6	14.1	4.8

Abbreviations: ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; NA, data not available; NSABP, National Surgical Adjuvant Breast and Bowel Project.

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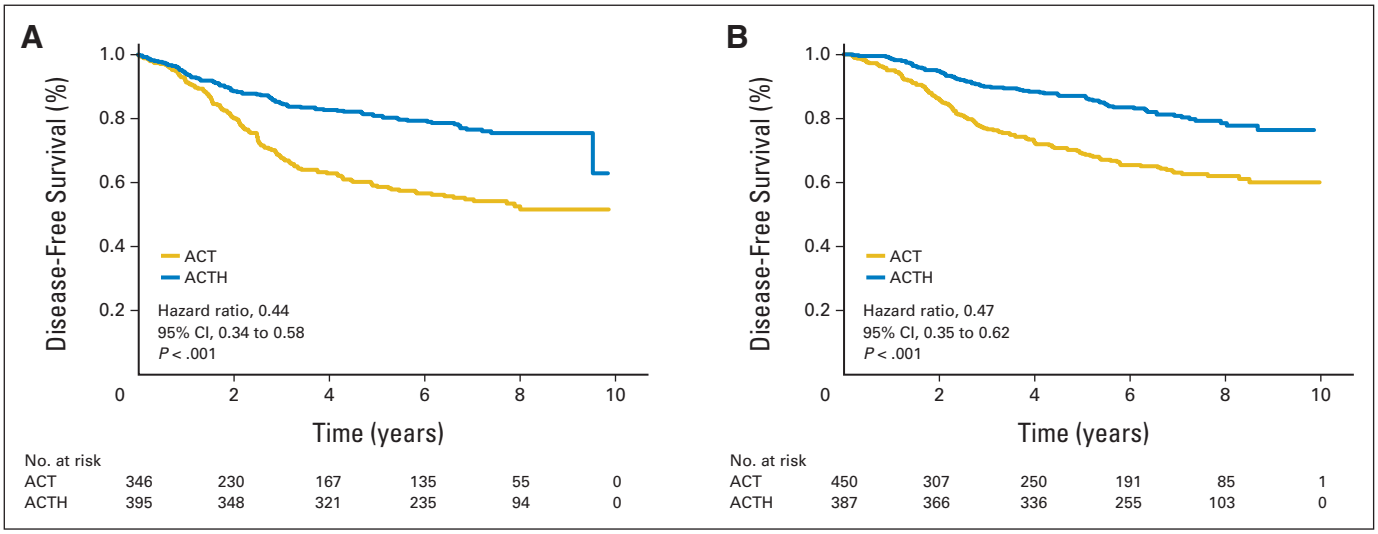

Fig 2. Kaplan-Meier plots for disease-free survival of (A) human epidermal growth factor receptor 2–enriched subtype and (B) other PAM50 intrinsic subtypes (including basal-like, luminal A, luminal B, and normal-like subtypes) by treatment received. The numbers of patients at risk are provided below the figure. ACT, doxorubicin and cyclophosphamide followed by paclitaxel; ACTH, doxorubicin and cyclophosphamide followed by paclitaxel plus trastuzumab.

mRNA expression was highest in the HER2E subtype, whereas *ESR1* mRNA expression was highest in luminal subtypes (Data Supplement). Also as expected, luminal B had a higher proliferation score than did luminal A (Data Supplement).

Having confirmed that research PAM50 subtyping performed as expected, we then checked whether there was heterogeneity of trastuzumab benefit among intrinsic subtypes. For DFS, which was the primary end point of the B-31 trial, there was no significant interaction between HER2E versus other subtypes and degree of benefit from trastuzumab (interaction $P = .67$). The HR for trastuzumab treatment in the HER2E subtype was 0.44 (95% CI, 0.34 to 0.58; $P < .001$), and the HR for other subtypes was 0.47 (95% CI, 0.35 to 0.62; $P < .001$). The Kaplan-Meier plots clearly show that both the HER2E subtype and the non-HER2E subtype receive benefit from trastuzumab (Fig 2).

Within each non-HER2E subtype, there was a trend for benefit from trastuzumab, although P values for basal-like and normal-like subtypes did not reach statistical significance because of small sample size. The HRs for luminal A, luminal B, basal-like, and normal-like subtypes were 0.42 (95% CI, 0.28 to 0.63; $P < .001$), 0.62 (95% CI, 0.37 to 1.04; $P = .072$), 0.43 (95% CI, 0.20 to 0.93; $P = .033$), and 0.45 (95% CI, 0.17 to 1.21; $P = .114$), respectively (Data Supplement). On the basis of these findings, we concluded that PAM50 intrinsic subtyping cannot identify a subset with no benefit from trastuzumab added to adjuvant chemotherapy. We also examined the PAM50 supervised predictors risk of recurrence and risk of recurrence subtype (RORS) and risk of recurrence proliferation (RORP) described by Parker et al[3] (Data Supplement). All subsets defined by RORS and RORP received statistically significant benefit from trastuzumab. Thus, these predictors also failed to identify a subset that did not benefit from trastuzumab.

Relationship Between PAM50 Intrinsic Subtypes and the 8-Gene Trastuzumab Predictive Signature

We have previously published an 8-gene predictive signature for the degree of benefit from trastuzumab.[5] Because these eight genes included ER- and HER2-related genes that play a major role in PAM50 intrinsic subtyping algorithm, we tested the relationship between the two signatures. As shown in the heat map of clustering results (Fig 3), the subset with no benefit defined by the 8-gene predictive signature consisted mostly of luminal subtypes defined by PAM50. However, all five PAM50 subtypes, including both HER2E and non-HER2E subtypes, are represented in the high- and moderate-benefit groups based on the 8-gene model.

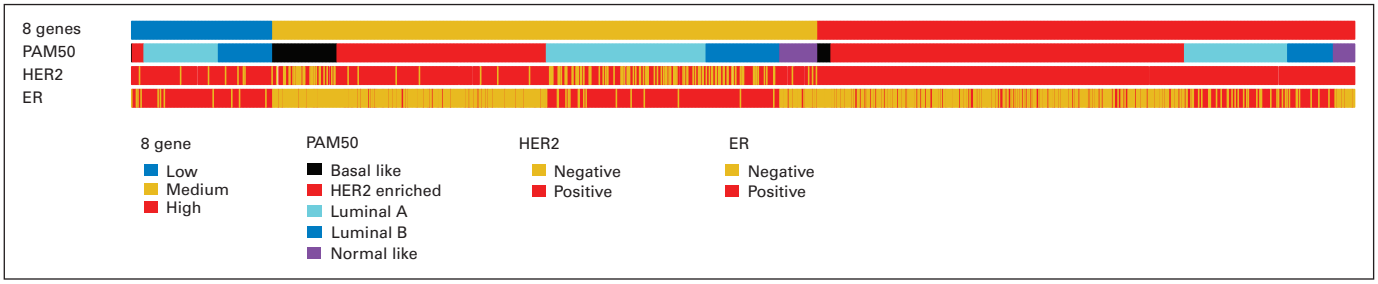

Fig 3. The distribution of subtypes based on the 8-gene predictive signature, PAM50, and estrogen receptor (ER) and clinical human epidermal growth factor receptor 2 (HER2) status is shown.[5]

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Table 2. Association of Clinical Variables With *PIK3CA* Mutations in NSABP B-31

Variable	*PIK3CA* Gene					Exon 9					Exon 20				
	WT		MT			WT		MT			WT		MT		
	No. of Patients	%	No. of Patients	%	*P*	No. of Patients	%	No. of Patients	%	*P*	No. of Patients	%	No. of Patients	%	*P**
Total	505	—	166	—		624	—	49	—		573	—	104	—	
Age, years					.81					.35					.76
< 60	423	83.8	141	84.9		522	83.7	44	89.8		484	84.5	86	82.7	
≥ 60	82	16.2	25	15.1		102	16.3	5	10.2		89	15.5	18	17.3	
Tumor size, cm					.03					0					.39
≤ 2	194	38.4	52	31.3		233	37.3	14	28.6		216	37.7	31	29.8	
2.1 to 5	260	51.5	102	61.4		331	53	32	65.3		302	52.7	64	61.5	
> 5	51	10.1	11	6.6		60	9.6	2	4.1		54	9.4	9	8.7	
NA	0	0	1	0.6		0	0	1	2		1	0.2	0	0	
No. of nodes					.37					.7					.53
1 to 3	282	55.8	103	62		356	57.1	31	63.3		325	56.7	65	62.5	
4 to 9	147	29.1	41	24.7		176	28.2	12	24.5		164	28.6	25	24.0	
≥ 10	76	15	22	13.3		92	14.7	6	12.2		84	14.7	14	13.5	
ER					.45					.26					.8
Negative	244	48.3	74	44.6		301	48.2	19	38.8		270	47.1	51	49.0	
Positive	261	51.7	92	55.4		323	51.8	30	61.2		303	52.9	53	51.0	
HER2					.45					.65					.83
Negative	42	8.3	18	10.8		54	8.7	6	12.2		51	8.9	9	8.7	
Positive	461	91.3	148	89.2		568	91	43	87.8		520	90.8	95	91.3	
NA	2	0.4	0	0		2	0.3	0	0		2	0.3	0	0	
PAM50†					.05					.17					.52
Basal like	37	9.1	3	2.3		40	8	0	0		37	8	3	3.8	
HER2 enriched	193	47.3	61	47.7		238	47.8	16	42.1		216	46.9	40	50.6	
Luminal A	101	24.8	41	32		130	26.1	12	31.6		119	25.8	24	30.4	
Luminal B	57	14	20	15.6		68	13.7	9	23.7		68	14.8	10	12.7	
Normal like	20	4.9	3	2.3		22	4.4	1	2.6		21	4.6	2	2.5	
DFS					.77					.44					.76
Censored	360	71.3	121	72.9		445	71.3	38	77.6		414	72.3	73	70.2	
Event	145	28.7	45	27.1		179	28.7	11	22.4		159	27.7	31	29.8	

Abbreviations: DFS, disease-free survival; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; MT, mutant; NA, data not available; NSABP, National Surgical Adjuvant Breast and Bowel Project; WT, wild type.
*P value was calculated using χ^2 test.
†Not all cases with PIK3CA mutation results have been profiled for PAM50.

PIK3CA *Mutations Are Not Associated With Resistance to Adjuvant Trastuzumab*

Of the randomly selected subset of 800 cases for *PIK3CA* mutation screening, 47 with thin blocks were excluded to preserve precious resources, leaving 753 for mutation analyses. From these, sufficient tumor DNA was obtained for 709 cases, and complete mutation results were obtained for 672 patients. Thirty-seven cases were excluded because of assay failures based on preset criteria of the presence of unextended primers in more than 25% of the area under the peaks.

The frequency and location of the *PIK3CA* mutations detected in B-31 tumors are similar to other studies of breast cancer *PIK3CA* mutations and in close agreement with other studies[18,19] (Data Supplement). Mutations in exons 9 and 20 were not statistically significantly associated with any of these clinical variables (Table 2). *PIK3CA* mutations were also not statistically significantly associated with clinical variables and HER2 status but were associated with age, although the absolute difference was small. In addition, PIK3CA mutations were significantly over-represented in the luminal A subtype compared with WT tumors (Table 2).

In univariate analyses, the DFS benefit from trastuzumab in *PIK3CA* mutant tumors (HR, 0.44; 95% CI, 0.24 to 0.82; $P = .009$) was similar to the benefit in *PIK3CA* WT tumors (HR, 0.51; 95% CI, 0.37 to 0.71; $P < .001$), as illustrated in Figure 4. These results did not change when adjusted for nodal status, ER status, age, and tumor size. In a Cox proportional hazards model controlling for nodal status, ER status, and tumor size, *PIK3CA* did not show a statistically significant interaction with trastuzumab benefit for DFS (interaction $P = .77$). We repeated the analyses with exclusion of central assay HER2-negative cases, and the results were nearly identical (data not shown).

Comparison of the 8-Gene Trastuzumab Predictive Model With Other Signatures

Forest plots of all markers that were tested (PAM50, RORS, RORP, *PIK3CA* mutation status, and the 8-gene predictive model, using only the validation cohort) with univariate and multivariate analyses are shown in Figure 5 and the Data Supplement, respectively. Perhaps it is not surprising that only the 8-gene model was able to identify a subset of patients with no benefit from trastuzumab in

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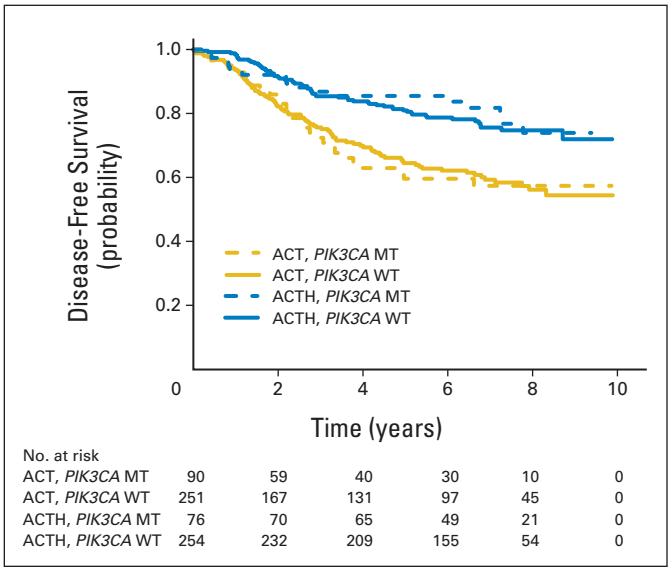

Fig 4. Disease-free survival for *PIK3CA* wild-type (WT) and mutant (MT) tumors for patients treated with doxorubicin and cyclophosphamide followed by paclitaxel (ACT) versus those treated with doxorubicin and cyclophosphamide followed by paclitaxel plus trastuzumab (ACTH). The numbers of patients at risk are provided below the figure.

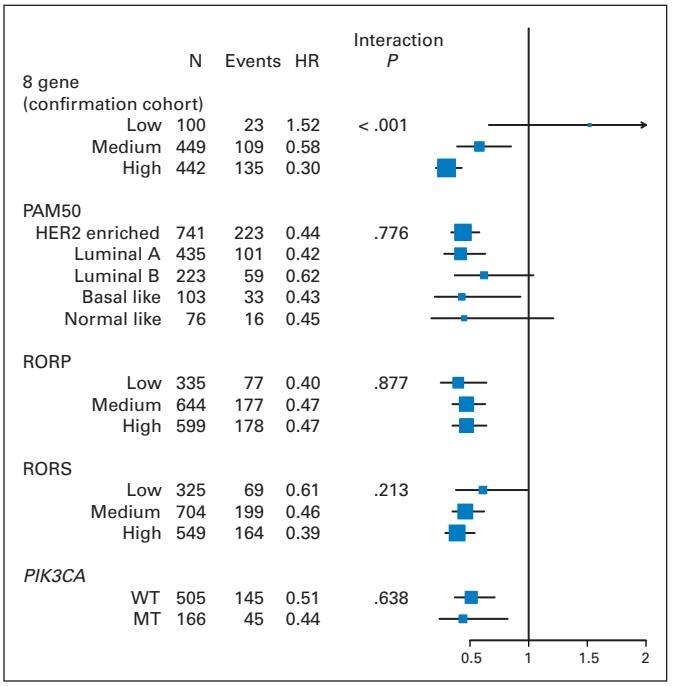

Fig 5. Forest plot of hazard ratios (HRs) for subtypes identified by the 8-gene predictive model,[5] PAM50, risk of recurrence proliferation (RORP), risk of recurrence subtype (RORS), and *PIK3CA* mutation status. HR and interaction *P* value were determined by univariate analysis. With multiple hypothesis adjustment, only the 8-gene model, which was analyzed in the confirmation cohort only, has significant treatment-group interaction. HER2, human epidermal growth factor receptor 2; MT, mutant; WT, wild type.

univariate and multivariate analyses, given the fact that the signature was developed to do just that within a discovery cohort of B-31.

Molecular Profiling Does Not Identify a Subset With Poor Prognosis After Adjuvant Trastuzumab

Recently, pertuzumab received accelerated approval based on the doubling of pCR compared with trastuzumab alone in the NeoSphere trial (NCT00545688),[20] but final approval is contingent on demonstration of DFS benefit in the APHINITY (Study of Pertuzumab in Addition to Chemotherapy and Herceptin [trastuzumab] As Adjuvant Therapy in Patients With HER2-Positive Primary Breast Cancer; NCT01358877) trial. Even if the trial shows positive results, absolute reduction in event rate is expected to be small. Therefore, it would be prudent to develop a prognostic model to identify patients with high residual risk after adjuvant trastuzumab who may need a combination of trastuzumab and pertuzumab. We examined prognosis based on PAM50 intrinsic subtypes, RORS and RORP algorithms, and *PIK3CA* mutations in trastuzumab-treated patients. We could not identify a subtype with a particularly worse prognosis after trastuzumab, although luminal A and normal-like subtypes trended to have a more favorable residual risk (see Data Supplement for Kaplan-Meir plots). Therefore, other molecular assays or clinical parameters such as anatomic burden may be required to identify patients who require additional treatment such as pertuzumab.

DISCUSSION

Although gene expression profiling confirmed molecular heterogeneity of HER2-positive tumors in which the PAM50 intrinsic subtypes were readily identified, there was no difference in the degree of benefit from adjuvant trastuzumab based on intrinsic subtypes. This contrasts with the data from the Cancer and Leukemia Group B 40601 trial, in which 80% of HER2E tumors had pCR on neoadjuvant chemotherapy with trastuzumab compared with a pCR rate of 25% to 32% in the other subtypes, which was similar to the pCR rate in the chemotherapy arm.[4]

In the NeoSphere, Neoadjuvant Lapatinib and/or Trastuzumab Treatment Optimisation (Neo-ALTTO; NCT00553358), and Gepar Quinto (NCT00567554) and Sixto (NCT01426880) neoadjuvant trials, which compared the pCR rate of *PIK3CA* mutant versus WT tumors in patients treated with anti-HER2 therapies, the pCR rate was lower in those with *PIK3CA*-mutated tumors than in those with WT *PIK3CA*.[21,22] In the metastatic setting, the CLEOPATRA (Clinical Evaluation of Pertuzumab and Trastuzumab) trial (NCT00567190) demonstrated that *PIK3CA* mutations were associated with a poorer prognosis for patients treated with anti-HER2 therapy, based on progression-free survival (PFS); however, both *PIK3CA* mutant and WT tumors showed a trend for benefit from pertuzumab.

In contrast, we have demonstrated that there was no interaction between *PIK3CA* hotspot activating mutations and the degree of benefit from trastuzumab in the adjuvant setting, confirming the report from the retrospective analysis of the FINHER trial by Loi et al.[10] Our results, together with the report by Perez et al,[11] which showed the lack of interaction between PTEN loss and the degree of benefit from trastuzumab in the NCCTG N9831 trial,[11] do not support the prevailing hypothesis that PI3K pathway activation either by *PIK3CA* mutation or PTEN loss results in a lack of benefit from trastuzumab in the adjuvant setting.

These data support our hypothesis that there is a fundamental difference between macro diseases (advanced and neoadjuvant) and the minimal residual disease setting for trastuzumab response.[5] End

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points for the evaluation of neoadjuvant and metastatic settings (pCR, PFS) require that large numbers of cancer cells be eliminated in the tumor. In contrast, in the adjuvant setting where the tumor has been surgically removed, improvement in DFS may largely depend on the elimination of a small number of cancer stem cells (CSCs). CSCs have been shown to depend on HER2 signaling in the absence of *HER2* gene amplification.[23] *HER2*-amplified disseminated tumor cells have been detected in clinically HER2-negative patients.[24] CSCs isolated from luminal subtype breast cancer cells have been found to express high levels of HER2 protein and were responsive to trastuzumab therapy.[23] Furthermore, bone metastases of breast cancer frequently overexpressed HER2 protein in the absence of *ERBB2* gene amplification.[23] Therefore, disseminated CSCs or bone micrometastatic cells may be responsive to trastuzumab treatment regardless of HER2 status of the primary tumor. Thus, patients with HER2-negative tumors could benefit from trastuzumab in the adjuvant setting but not in the neoadjuvant or metastatic setting when pCR or PFS is the clinical end point. Benefit from trastuzumab in patients with *HER2*-nonamplified early breast cancer will be assessed in the ongoing NSABP B-47 trial.

However, the fact that *PIK3CA* mutations were not shown to be resistance markers to trastuzumab in NSBAP B-31 should not be interpreted as a contraindication to the clinical development of PI3K pathway inhibitors in HER2-positive breast cancer. A study using a global knockout screen of a single *HER2*-amplified breast cancer cell line demonstrated *PIK3CA* mutation or PTEN loss as a potential mechanism of resistance to trastuzumab.[6] However, many *ERBB2*-amplified cell lines were highly sensitive to *PIK3CA* small interfering RNA, regardless of the presence or absence of mutations in the *PIK3CA* gene.[25] The additive effect of combining trastuzumab with a *PIK3CA* inhibitor was observed in both trastuzumab-sensitive and -resistant cell lines,[26] suggesting that signaling through multiple pathways converge on PI3K in HER2-positive cancer cells and supporting the clinical development of PI3K pathway (*PIK3CA*, AKT, mTOR) inhibitors in HER2-positive breast cancer regardless of *PIK3CA* mutation status.

In conclusion, failure of PAM50 and *PIK3CA* mutations to define subsets of patients with differential benefit from trastuzumab in the adjuvant setting suggests that the predictive markers from metastatic or neoadjuvant studies cannot be applied directly to adjuvant studies, at least for HER2-positive breast cancer.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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GLOSSARY TERMS

estrogen receptor (ER): ligand-activated nuclear proteins, belonging to the class of nuclear receptors, present in many breast cancer cells that are important in the progression of hormone-dependent cancers. After binding, the receptor-ligand complex activates gene transcription. There are two types of estrogen receptors (ERα and ERβ). ERα is one of the most important proteins controlling breast cancer function. ERβ is present in much lower levels in breast cancer, and its function is uncertain. Estrogen receptor status guides therapeutic decisions in breast cancer.

neoadjuvant therapy: the administration of chemotherapy prior to surgery. Induction chemotherapy is generally designed to decrease the size of the tumor prior to resection and to increase the rate of complete (R0) resections.

PIK3CA: the catalytic subunit of phosphatidylinositol 3-kinase involved in the generation of PIP3 which, in turn, leads to the activation of AKT and other oncogenic kinases. Mutations in the PIK3CA gene have been found in several cancers, including ovarian, breast, colon, and lung carcinomas. See PI3K and AKT/PKB.

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