VOLUME 27 · NUMBER 24 · AUGUST 20 2009

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

From the Department of Molecular Pathology and Taussig Cancer Center, Cleveland Clinic, Cleveland; and Cancer and Leukemia Group B, Ohio State University, Arthur James Cancer Center Hospital, Columbus, OH; Cancer Research and Biostatistics; Fred Hutchinson Cancer Research Center; and Phenopath Laboratories, Seattle, WA; Department of Pathology, University of Texas, Health Science Center at San Antonio, San Antonio, TX; Eastern Cooperative Oncology Group, Cancer Pavilion, Indiana University Medical Center, Indianapolis, IN; North Central Cancer Treatment Group, Medical Oncology, Mayo Clinic, Rochester, MN; University of California, Los Angeles Medical Center, Santa Monica, CA; Loyola University Medical Center, Maywood, IL; Arizona Cancer Center, Tucson, AZ; and University of Michigan Comprehensive Cancer Center, Ann Arbor, MI.

Submitted September 17, 2008; accepted February 23, 2009; published online ahead of print at www.jco.org on July 20, 2009.

Supported in part by Public Health Service Cooperative Agreement Grants No. CA32102, CA38926, CA49883, CA21155, CA77658, CA25224, CA31946, CA32291, CA37891, CA35431, CA45377, CA58416, CA22433, CA58686, CA46113, CA04919, CA46441, CA58861, CA46282, CA35261, CA27057, CA76132, CA35192, CA76447, CA76462, CA45450, CA76429, CA63845, CA12644, CA20319, CA63844, CA45560, CA58415, CA14028, CA58658, CA42777, CA35119, CA35090, CA35117, CA13612, CA16385, CA67575, CA68183, CA46368, CA04920, CA74647, and CA52654, awarded by the National Cancer Institute, Department of Health and Human Services; by Amgen; and by the Breast Cancer Research Foundation.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Corresponding author: Raymond R. Tubbs, DO, Department of Molecular Pathology, Cleveland Clinic, Lerner College of Medicine, 9500 Euclid Ave, L30, Cleveland, OH 44195; e-mail: TubbsR@ccf.org.

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

0732-183X/09/2724-3881/$20.00

DOI: 10.1200/JCO.2008.20.1566

Outcome of Patients With Early-Stage Breast Cancer Treated With Doxorubicin-Based Adjuvant Chemotherapy As a Function of *HER2* and *TOP2A* Status

Raymond Tubbs, William E. Barlow, G. Thomas Budd, Eric Swain, Peggy Porter, Allen Gown, I-Ten Yeh, George Sledge, Charles Shapiro, James Ingle, Charles Haskell, Kathy S. Albain, Robert Livingston, and Daniel F. Hayes

ABSTRACT

Purpose
Amplification and deletion of the *TOP2A* gene have been reported as positive predictive markers of response to anthracycline-based therapy. We determined the status of the *HER2* and *TOP2A* genes in a large cohort of breast cancer patients treated with adjuvant doxorubicin (A) and cyclophosphamide (C).

Patients and Methods
TOP2A/CEP17 and *HER2*/CEP17 fluorescent in situ hybridization (FISH) were performed on tissue microarrays (TMAs) constructed from 2,123 of the 3,125 women with moderate-risk primary breast cancer who received equivalent doses of either concurrent adjuvant chemotherapy with A plus C (n = 1,592) or sequential A followed by C (n = 1,533).

Results
An abnormal *TOP2A* genotype was identified for 153 (9.4%) of 1,626 patients (4.0% amplified; 5.4% deleted). An abnormal *HER2* genotype was identified for 303 (20.4%) of 1,483 patients (18.8% amplified; 1.6% deleted). No significant differences in either overall survival (OS) or disease-free survival (DFS) were identified for *TOP2A*. In univariate analysis, OS and DFS rates were strongly and adversely associated only with higher levels of *HER2* amplification (ratio ≥ 4.0). Survival was not associated with low-level *HER2* amplification (ratio ≥ 2; OS hazard ratio [HR], 1.14; $P = .39$; DFS HR, 1.07; $P = .62$), but it was associated for a ratio ≥ 4 (OS HR, 1.45; $P = .03$; DFS HR, 1.38; $P = .033$), in which analysis was adjusted for menopausal status, hormone receptor status, treatment, number of positive nodes, and tumor size.

Conclusion
In this population of patients with early-stage breast cancer who were treated with adjuvant AC chemotherapy, *TOP2A* abnormalities were not associated with outcome. *HER2* high-level amplification was a prognostic marker in anthracycline-treated patients.

J Clin Oncol 27:3881-3886. © 2009 by American Society of Clinical Oncology

INTRODUCTION

Results of several clinical trials have documented the improved outcome of patients with breast cancer who were treated in the adjuvant setting with anthracycline-based chemotherapy.[1,2] Although anthracycline therapy improves the outcome of treated patients, it is associated with occasional life-threatening toxicities, such as congestive heart failure and acute leukemia, as well as with more common but annoying adverse effects, including nausea and vomiting, mucositis, alopecia, and fatigue. Management of patients in this clinical setting could be enhanced through selective use of these regimens via identification of specific immunophenotypic or molecular markers predictive of response (or absence of response) to the agents employed. Addition of a taxane either concurrently or sequentially to anthracycline-based therapy has been shown to additionally improve patient outcomes,[3,4] but this strategy also is encumbered with additional toxicities. Identification of a group of patients who have a low residual risk after treatment with preceding anthracycline-based therapy might spare them the toxicity of requiring subsequent taxane chemotherapy.

Several studies have suggested that amplification and/or overexpression of the *ERBB2* (*HER2*) gene in primary breast cancer tissue may identify a subgroup of patients who are more likely to benefit

from anthracyclines than those who have tumors that have normal *HER2*.[5,6] In approximately 35% to 40% of patients with breast carcinoma that demonstrates *HER2* gene amplification, topoisomerase II α (*TOP2A*) is coamplified.[7] *TOP2A* encodes for an enzyme that plays a key role in DNA replication, and it serves as a molecular target for many antineoplastic agents. The gene that encodes *TOP2A* is located at chromosome 17q 12-q21, in proximity to *HER2*. Several studies have suggested that, rather than *HER2*, *TOP2A* amplification or overexpression is predictive of favorable response to anthracycline-based chemotherapy.[8-18] Enigmatically, other reports have demonstrated that both amplification and deletion of *TOP2A* are related to the sensitivity to anthracycline therapy.[19-21] Thus, the simultaneous amplification of *HER2* and *TOP2A* has been proposed as a molecular predictor of response to anthracycline-based regimens.[22]

In Southwest Oncology Group Protocol S9313 (Intergroup Protocol 0137), patients with either high-risk node-negative or low-risk node-positive breast cancer were randomly assigned to one of two schedules of doxorubicin (A) and cyclophosphamide (C) chemotherapy (combined as AC). Overall results failed to demonstrate any difference in disease-free or overall survival for either of the two tested schedules of AC chemotherapy.[23] We hypothesized that patients with *TOP2A* amplification or deletion would have an outcome superior to patients without such abnormalities when treated with anthracyline-based therapy.

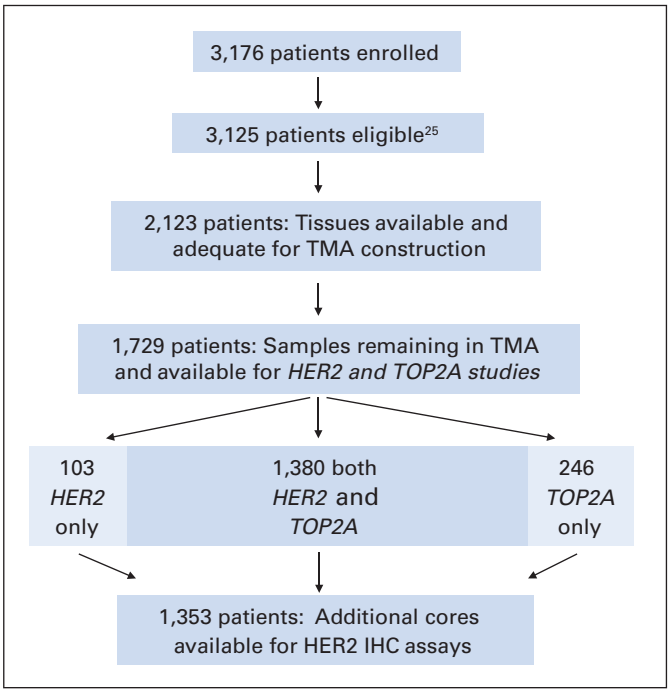

Fig 1. REMARK diagram detailing the materials used for this study. Absence of cores, core loss, absence of invasive carcinoma, and insufficient visualization of fluorescent in situ hybridization signals all contributed to absence of scores for cores within the tissue microarrays (TMAs). IHC, immunohistochemistry.

PATIENTS AND METHODS

Patients

Patient selection, assay performance, and data analysis are reported according to the REMARK criteria.[24] Tissue microarrays (TMAs) that had been prepared with paraffin blocks collected prospectively from patients who participated in SWOG S9313/Int0137 were used for this study. SWOG S9313 was an adjuvant chemotherapy trial that accrued 3,125 eligible women with early-stage breast cancer from April 1994 through May 1997.[23] Participants were required to have one to three nodes involved or to have high-risk node-negative breast cancer, which was defined as primary tumors greater than 2 cm in size or greater than 1 cm for tumors that were both estrogen- and progesterone-receptor negative. Patients were randomly assigned to treatment with one of two alternative dose schedules of AC. As previously reported, there was no difference in disease-free or overall survival for patients treated on the two arms, though the sequential arm (arm 2) produced more myelosuppression and complications related to myelosuppression.[23]

Construction of TMAs

TMAs were constructed from tumor tissue blocks from 2,123 (67%) of the 3,125 patients on S9313.[25] Inclusion of tissue for this study is illustrated in Figure 1. All patients provided written informed consent to participate on S9313 as well as to collect blocks for correlative studies.

TMA Core Tracking and Fluorescent In Situ Hybridization

Automated TMA core tracking and scoring was performed by using modifications of previously described methods.[26] Twenty-eight TMA blocks that contained normal and tumor cores were evaluated by dual-color, direct-label fluorescent in situ hybridization (FISH) by using the *TOP2A*/CEP17 probe set (Abbott Molecular/Vysis, Des Plaines, IL). Unstained sections from 27 of the TMA blocks were available for evaluation with the *HER2*/CEP17 probe set by using staining methods previously described.[26,27] Absence of cores in TMA blocks, core loss during preparation, absence of invasive carcinoma in cores, and insufficient visualization of signals all contributed to the exclusion of some cores from analysis. The FISH results for available cores that could be scored were averaged, and the mean was used as the FISH score of record.

Automated Scoring of FISH Results

The Metasystems Metafer Metacyte v4.3.1.133 scanning system (Metasystems, Altlussheim, Germany) was used to scan slides and to track TMA cores. The Micro Array Tool (MAT) interface window was used to manually assign each core an identification and to establish coordinate positions for every core, thereby creating a position list for each TMA slide. By using the spot center and relocation functions from the interface window, the core center coordinates were marked and aligned in the overview image and were assigned a core identification from the array map.

Immunohistochemistry

Tissue sections were deparaffinized and rehydrated before they were incubated in 0.01M citrate buffer at pH 6.0 in a steamer for 40 minutes at greater than 95°C. All immunohistochemical procedures were performed on a Dako Autostainer (Dako, Carpinteria, CA). A polyclonal antibody to *HER2* (A0485; Dako) was applied at a 1:200 dilution in phosphate-buffered saline (PBS) to sections and was incubated for 40 minutes at room temperature. With intervening wash steps in PBS, slides were incubated for 30 minutes at room temperature in a rabbit-specific, labeled polymer (EnVision+; Dako), which was followed by 10 minutes at 37°C in a solution that contained 3% hydrogen peroxide and 3,3′ -diaminobenzidine. Slides were counterstained with hematoxylin.

Normalized Immunohistochemical Scoring Methodology

Immunostained slides were scored according to a modification of the scoring system approved by the US Food and Drug Administration.[28] Only invasive carcinoma was scored among the neoplastic cells. For tumor cells, only membrane staining intensity and pattern were evaluated by using the semiquantitative scale of 0 to 3+. The non-neoplastic epithelium was scored on a scale of 0 to 3+ by using identical criteria. The normalized *HER2* score subtracted the score on the benign cells from that on the tumor cells.

Statistical Analysis

Disease-free survival was defined as the time from registration to first recurrence (local, regional, or distant), to new primary cancer in the contralateral breast, or to death as a result of any cause, as per the clinical protocol.

Overall survival was defined as time from registration to death as a result of any cause. Patients were censored on the date of last contact if a failure event had not been observed. Unadjusted survival was assessed by the Kaplan-Meier method. Cox regression analysis was used to estimate hazard ratios (HRs) and their 95% CIs; this analysis included possible adjustment for treatment assignment, tumor size (< 2 cm, 2 to 5 cm, or > 5 cm), number of positive lymph nodes (0, 1, 2, or 3), menopausal status, and hormone receptor status (both estrogen- and progesterone-negative status versus either positive by local institutional standards). All reported *P* values and CIs were from two-sided tests. Statistical testing was done with different cutoffs, as described in the Normalized Immunohistochemical Scoring Methodology section, to determine the sensitivity of the results to the choice of cut point.

To test agreement between immunohistochemical and FISH evaluations of *HER2* positivity, we used the κ statistic that corrects for agreement as a result of chance and then determines whether the remaining agreement is significantly greater than chance.[29]

RESULTS

Patient Demographics

Patients with TMA cores who were included on this study did not differ significantly from those without tissue samples on age, menopausal status, or receptor status. Included patients had slightly larger tumors ($P = .049$) and slightly more positive nodes ($P = .033$) than patients without tissue samples. For those with markers, 45% were receptor negative, and 53% had one or more positive nodes. Disease-free survival and overall survival did not differ after adjustment for prognostic factors, so the sample is representative of the entire trial population as described by Linden et al.[23]

Originally, 2,123 patients (67%) had banked tissue from which adequate TMAs could be constructed.[25] After some depletion of the tissue, a total of 11,114 TMA cores from 1,729 patients were sufficiently intact and yielded enumerable FISH signals for scoring (Fig 1). Absence of cores in the TMA blocks, core loss, absence of invasive carcinoma, and insufficient visualization of FISH signals contributed to the exclusion of cores for analysis. *HER2* and *TOP2A* FISH data were available for 1,483 (86%) and 1,628 (94%) women, respectively. The reduced number for *HER2* was due to a processing error on one of the 28 TMAs. Both assays could be performed for 1,380 women (80%), whereas the remaining 349 women (20%) had only one assay, not both, completed.

Association of Outcomes With HER2 Abnormalities

An abnormal *HER2* genotype was identified for 303 (20.4%) of 1,483 women (18.8% amplified; ratio $\geq$ 2.0; 1.6% deleted; ratio $\leq$ 0.70). Disease-free and overall survival rates were marginally worse with *HER2* amplification by classic criteria, but these data were not statistically significant after analysis was adjusted for hormone receptor status, tumor size, number of positive nodes, menopausal status, and randomly assigned treatment (Table 1). Clinical outcomes were not associated with *HER2* amplification when considered by consensus agreement for positivity. However, in an exploratory analysis, we observed that both disease-free survival (adjusted HR, 1.38; 95% CI, 1.03 to 1.85) and overall survival (adjusted HR, 1.45; 95% CI, 1.04 to 2.03) were statistically significantly shorter in patients with *HER2*/CEP17 ratios of greater than 4.0 (Table 1, Fig 2). As the criterion for the *HER2* ratio increased, disease-free and overall survival HRs increased, though only those for disease-free survival were statistically significant. No association with survival was detected when patients who had *HER2* deletion were compared with those who had normal *HER2* or with those who had amplified *HER2*.

Association of Outcomes With TOP2A Abnormalities

An abnormal *TOP2A* genotype was identified in 153 (9.4%) of 1,626 women (4.0% amplified; 5.4% deleted). All but one patient with

Table 1. *HER2* and *TOP2A* Gene Amplification Associations

Genotype	Patient Data		Disease-Free Survival			Overall Survival		
	No.	%	HR	95% CI	*P*	HR	95% CI	*P*
HER2/CEP17	1,483							
≥ 2.0	279	19	1.07	0.83 to 1.37	.62	1.14	0.85 to 1.52	.39
≥ 4.0	149	10	1.38	1.03 to 1.85	.033	1.45	1.04 to 2.03	.03
≥ 6.0	51	3.4	1.76	1.14 to 2.73	.01	1.58	0.95 to 2.63	.08
≥ 8.0	16	1.1	2.48	1.27 to 4.82	.008	1.75	0.72 to 4.28	.22
≤ 0.70 or ≥ 2.0	303	20	1.11	0.87 to 1.42	.39	1.21	0.91 to 1.60	.19
TOP2A/CEP17								
≥ 2.0	65	4.0	0.98	0.60 to 1.59	.92	1.10	0.64 to 1.89	.72
≥ 4.0	13	0.8	0.52	0.13 to 2.08	.35	0.77	0.19 to 3.11	.72
≥ 6.0	4	0.3	NE	NE	NE	NE	NE	NE
≤ 0.70 or ≥ 2.0	153	9.4	1.05	0.77 to 1.45	.75	1.24	0.87 to 1.78	.23
TOP2A copy								
≥ 4.0	195	12	1.07	0.80 to 1.44	.64	1.10	0.78 to 1.56	.58
≥ 6.0	48	3.0	0.70	0.38 to 1.32	.27	0.87	0.45 to 1.69	.68
HER2/CEP17 > 4.0 subset	140							
TOP2A/CEP17								
≥ 2.0	33	24	0.79	0.40 to 1.59	.51	0.81	0.37 to 1.78	.61
≥ 4.0	7	5	0.62	0.15 to 2.62	.51	0.82	0.19 to 3.57	.79
≤ 0.70 or ≥ 2.0	51	36	0.78	0.43 to 1.41	.41	0.72	0.36 to 1.43	.34

NOTE. Analysis was adjusted for hormone receptor status, tumor size, number of positive nodes, menopausal status, and randomized treatment assignment.
Abbreviations: HR, hazard ratio; NE, not estimable.

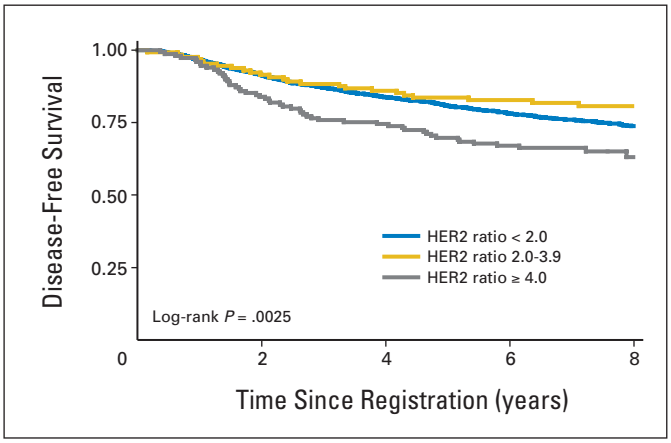

Fig 2. The effect of *HER2* gene amplification on disease-free survival. Significantly shortened disease-free survival was observed only for high-level amplification (*HER2*/CEP17 ratio > 4.0).

amplified *TOP2A* were coamplified for *HER2*. No significant associations with either disease-free or overall survival were identified for *TOP2A* amplification and deletion. Both amplification by *TOP2A*/CEP17 ratio and *TOP2A* copy number were considered, though neither were associated significantly with survival.

Because *TOP2A* amplification occurred almost solely in patients with *HER2* amplification, we also considered the potential interaction of dual gene amplification—the influence of *TOP2A* deletion and/or amplification for women with amplified *HER2* (ratio ≥ 2.0) and for women who displayed high-level *HER2* amplification (ratio ≥ 4.0). As summarized in Table 1 and as illustrated in Figure 3, a statistically significant association was not identified between *TOP2A* deletion and/or amplification in the context of *HER2* amplification, even when patients were segregated by high-level *HER2* amplification and by both *TOP2A*/CEP17 ratio and *TOP2A* copy number.

Analysis of Outcomes According to Treatment Arm

As noted, there was no difference in disease-free and overall survival in the parent trial in patients randomly assigned to sequential

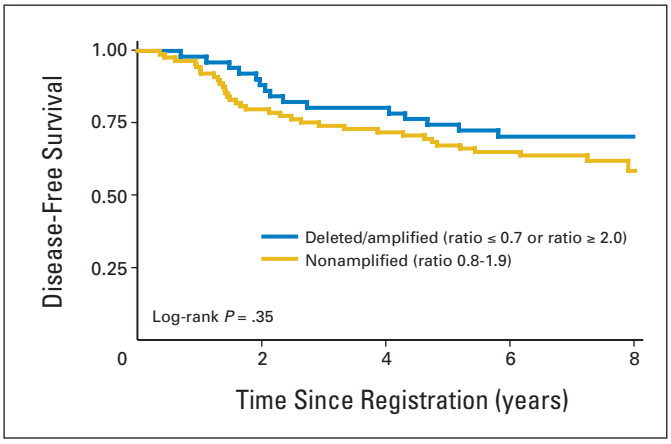

Fig 3. The effect of any *TOP2A* abnormality (either *TOP2A* deletion or *TOP2A* amplification) on disease-free survival for high-level *HER2*-amplified instances (*HER2*/CEP17 ratio > 4.0). Similarly, no significant differences in overall survival were observed for *TOP2A* and *HER2* coamplified instances, and neither overall nor disease-free survivals were significantly different when the analysis was segregated for *TOP2A* deletion or *TOP2A* amplification within the *HER2*-amplified group.

Table 2. Correlations of *HER2* Determinations by IHC and FISH

	HER2/CEP17 Ratio					
	< 2.0		≥ 2.0		Total	
IHC	No.	%	No.	%	No.	%
0	394	97.6	14	3.4	408	100
1	416	95.2	21	4.8	437	100
2	236	79.7	60	20.3	296	100
3	42	19.8	170	80.2	212	100
Total	1,088	80.4	265	19.6	1,353	100

Abbreviations: IHC, immunohistochemistry; FISH, fluorescent in situ hybridization.

or concurrent AC. In this correlative study, we did not detect any significant differences between these two treatment arms according to *HER2* status, even when data were evaluated by using a *HER2*/CEP17 ratio ≥ 4.0 and *TOP2A*/CEP17 normal ratio (ie, 0.80 to 1.9; data not shown). Similarly, outcomes in the two arms were similar when analyzed by using *HER2*/CEP17 ratio ≥ 4.0 and by either *TOP2A* deletion (ratio ≤ 0.7) or amplification (ratio ≥ 2.0; data not shown).

Correlation of HER2 *Amplification by FISH and Immunohistochemical* HER2 *Status*

Tables 2 and 3 summarize the agreement between the *HER2*/CEP17 ratio and *HER2* status by immunohistochemical assay. Among the 845 tumors (62.5%) that stained 0 to 1+ on immunohistochemistry for *HER2*, 4.1% showed *HER2* amplification by FISH. A total of 20.3% of tumors that stained 2+ on immunohistochemistry were found to be *HER2* amplified, whereas 80.2% of tumors that stained 3+ on immunohistochemistry showed *HER2* amplification by FISH. Statistical comparison of agreement between immunohistochemistry (categorized as 0 to 2+ *v* 3+) and the *HER2*/CEP17 ratio (categorized as < 2.0 *v* ≥ 2.0) showed 89.9% agreement ($\kappa = 0.65$; $P < .001$ for testing against chance agreement).

Association of Outcome With Chromosome 17 Copy Number

Patients who had tumors with aneusomy/polysomy 17 (defined as tumors that had a mean of greater than three CEP17 signals per nucleus) had a significantly better disease-free survival (log-rank $P = .016$) and overall survival (log-rank $P = .028$) than did patients with eusomic tumors (defined as having a mean of ≤ three CEP17 signals per nucleus). In an unadjusted Cox model, patients who had tumors with aneusomy/polysomy 17 had significantly better disease-free survival than did patients with eusomy 17 (Cox model HR, 0.84;

Table 3. Correlation of *HER2* Determinations by Combined IHC Scores

	HER2/CEP17 Ratio		
IHC	< 2.0	≥ 2.0	Total
0-2	1,046	95	1,141
3	42	170	212
Total	1,088	265	1,353

NOTE. IHC was combined into the following categories: 0-2+ and 3+. Statistics were as follows: κ, 0.65; standard error, 0.02; $P < .0001$.
Abbreviation: IHC, immunohistochemistry.

$P = .033$ for a one-unit change in CEP17). After analysis was adjusted for receptor status, tumor size, menopausal status, and number of positive nodes, the adjusted HR was 0.86, and $P = .075$ for a one-unit change in CEP17. For overall survival, an unadjusted Cox model produced an HR of 0.81 and $P = .032$ for a one-unit change in CEP17. After analysis was adjusted for these same factors, the overall survival HR was 0.83 and $P = .071$ for a one-unit change in CEP17. When the data were analyzed as described by Reinholz et al,[30] in which aneusomy was defined as greater than 30% of nuclei having three or more CEP17 signals, the log-rank P for disease-free survival = .12, though $P = .007$ if the proportion of aneusomic cells was analyzed as a continuous variable in a Cox regression analysis.

DISCUSSION

In this study, we failed to detect any prognostic effect for classically determined *HER2* gene amplification or for *TOP2A* gene amplification or deletion in tissues collected from a large group of women with modest-risk, stages I and II breast cancer who were all treated with nearly identical adjuvant AC chemotherapy regimens. The correlation between *HER2* status as determined by immunohistochemistry and by FISH is similar to that reported by others.[29] In an exploratory analysis, we did observe that patients with particularly high levels of *HER2* amplification had worse DFS. Reinholz et al[30] have previously reported that patients treated with anthracycline- and taxane-based chemotherapy alone (without trastuzumab) whose tumors were *HER2* amplified and were polysomic for chromosome 17 had a superior disease-free survival compared with patients with *HER2*-amplified tumors that were not polysomic for chromosome 17. Too few patients with *HER2*-nonamplified tumors were present in that group to draw clear conclusions regarding the majority of patients whose tumors are not amplified for *HER2.* We examined the data in this study in a *HER2*-unselected population to determine if this finding could be replicated. Although these results are not robust and should not be used to justify the selection of therapy in any patient group, they appear consistent with the report of Reinholz et al.[30] Although it is tempting to invoke an explanation for this observation on the basis of topoisomerase II copy number in the tumors with aneusomy/polysomy 17, this explanation is made less tenable by the observations in this study for patients with *TOP2A* gene amplification, as discussed in the Discussion section; additional studies are needed to additionally explore the relationship between chromosome 17 copy number and outcome and of the biology underlying this apparent association. The analysis was performed by using Cox regression hazard risk adjusted for treatment, menopause, tumor size, the number of positive lymph nodes, and hormone receptor status.

Our results are, at first glance, perplexing. In retrospect, however, we believe they may represent the mixed prognostic and predictive role of *HER2* and *TOP2A*. Several studies have suggested that *HER2* amplification is associated with worse prognosis in breast cancer, so one might expect lower disease-free and overall survival in patients with this abnormality.[31] Conversely, *HER2* and *TOP2A* appear to be favorable predictive factors for a benefit from anthracycline-containing therapy. Thus, in this population of patients who all received an equivalent regimen of AC, one might expect that patients whose tumors are *HER2* and *TOP2A* positive would have the same outcomes as those whose tumors are negative for these two markers. Their overall prognosis would be worse in the absence of chemotherapy, but they might be expected to achieve more benefit from doxorubicin-based therapy. It has been hypothesized that topoisomerase II abnormalities, which occur almost exclusively in *HER2*-amplified tumors, are responsible for the previously described association between *HER2* amplification and anthracycline sensitivity.[8-18] It would be expected that anthracyclines would be of particular benefit in the *HER2*-amplified tumors that were also amplified or deleted for *TOP2A*, but not in the *HER2*-amplified tumors with the normal copy number of *TOP2A*. The observation that outcome among patients with *HER2*-amplified tumors did not differ according to *TOP2A* status suggests that *TOP2A* amplification or deletion is not the sole explanation for the observation that *HER2*-amplified tumors benefit from anthracycline-based chemotherapy.

These data do not provide additional insights into how better to treat patients with stages I and II cancer who have modestly high risk for recurrence, if it is assumed that they will receive a doxorubicin-based regimen. Several studies have reported that addition of a taxane to anthracycline-based therapy additionally decreases the risk of recurrence and death,[4] although neither increased dose of C or A appears beneficial.[4,32,33] A recently reported study from the Cancer and Leukemia Group B (CALGB) has suggested that *HER2* amplification and/or overexpression may identify those patients most likely to benefit from addition of pactlitaxel, but there was no detectable interaction between *HER2* and D dose greater than 60 mg/m.[34] However, in this study, neither *HER2* nor *TOP2A* abnormalities identified a group of patients who might have done so well with AC alone that they would forego paclitaxel. Likewise, with the possible exception of very high *HER2* amplification, neither marker distinguished a group of patients whose prognoses appears substantially worse than other groups to the extent that a different treatment strategy would be justified.

HER2 and *TOP2A* copy number alterations are not associated with outcome in patients who are treated with standard doses of AC given either concurrently or sequentially. Although some studies suggest that these markers might be used to select patients for anthracycline-based therapies,[8-17,19-22,35] this finding has not been universal[36]; because all of the patients on this study were treated with anthracyclines, these data do not bear directly on this question. *HER2* is an important predictive marker for selection of anti-*HER2*–based therapies, such as trastuzumab and lapatinib, and possibly for selection of paclitaxel after adjuvant AC chemotherapy. However, these markers have not been definitively shown to be otherwise useful in selecting additional therapies for patients who receive regimens similar to those received by the patients in this trial.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Conception and design: Raymond Tubbs, William E. Barlow, G. Thomas Budd, Allen Gown, Robert Livingston, Daniel F. Hayes
Administrative support: Raymond Tubbs, William E. Barlow, G. Thomas Budd, Daniel F. Hayes
Provision of study materials or patients: Raymond Tubbs, William E. Barlow, G. Thomas Budd, Peggy Porter, I-Ten Yeh, George Sledge,

Charles Shapiro, James Ingle, Charles Haskell, Robert Livingston, Daniel F. Hayes

Collection and assembly of data: Raymond Tubbs, William E. Barlow, G. Thomas Budd, Eric Swain, Allen Gown

Data analysis and interpretation: Raymond Tubbs, William E. Barlow, G. Thomas Budd, Allen Gown, Kathy S. Albain, Robert Livingston, Daniel F. Hayes

Manuscript writing: Raymond Tubbs, William E. Barlow, G. Thomas Budd, Eric Swain, Allen Gown, Daniel F. Hayes

Final approval of manuscript: Raymond Tubbs, William E. Barlow, G. Thomas Budd, Eric Swain, Peggy Porter, Allen Gown, I-Ten Yeh, George Sledge, Charles Shapiro, James Ingle, Charles Haskell, Kathy S. Albain, Robert Livingston, Daniel F. Hayes

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