Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

p53 Expression in Node-Positive Breast Cancer Patients: Results from the Cancer and Leukemia Group B 9344 Trial (159905)

Jonathan F. Lara[1], Ann D. Thor[2], Lynn G. Dressler[3], Gloria Broadwater[5], Ira J. Bleiweiss[6], Susan Edgerton[2], David Cowan[4], Lori J. Goldstein[8], Silvana Martino[9], James N. Ingle[10], I. Craig Henderson[11], Larry Norton[7], Eric P. Winer[12], Clifford A. Hudis[7], Matthew J. Ellis[13], Donald A. Berry[14], and Daniel F. Hayes[15] for the Cancer and Leukemia Group B

Abstract

Purpose: p53 as a prognostic and predictive factor in early-stage breast cancer has had mixed results. We studied p53 protein expression, by immunohistochemistry, in a randomized clinical trial of stage II patients treated with adjuvant doxorubicin and cyclophosphamide with or without paclitaxel [Cancer and Leukemia Group B (CALGB) 9344, INT0148].

Patients and Methods: Epithelial p53 expression was evaluated using two immunohistochemical antibodies (DO7 and 1801) in formalin-fixed, paraffin-embedded tissue from patients with node-positive breast cancer who were randomized to four cycles of cyclophosphamide and one of three doses of doxorubicin (60, 75, or 90 mg/m^2; AC) and to receive four subsequent cycles of paclitaxel (T) or not. Prognostic and predictive value of p53 protein expression was assessed, independent of treatment assignment, for escalating doses of doxorubicin or addition of T with endpoints of relapse-free (RFS) and overall survival (OS).

Results: Of 3,121 patients, 1,887 patient specimens treated on C9344 were obtained, passed quality control, and evaluated for p53 expression. Expression was 23% and 27% for mAbs 1801 and D07, respectively, with 92% concordance. In univariate analysis, p53 positivity was associated with worse OS with either antibody, but only p53 staining with monoclonal antibody 1801 had significantly worse RFS. In multivariate analysis, p53 was not predictive of RFS or OS from either doxorubicin dose escalation or addition of paclitaxel regardless of the antibody.

Conclusion: Nuclear staining of p53 by immunohistochemistry is associated with worse prognosis in node-positive patients treated with adjuvant doxorubicin-based chemotherapy but is not a useful predictor of benefit from doxorubicin dose escalation or the addition of paclitaxel. *Clin Cancer Res; 17(15); 5170–8. ©2011 AACR.*

Authors' Affiliations: [1]Department of Pathology, Saint Barnabas Medical Center, Livingston, New Jersey; [2]Department of Pathology, University of Colorado, Denver, Colorado; [3]Institute of Pharmacogenomics and Individualized Therapy, Eshelman School of Pharmacy, [4]Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill; [5]Cancer and Leukemia Group B Statistical Center, Duke University Medical Center, Durham, North Carolina; [6]Department of Pathology, Mount Sinai Medical Center; [7]Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York; [8]Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, Pennsylvania; [9]Department of Medical Oncology, The Angeles Clinic and Research Institute, Santa Monica, California; [10]Department of Medical Oncology, Mayo Clinic, Rochester, Minnesota; [11]Department of Medical Oncology, University of California, San Francisco, California; [12]Department of Medical Oncology, Dana Farber Cancer Institute, Boston, Massachusetts; [13]Department of Medical Oncology, Washington University School of Medicine, St. Louis, Missouri; [14]Department of Biostatistics, MD Anderson Cancer Center Statistical Center, Houston, Texas; and [15]University of Michigan Comprehensive Cancer Center, Ann Arbor, Michigan

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Corresponding Author: Jonathan F. Lara, Department of Pathology, Saint Barnabas Medical Center, 94 Old Short Hills Road, Livingston, NJ 07039. Phone: 973-322-5762; Fax: 973-322-5564; E-mail: jlara@sbhcs.com

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Introduction

p53 is a vital regulator of genomic stability by controlling the cell cycle and inducing apoptosis when cell damage is beyond repair (1–3). The *p53* gene is located on the short arm of chromosome 17 (17p13.1) and encodes a 375-amino acid nuclear phosphoprotein that prevents propagation of genetically altered cells (4). In normal cells, p53 protein has a very short half-life, expressed in minutes, by virtue of ubiquitylation and proteasome degradation, mediated by MDM2 (5, 6). However, missense mutations within the *p53* gene result in protein that is stabilized through posttranscriptional modification and accumulation in the cell nucleus.

p53 protein expression has been related to poor outcome in breast cancer (1, 7–16). However, its utility as a prognostic marker is controversial, and p53 determination is not recommended for routine clinical use in newly diagnosed breast cancer patients (2, 3, 6, 17–24). The mixed results for epithelial p53 and breast cancer prognosis may reflect, in part, the pleiotropic functions

Translational Relevance

The findings of this study will further refine the direction of evaluation and treatment of new breast cancers, specifically whether or not to include p53 in initial evaluation as a breast prognostic factor.

of p53, which are mediated by different domains of the protein. In this regard, p53 might confer both prognostic and predictive effects, depending on whether and what systemic therapy is applied. Predictive factors are best considered in the context of prospective randomized trials that have addressed the specific utility of the treatment in question (25, 26). Therefore, studies that do not take systemic therapies into consideration are likely to be highly confounded.

The Cancer and Leukemia Group B (CALGB) has previously reported that increasing doses of a doxorubicin-based regimen (doxorubicin doses from 30–60 mg/m^2) improved both relapse-free and overall survival (RFS and OS, respectively; ref. 27). The results from a subsequent study, CALGB 9344 (North American Intergroup 0148), showed no evidence of benefit from further escalation of doxorubicin above 60 mg/m^2, when applied with a fixed dose of cyclophosphamide ("AC" chemotherapy) but a statistically significant and clinically important benefit with addition of paclitaxel after AC (28). We have also previously reported that HER2 amplification and/or overexpression is a strong predictive factor of outcome in patients receiving paclitaxel after AC in C9344 (29). We hypothesized that p53 abnormalities, as indicated by staining with immunohistochemistry might also predict benefit from either increasing doses of doxorubicin or from addition of paclitaxel after 4 cycles of AC. In the present study, we report the results of analysis of C9344 according to p53 protein expression as determined by immunohistochemistry with 2 different monoclonal antibodies (mAb).

Materials and Methods

Patients

The CALGB Study 9344, a Phase III Intergroup Study (INT-0148, CALGB 9344, ECOG C9344, NCCTG 94-30-51, and SWOG 9410) was the source of the patient material used in this analysis. Prior analyses of the main effects and of subgroup analyses according to HER2 status have been published by the CALGB (28, 29) and others (30). CALGB/INT 0148 was a 2 × 3 factorial design in which patients were randomly assigned to 1 of 6 possible treatment combinations. All patients received 4 cycles of doxorubicin (Adriamycin, A) and cyclophosphamide (C) given every 3 weeks. The latter was given at a fixed dose of 600 mg/m^2, whereas patients were randomly assigned to 1 of 3 doses of doxorubicin (60, 75, or 90 mg/m^2). All patients were also randomly assigned to either receive 4 cycles of paclitaxel (Taxol, T) every 3 weeks following the AC, or no further chemotherapy. A total of 3,121 patients were accrued to C9344. All patients signed written informed consent and the protocol was approved through individual Institutional Review Boards (IRB).

Tissue procurement and utilization

Approximately 90% of patients accrued to C9344 provided written informed consent for collection and submission of formalin-fixed, paraffin-embedded blocks or unstained tissue sections on slides. The informed consent was approved by all participating IRBs. Permission to receive and study the individual tissue sections was provided by the IRBs of the respective investigational laboratories. All processing, staining, and statistical evaluation was prospectively described in a written protocol (CALGB 159905). Tissue specimens from the patients' primary institution were submitted to the Pathology Coordinating Office (PCO) of each of the participating groups and then submitted for storage and processing at the CALGB PCO. Sections were logged, coded, and reviewed for sufficient invasive cancer by the study pathologist (I.J. Bleiweiss) and sent to 2 different laboratories (A.D. Thor and L.G. Dressler) for p53 analysis using 2 different but previously described antibodies (p1801 and DO7, respectively) and methodology. Standard sections were prepared using formalin-fixed, paraffin-embedded sections cut on a microtome at standard 5- or 6-micron sections and deparaffinized and placed on charged slides.

Immunohistochemical analysis

p53 status was determined by immunohistochemical (IHC) analyses with 2 separate monoclonal antibodies: p1801 (Genesis Bio-Pharmaceuticals, Inc.) and D07 (Biogenix).

mAB p1801. IHC staining and analysis of mAB p1801 was conducted in the laboratory of A.D. Thor, on formalin-fixed, paraffin-embedded sections as described previously (12).

mAB DO7. IHC staining and analyses of mAB D07 was conducted in the laboratory of L.G. Dressler using a steam antigen retrieval system. mAb D07 was prepared in a 1:1,000 dilution and formalin-fixed, paraffin-embedded sections cut at 5 microns were prepared on charged slides, stained with the diluents, and prepared according to manufacturer's guidelines (followed instructions in package insert).

All slides prepared were scored by pathologists (A.D. Thor and J.F. Lara). A visual score of 10% or greater positive nuclear staining of invasive cancer cells was considered positive for both antibody analyses, a cutoff point used for previous CALGB p53 studies (19). All IHC analyses were conducted blindly at the respective laboratories and data were submitted to the CALGB statistical center for correlation with clinical outcomes.

Statistical analysis

Statistical analyses were conducted by the CALGB statistical center (G. Broadwater) using the SAS software package. The primary objective of this study was to evaluate whether RFS differed on the basis of p53 protein expression status and whether there was a detectable interaction between p53 protein status and either escalating doses of doxorubicin (60, 75, or 90 mg/m^2 per cycle) when administered with a fixed dose of cyclophosphamide, and/or between p53 protein status and administration of 4 cycles of paclitaxel after AC chemotherapy. Correlation of p53 staining with OS was a secondary objective.

To preserve tissue and resources, a sampling scheme was prospectively developed to test the relative worth of potential predictive markers in CALGB 9344, including p53 protein expression as described (29). By direction of the statistical center of the PCO, an initial sample study was recommended. First, a test set of specimens was randomly requested from 750 of the 3,121 patients who participated in C9344. This set was balanced, compared with the remaining patients from whom specimens were not requested, on potential prognostic factors, such as number of positive lymph nodes, estrogen receptor (ER) status, age, and randomized treatment assignment. This first randomly selected group of tumors was assayed by both the mAb p1801 and the mAb DO7. Two subsequent sets of specimens, each containing 800 cases, were requested. These sets were distinct from each other, and each was stained with 1 of the 2 antibodies.

The χ^2 test was used for comparing patient characteristics of those with p53 assessment to those without. The log-rank test compares 2 or more survival distributions. Cohen's kappa coefficient was used to show level of agreement between the 2 methodologies used to assess p53 (IHC with mAbs D07 and 1801). McNemar's test was used to compare levels of disagreement between the methods. Cox proportional hazard regression models were used to determine significance of the interaction of treatment (doxorubicin dose escalation; addition of paclitaxel) and p53 protein status on RFS and OS after adjusting for clinical characteristics including number of positive lymph nodes, tumor size, hormone receptor status, patient age upon admission to study, dose of doxorubicin, and administration of paclitaxel. Kaplan–Meier survival curves visually displayed the interaction effect of either doxorubicin or paclitaxel and p53 status. Results of this study are presented in accordance with REMARK criteria for tumor marker results reporting (31)

As part of the quality assurance program of the CALGB, members of the audit committee visit all participating institutions at least once every 3 years to review source documents. The auditors verify compliance with federal regulations and protocol requirements, including those pertaining to eligibility, treatment, adverse events, tumor response, and outcome in a sample of protocols at each institution. Such on-site review of medical records was conducted for a subgroup of 14 patients (4%) of the 3,170 patients under this study.

Results

Patient selection and demographics

A total of 3,121 patients were enrolled into the C9344 trial and approximately 90% (2,880) of these patients had representative paraffin blocks or unstained slides of their tumors submitted to the PCO and were eligible for inclusion in this study. From this accrued group, 3 separate sets of specimens were requested as described in Methods. Of the 750 specimens in set 1, 641 and 629 were available, passed internal quality control (QC) for IHC staining and were successfully evaluated for p53 by antibodies 1801 and D07, respectively. Of the 800 specimens that were requested for each of the second and third sets, 680 and 566 were available, passed QC and were successfully analyzed for 1801 and D07, respectively. In total, 1,321 (85% of requested, 42% of total accrued) and 1,195 (77% of requested, 38% of accrued) specimens were successfully analyzed for staining with mAbs 1801 and D07, respectively, and 1,887 patients were evaluable by at least one of the mAbs (1801 or D07). These cases represent the analyzable cohort (Table 1). These results are also provided in the REMARK diagram (Fig. 1). The median follow-up for the 1,887 patients was 11.2 years (134 months). Of these patients, 671 (36%) have died, whereas 819 (43%) experienced failure events (recurrence or metastasis). There were no statistically significant or appreciable differences, in any of the demographic or outcomes data between those cases selected for the current study and the total group of patients, or those not selected; however, there was a difference in P value between RFS and OS (RFS, $P = 0.075$ vs. OS, $P = 0.053$; Table 1).

*p*53 protein expression

Of the 629 specimens successfully stained with both antibodies in set 1, 155 (25%) and 180 (29%) were p53 immunopositive (as defined in Methods) for mAbs 1801 or D07, respectively (Table 2). Concordance between staining for mAbs 1801 and D07 was evaluated and seen in 580 of 629 cases (92.2%) with a kappa statistic of 80% ± 2.7% (Table 2). Of the discordant cases, more cases were p53 positive using the DO7 antibody and negative with the p1801 antibody than vice versa (76% vs. 24%, respectively; McNemar's test; $P = 0.0004$).

*p*53 protein expression and outcomes

p53 staining as a prognostic factor. When all cases were analyzed, irrespective of treatment assignment, p53 staining was found to be an adverse prognostic factor (Fig. 2). Association of *p*53 staining with worse RFS was significant for mAB p1801 alone ($P = 0.007$), but not for mAb D07 alone ($P = 0.22$), and only marginally when mAbs 1801 and D07 were considered together ($P = 0.05$). Univariate

Table 1. Patient characteristics: comparison of all patients enrolled in CALGB 9344 versus cases selected for p53 IHC staining versus those not selected

	All treated patients ($n = 3,121$)	Patients with p53 by D07 or 1801 ($n = 1,887$)	Others ($n = 1,234$)	P[a]
Treatment arm of 9344				
CA 600/60 + Paclitaxel	533 (17)[b]	321 (17)	212 (17)	0.99
CA 600/60	515 (16)	306 (16)	209 (17)	
CA 600/75 + paclitaxel	517 (17)	316 (17)	201 (16)	
CA 600/75	523 (17)	316 (17)	207 (17)	
CA 600/90 + paclitaxel	520 (17)	313 (17)	207 (17)	
CA 600/90	513 (16)	315 (17)	198 (16)	
Age, y				
<40	636 (20)	376 (20)	260 (21)	0.37
40–49	1,248 (40)	741 (39)	507 (41)	
50–59	843 (27)	530 (28)	313 (25)	
60+	394 (13)	240 (13)	154 (12)	
Race				
White	2,611 (84)	1,586 (84)	1,025 (83)	0.53
Hispanic American	127 (4)	82 (4)	45 (4)	
African American	296 (9)	160 (9)	136 (11)	
Asian	52 (2)	34 (2)	18 (1)	
Other	35 (1)	25 (1)	10 (1)	
Menopausal status				
Premenopausal	1,925 (62)	1,151 (61)	774 (63)	0.33
Peri/postmenopausal	1,196 (38)	736 (39)	460 (37)	
Pathologic tumor size,[c] cm				
≤2	1,096 (35)	639 (34)	457 (37)	0.072
>2	2,008 (65)	1,237 (66)	771 (63)	
ER status[c]				
Positive	1,840 (59)	1,120 (60)	720 (59)	0.66
Negative	1,263 (41)	759 (40)	504 (41)	
Number positive lymph nodes[c]				
1–3	1,450 (46)	885 (47)	565 (46)	0.81
4–9	1,310 (42)	784 (42)	526 (43)	
10+	360 (12)	217 (12)	143 (12)	
Primary treatment mastectomy	2,177 (70)	1,331 (71)	846 (69)	0.71
5-year survival				
Relapse-free	67% (66–69)[d]	69% (67–71)	66% (63–68)	0.075
Overall	78% (77–80)	79% (77–81)	76% (74–79)	0.053

[a]Comparing cases selected versus those not selected.
[b]Number (% of all).
[c]There were a small number of patients where these results were not known.
[d]Percentage free of event (95% CI).

analysis of p53 positivity was associated with significantly worse OS when analyzed by either antibody alone (mAb p1801, $P < 0.0001$; mAb D07, $P = 0.002$) or both together ($P < 0.0001$).

p53 staining as a predictive factor. In multivariate analysis, there was no significant interaction between p53 protein expression status and dose of doxorubicin (Fig. 3A) or with addition of paclitaxel (Fig. 3B) with respect to either RFS or OS. (Table 3). There was no apparent relationship between dose level of doxorubicin and p53 status or between paclitaxel and p53 status by IHC. The benefit from paclitaxel seen in the overall cohort was equally observed in both p53 positive and negative cases, regardless of the antibody used.

We also conducted several exploratory analyses. Previously, we had reported that the addition of paclitaxel

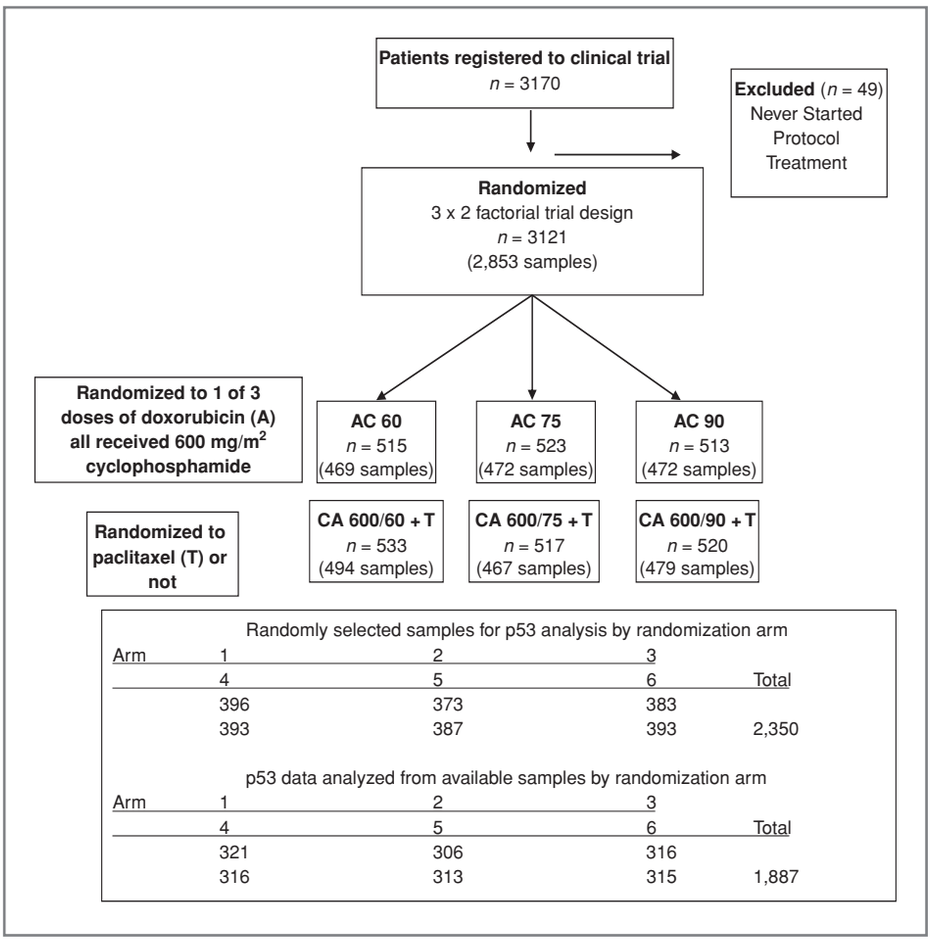

Figure 1. REMARK diagram of case selection for p53 staining. Treatments: C, cyclophosphamide; A, doxorubicin; and T, paclitaxel. Arm/treatment: 1, CA 600/60 + T; 2, CA 600/60; 3, CA 600/75 + T; 4, CA 600/75; 5, CA 600/90 + T; and 6, CA 600/90.

to AC adjuvant chemotherapy appeared to be of little, if any, value in patients who participated in CALGB 9344 who had ER-positive, HER2-negative cancers (29). In the current study, we did not detect any statistically significant interactions between p53 and the addition of paclitaxel by ER status for either RFS or OS. Also, in an exploratory Cox proportional hazards model analysis, we did not observe a significant interaction of p53 status and dose of doxorubicin in predicting RFS, interaction ($P = 0.69$), when adjusting for covariates.

Discussion

In this study, we observed that while p53 protein expression, presumably reflecting p53 abnormality, was associated with worse overall survival, it did not predict

Table 2. Comparison of p53 staining by mAb 1801 and mAb DO7

		p53 D07		
		Negative[a]	**Positive**	**Total**
p53 1801	Negative	437 (69%)[b]	37 (6%)	474 (75%)
	Positive	12 (2%)	143 (23%)	155 (25%)
	Total	449 (71%)	180 (29%)	629 (100%)

[a]Negative: <10% nuclear staining; positive: ≥10% nuclear staining.
[b]Number of patients (% of total).

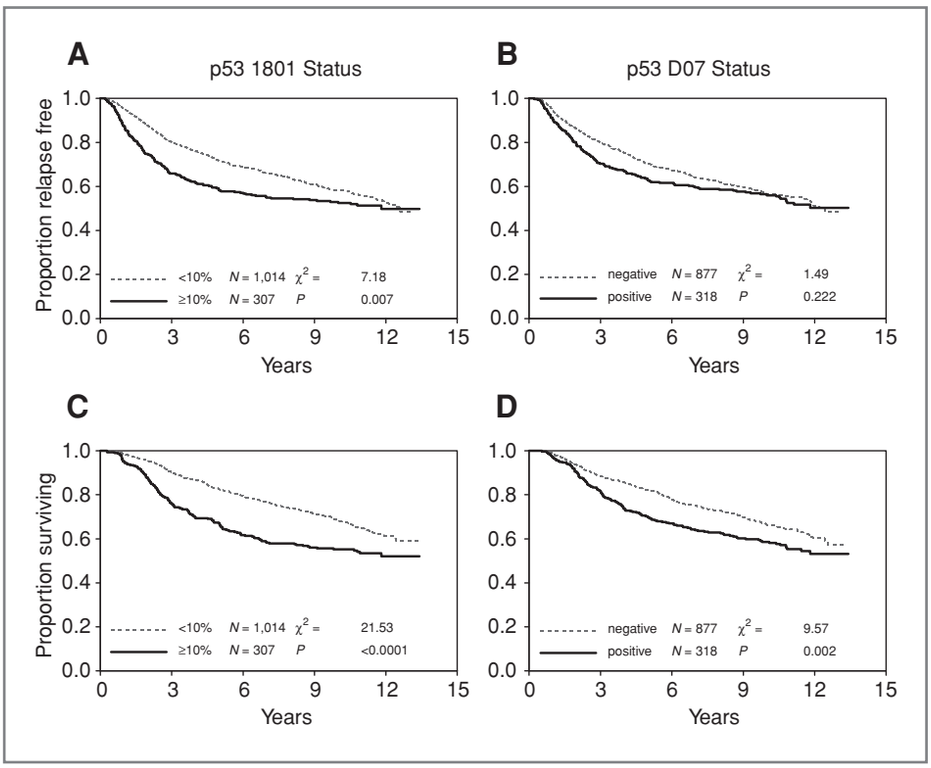

Figure 2. RFS and OS by p53 status. RFS (A and B) and OS (C and D) were determined according to p53 staining with either mAb 1801 (A and C) or D07 (B and D) in 1,195 patients with node-positive breast cancer treated within CALGB 9344 (see Methods). Negative: <10% nuclear staining. Positive: ≥10% nuclear staining.

benefit from escalating doses of doxorubicin above the standard used 60 mg/m^2, or from the addition of paclitaxel in patients with node-positive, newly diagnosed breast cancer. This observation was made regardless of IHC methodologies using either mAbs 1801 or D07 which collectively represent the most frequently used p53 IHC antibodies currently in standard everyday practice.

Our observation of a marginal prognostic and nonpredictive effect of p53 in this study differs from some (3, 5, 14, 20, 21) but not all prior investigations (10, 23, 30). However, our data represent a strong and definitive statement due to the large cohort and lengthy follow-up presented. Indeed, in our own preliminary results from CALGB protocol 8541, we observed that p53 staining with mAb p1801, especially when combined with HER2 status, was predictive of benefit from increasing doses of doxorubicin from very low (30 mg/m^2) to what is now considered standard (60 mg/m^2) dose (19). Prior to this current study, we hypothesized that positive p53 staining, coupled with HER2 positivity, might predict for even higher escalation of doxorubicin dose, which was escalated up to 90 mg/m^2 in CALGB 9344. However, we did not observe such an effect in this large correlative study.

There are a number of possible reasons why we did not detect a more substantial effect of p53. Overall, the literature supports our observation (Fig. 2) that p53 expression is associated with more aggressive behavior in many cancers including breast cancer. However, previous studies have provided highly contradictory results about the predictive role of p53 expression for beneficial effects of chemotherapy. Our data, taken from a prospective randomized clinical trial, provide high levels of evidence that p53 expression does not predict benefit from either escalation of doxorubicin dose above 60 mg/m^2, perhaps because this represents an optimal or threshold dose for this agent, or addition of paclitaxel after 4 cycles of AC chemotherapy. It is possible that our data represent a false-negative observation. However, in this regard, we have previously reported a substantial and significant predictive effect for benefit from addition of paclitaxel, but not doxorubicin dose escalation, in patients whose tumors are either ER negative or HER2 positive or both (29), suggesting that this data set is adequately powered to detect an important biomarker–treatment interaction.

Technical concerns may also account for why we did not observe any association between p53 positivity and benefit from doxorubicin dose escalation or paclitaxel. In our study, we assessed p53 by immunohistochemistry. We detected similar, although not identical, effects with each of the 2 antibodies we incorporated into this study (mAbs D07 and 1801), which have been widely used and validated in prior studies, including CALGB 8541 (19). It is noteworthy that for this study, we report only epithelial p53 staining patterns, in particular nuclear staining characteristics, and did not report stromal staining characteristics of invasive carcinomas relative to p53 staining which has recently been observed and noted to be of clinical significance (32). Several studies have shown that IHC

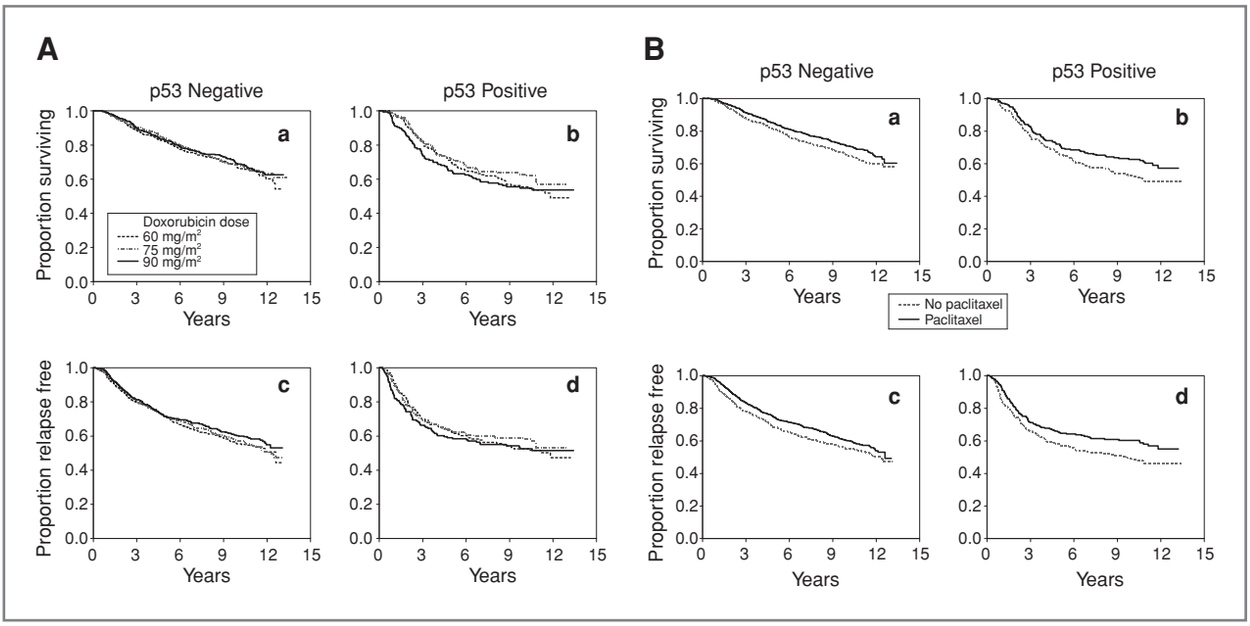

Figure 3. A, RFS and OS according to p53 status by doxorubicin treatment arm. RFS (a and b) and OS (c and d) were determined according to p53 staining with both mAb 1801 and D07 in 1,887 patients with node-positive breast cancer treated within CALGB 9344. Positive (b and d) or negative (a and c) refers to both mAb 1801 and D07. B, RFS and OS according to p53 status by paclitaxel treatment arm. OS (a and b) and RFS (c and d) were determined according to p53 staining with both mAb 1801 and D07 in 1,887 patients with node-positive breast cancer treated within CALGB 9344. Positive (b and d) refers to both mAb 1801 and D07 positive; negative (a and c) is otherwise.

staining for p53 does not always detect all mutations, it does not identify all deletions, and it may detect stabilized, but wild-type, p53 protein (7, 33). p53 is a highly pleiotropic molecule, with several different cellular and biological functions and each of these is associated with one or more different domains in the protein (4, 19). Of the 2 antibodies used in this study, the mAb1801 covers epitopes 32 to 79, whereas DO7 covers epitopes 37 to 45 (34). Most mutations of p53 occur within exons 4 to 8, which are generally the areas of the proteins for which these 2 antibodies test, although of course it is possible that either or both antibodies might not detect protein with specific mutations in these regions.

IHC staining may miss important activating or inactivating abnormalities within the *p53* gene that might mediate sensitivity or resistance to all, or specific types, of chemotherapies. In addition, as the functions of p53 include, but are likely not limited to, ubiquitylation and sumoylation, there are various pathways through which this complex tumor suppressor carries out its functions.

Table 3. Survival according to p53 status

Variable	RFS			OS		
	HR (confidence limits)	Likelihood-ratio tests, χ^{2a}	*P*	HR (confidence limits)	Likelihood-ratio tests, χ^{2a}	*P*
Number of positive nodes (square root)	1.43 (1.33–1.54)	94.8	<0.0001	1.45 (1.34–1.57)	87.9	<0.0001
Tumor size (≤2 vs. >2 cm)	1.45 (1.24–1.70)	21.7	<0.0001	1.49 (1.25–1.78)	20.0	<0.0001
ER status (positive vs. negative)	0.70 (0.60–0.81)	22.6	<0.0001	0.58 (0.49–0.68)	44.7	<0.0001
Age at study entry	0.99 (0.99–1.01)	0.30	0.58	1.00 (0.99–1.01)	1.09	0.30
Paclitaxel (yes vs. no)	0.84 (0.72–0.99)	9.84	0.0017	0.80 (0.67–0.97)	11.2	0.0008
p53 1801 or D07 (either positive vs. other)	1.19 (0.96–1.47)	1.14	0.29	1.34 (1.07–1.68)	7.04	0.008
Interaction of paclitaxel and p53	0.83 (0.61–1.14)	1.35	0.25	0.87 (0.63–1.22)	0.64	0.42

[a]Multivariate Cox proportional hazards model for prediction of RFS and OS by p53 1801 or p53 D07 status and paclitaxel interaction; $n = 1{,}868$ of 1,887 patients with p53 evaluations are included in this model. (p53 status is considered positive if either D07 or 1801 is positive.)

Furthermore, certain p53-mediated pathways may differ among even similar appearing tumor types and in their response to various therapeutic agents. (35)

Although *p53* gene sequencing might provide additional or alternative results about prediction of benefit from doxorubicin dose or addition of paclitaxel, this study was not designed nor intended to be a comparative analysis of IHC versus gene sequencing. Gene sequencing is not a practical everyday test that can be carried out readily in most laboratories and it is currently cost prohibitive. Moreover analysis of gene sequencing using formalin-fixed, paraffin-embedded tissue is not optimal and results may be unpredictable. Most studies to date using gene sequencing have been of relatively small cohorts from single institutions in which processing methods can be more easily standardized and regulated. The likelihood of maintaining such stringent QC standards in a multi-institutional group study would be precarious at best. Finally, sequencing studies may not necessarily identify small nuances among the myriad of potential alternate pathways that continue to be identified in sporadic cases.

In conclusion, our data, produced from archived specimens from a prospective randomized clinical trial, do not support routine use of IHC to determine epithelial p53 protein status in patients who will benefit from higher doses of doxorubicin those that are recommended for standard clinical care, nor from taxane-based adjuvant chemotherapy. We did observe statistically significant prognostic differences overall for p53 positivity. Although these differences suggest clinical validity of p53 staining, these findings do not have clinical utility other than to predict a more aggressive tumor (26, 33). The magnitude of differences in OS between those for whom p53 was positive nor negative was insufficiently substantial to change practice, nor did p53 negativity did not identify a group of patients with such a favorable prognosis that further therapy, if available, would not be indicated. Nevertheless, they do suggest that p53 plays an important role in the biology of breast cancer and for that reason is felt to still be an important mechanism in the pathway of carcinogenesis and worthy of continued study to further our understanding of cancer genesis and biology.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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