

## The Combination of *p53* Mutation and *neu/erbB-2* Amplification Is Associated With Poor Survival in Node-Negative Breast Cancer

Shelley B. Bull, Hilmi Ozcelik, Dushanthi Pinnaduwa, Martin E. Blackstein, Donald A.J. Sutherland, Kathleen I. Pritchard, Anjela T. Tzontcheva, Saul Sidlofsky, Wedad M. Hanna, Ali H. Qizilbash, Mary E. Tweeddale, Sheldon Fine, David R. McCready, and Irene L. Andrulis

From the Samuel Lunenfeld Research Institute and Departments of Medicine, Pathology and Laboratory Medicine, and Surgery, Mount Sinai Hospital; Toronto-Sunnybrook and Women's Health Science Center; North York General Hospital; Toronto Hospital; Departments of Public Health Sciences, Laboratory Medicine and Pathobiology, Medicine and Anatomy, Medical Genetics, and Microbiology, University of Toronto, Toronto; and Credit Valley Hospital, Mississauga, Ontario, Canada.

Submitted September 24, 2002; accepted October 24, 2003.

Supported by grants from the Canadian Breast Cancer Research Initiative and the Canadian Breast Cancer Foundation. S.B.B. is a Senior Investigator, Canadian Institutes for Health Research.

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

Address reprint requests to Irene L. Andrulis, PhD, Fred A. Litwin Centre for Cancer Genetics, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Ave, Toronto, Ontario M5G 1X5, Canada; e-mail: Andrulis@mshri.on.ca.

© 2004 by American Society of Clinical Oncology

0732-183X/04/2201-86/\$20.00

DOI: 10.1200/JCO.2004.09.128

### A B S T R A C T

#### Purpose

Increases in *neu/erbB-2* have been implicated in breast cancer prognosis, but do not predict all recurrences. On the basis of evidence that *p53* mutation is involved in the development of human neoplasia, we examined the prognostic value of *p53* alterations in combination with *neu/erbB-2* amplification.

#### Patients and Methods

A consecutive series of women were observed for recurrence and death (median follow-up of 85 months) and tumors from 543 individuals were analyzed for *p53* mutation status and *neu/erbB-2* amplification. Exons 4 through 10 of the *p53* gene were analyzed by single-stranded conformational polymorphism and mutations were confirmed by DNA sequencing. The association of *p53* mutation status and *neu/erbB-2* amplification with risk of recurrence and death was examined in survival analyses with traditional and histologic markers as prognostic factors.

#### Results

*p53* mutations occurred in 24.5% of the axillary node-negative breast carcinomas. Mutations were more frequent in carcinomas with *neu/erbB-2* amplification: 38.9% compared with only 20.9% in those without *neu/erbB-2* amplification. We found elevated risks of disease recurrence and overall mortality in patients with both *p53* mutation and *neu/erbB-2* amplification in their tumor compared with patients with neither or only one of the alterations. This increase persisted with adjustment for other prognostic factors (relative risk, 2.32;  $P = .002$  for recurrence; relative risk, 2.22;  $P = .004$  for death).

#### Conclusion

Evaluation of tumors for *p53* mutations may be beneficial to identify women at higher risk of disease recurrence and death when the tumor has *neu/erbB-2* amplification, but in the absence of *neu/erbB-2* amplification, the presence of *p53* mutation may not provide additional independent prognostic information.

*J Clin Oncol* 22:86-96. © 2004 by American Society of Clinical Oncology

### INTRODUCTION

As a group, women with axillary node-negative (ANN) breast cancer have a good prognosis; however, approximately 20% of individuals will experience a recurrence and die from systemic disease. Although some ANN patients may benefit from adjuvant chemotherapy or hormonal therapy [1-5], large numbers of women (including those in whom no recurrence will occur) must be treated to benefit those destined to experience relapse. Identification of tradi-

tional, histopathologic, and molecular prognostic markers would be of value in individualizing therapy.

Amplification and overexpression of the *neu/erbB-2* proto-oncogene have been found to be of prognostic significance in a number of studies [6,7] (reviewed previously [8-10]) including our own [11]. However, like any single prognostic marker, *neu/erbB-2* amplification does not predict all recurrences and the importance of other molecular alterations remains to be determined.

Because of the role of p53 in cell cycle control, apoptosis, and response of cells to cytotoxic agents, there has been extensive investigation of the prognostic value of p53 in human breast cancer (reviewed previously [8-10,12-14]). Many studies have shown that alterations in p53 are associated with poor prognosis in breast cancer, including node-negative disease (reviewed previously [13,14]); however, others have not detected a significant association. The lack of agreement may be due to different methods of detection of p53 alterations [12]. The majority of investigations have used immunohistochemical detection of stabilized mutant forms of p53 protein in archival breast tumor specimens [13]. Others have conducted more complete molecular analysis with determination of specific DNA alterations [14]. Individual studies often have the disadvantage of being retrospective and consisting of small sample size, and most lack long-term follow-up. More importantly, the prognostic value of p53 mutation in combination with traditional histopathologic tumor characteristics and molecular markers, such as *neu/erbB-2* amplification, has not been addressed in a prospective study.

In a multicenter study of 580 newly diagnosed ANN breast cancer patients, we had previously found *neu/erbB-2* amplification to be an independent predictor of recurrence [11]. We have continued to observe this group for recurrence and death (median follow-up, 85 months) and to evaluate the importance of additional molecular alterations. In this prospective study, we considered whether p53 mutation detected by single-stranded conformation polymorphism (SSCP) analysis of exons 4 through 10 would provide additional prognostic information in this group. Our primary objective was to evaluate the contribution to disease-free survival (DFS) of a second molecular prognostic factor, p53 mutation, in addition to *neu/erbB-2* amplification and traditional factors. A second objective was to determine whether associations detected with DFS persisted for overall survival (OS).

## PATIENTS AND METHODS

### Patient Eligibility

In this prospective study, women with node-negative breast cancer were enrolled from September 1987 until March 1993. A consecutive series of individuals were accrued at each of eight Toronto hospitals. All women who had node-negative invasive breast cancer pathologically confirmed at the participating centers were potentially eligible, whether or not we had received a tumor specimen for analysis. The pathology report was used to determine the initial eligibility (which required clear resection margins and at least four lymph nodes sampled), pathologic size of the invasive component (centrally reviewed), histologic grading, nuclear grading, presence of vascular or lymphatic invasion by tumor cells, estrogen receptor (ER) status, progesterone receptor (PgR) status, and histologic subtype of the invasive and intraductal components (if present). Imaging (bone scan and abdominal ultrasound or abdominal computed tomography scan) and chest x-ray were

required for patients with T2 tumors. If the patient was eligible on the basis of pathology, staging, and age (between 18 and 75 years inclusive), the surgeon invited the patient to participate and provide a signed consent form (approved by the various institutional review boards), with final eligibility determined by chart review. Exclusion criteria were inadequate staging (ie, Tx, Nx, Mx), synchronous breast primary tumors, surgeon or patient refusal, or prior malignancies (excluding nonmelanoma of skin and carcinoma-in-situ of the cervix).

### Molecular Analyses

After frozen-section diagnosis of invasive cancer, tumor specimens were sampled by the pathologists, immediately snap frozen, and stored in liquid nitrogen until subjected to DNA and RNA extraction by conventional techniques [15]. DNA copy number of *neu/erbB-2* was measured as previously described [11]. Tumors that exhibited a two-fold or greater increase in copy number relative to control unamplified DNA were considered to be amplified. In 56 of 635 available specimens, an amplification result could not be obtained because of insufficient or poor-quality DNA.

### p53 Mutation Analysis

SSCP analysis was used to analyze exons 4 through 10 of the p53 gene for sequence alterations. Genomic DNA was used as a template for polymerase chain reaction amplification of fragments containing an exon, and its adjacent intronic boundaries. The sequences of each primer set used to amplify exons 5 to 9 were taken from Ozcelik et al [16]. We designed three sets of primers for amplification of exons 4 and 10 (4AF: GGAAGGGACAGAAGATGACA, 4AR: TCCTCTGACTGCTTTTTTC; 4BF: CCCCTGCAC-CAGCAGCTCCTA; 4BR: CAGGCATTGAAGTCTCATGG, 10F: AACTCAGGT ACTGTGAATATACT, 10R: TTACTIONGGCCCT-ACTCCCCTG). Some of the exons were multiplexed (exons 4A and 10, and exons 6 and 8). One hundred nanograms of tumor DNA was added to reaction buffer containing 10 mmol/L Tris (pH 8.3), 50 mM KCl, 0.8 to 1.2 mmol/L MgCl<sub>2</sub>, 100 μM of each deoxynucleotide triphosphate, 6 μM of each primer, 1 μL phosphorus-33 (<sup>33</sup>P)-adenosine triphosphate (10 mCi/μL), and 2 units of AmpliTaq (Perkin Elmer, Norwalk, CT). <sup>33</sup>P-adenosine triphosphate-incorporated polymerase chain reaction products were heat denatured and electrophoresed on a native polyacrylamide gel containing 10% glycerol. Sequence alterations, detected as electrophoretic mobility shifts on SSCP gels, were confirmed and characterized independently by direct sequencing using the same SSCP primers (Thermo Sequenase cycle sequencing kit; Amersham Biosciences, Baie d'Urfé, Canada). A mutation result could not be obtained in 63 of 635 specimens because of insufficient or poor-quality DNA.

### Clinical Follow-Up

Patient follow-up data and information on adjuvant treatment received were obtained by chart review in a standardized fashion and without knowledge of the molecular analyses. Charts were reviewed every 3 months in the first 2 years after diagnosis, every 6 months until 5 years after diagnosis, and annually thereafter. Confirmation of local (within the breast) or distant relapse was by review of original reports or imaging (by M.E.B). Recurrences were considered to have occurred if there was radiologic evidence of disease or clinically apparent disease with biopsy confirmation. However, recurrence did not include relapse in the ipsilateral breast in patients who underwent less than mastectomy, and these patients continued to be monitored for nonbreast relapse. DFS

was taken as the time between diagnosis and the confirmation of nonbreast recurrence. All patients were monitored for death whether or not they experienced disease recurrence. OS was taken as the time between diagnosis and death as a result of any cause, regardless of recurrence events.

Patient status on November 30, 1998, determined survival times (DFS and OS) and censoring status using clinical follow-up data. Follow-up data were monitored for an additional 6 months (up to June 4, 1999) to confirm patient status at the termination date. We observed 96 disease recurrences among patients with molecular results. Calculations made at *p53* study initiation indicated that a minimum of 91 recurrences would need to be observed to have 80% power to detect a relative risk (RR) of recurrence of 2.0 associated with *p53* mutation, taking into account the contribution of *neu/erbB-2* amplification and the other clinical prognostic factors [17,18]. Patients with disease-free status were censored at the termination date, and patients lost to follow-up evaluation ( $n = 7$ ) were censored at the last known follow-up time. One patient without follow-up data did not contribute to the survival analysis. In the DFS analysis, the actual date of disease recurrence was used for patients who subsequently died with disease, and patients who died without recurrence of breast disease (ie, other causes) were censored at the date of death ( $n = 33$ ). Excluding the patients lost to follow-up and those with recurrences, the minimum follow-up time was 55 months after surgery and the median follow-up time was 85 months.

### Statistical Analysis

A total of 1,001 patients met the eligibility criteria: specimens were received from 635 of these patients, and 543 patients (with 96 recurrences and 104 deaths) had both *neu/erbB-2* DNA amplification and *p53* mutation results. As reported previously [11], the most frequent reason for lack of available specimen was small tumor size, and patients for whom a specimen was not obtained had longer DFS, smaller tumors, more tumors of histologic grade 1, and were less likely to have hormone receptor levels and nuclear grade determined. To assess generalizability further, characteristics of the 543 patients with molecular results available were compared with the remaining 92 patients with a specimen, and differences in survival were examined by the log-rank test.

### Group Analyzed for *p53* Mutation and *neu/erbB-2* Amplification

Baseline analysis was descriptive, comparing frequency distributions of known prognostic factors among groups defined by *neu/erbB-2* amplification status (ie, relative copy number increase  $\geq 2$  v  $< 2$ ) and *p53* mutation (*p53* mutation v *p53* wild type). Univariate survival analysis (DFS and OS) of *p53* mutation status, *neu/erbB-2* amplification status, and each of the prognostic factors was by the log-rank test with Kaplan-Meier survival curves and by the Cox proportional hazards model [19]. Categorical or continuous coding schemes for the prognostic factors were selected before the analysis, on the basis of previous studies or clinical convention.

Multivariate analysis (DFS and OS) to assess the contribution of *p53* mutation in addition to *neu/erbB-2* amplification status and the traditional variables was by the Cox proportional hazards model. Prognostic factors included in the analysis of the cohort of 543 patients were menopausal status, tumor size, ER and PgR status, age, and adjuvant therapy received. Histopathologic tumor characteristics (ie, histologic grade, tumor subtype, lymphatic invasion, and type of intraductal component) were also examined in

full multivariate analyses including all prognostic factors. The choice of factors included in a summary model was based on a priori clinical importance and on the results from the full multivariate model. The prognostic importance of each factor was summarized by the RR, as estimated by the hazard ratio in the Cox proportional hazards model. To verify the proportional hazards assumption, scaled Schoenfeld residuals with lowess smoothing were plotted against survival time for each factor [20]. Formal tests for suspected time-dependent factors were conducted in the Cox model by adding a linear term for interaction with follow-up time [20]. Analyses for short-term effects were conducted similarly by estimating a separate RR for the first 36 months of follow-up.

## RESULTS

### Patient and Tumor Characteristics

**Eligible patients.** All node-negative patients who consented to participate in the study were observed regardless of whether a tumor specimen was available for molecular analyses ( $N = 1,001$ ). Treatment decisions were made without knowledge of the patient's *p53* mutation or *neu/erbB-2* amplification status. During the accrual period, the likelihood of receiving treatment increased: in 1987 to 1989, before adjuvant treatment was widely recommended for node-negative patients, 28% were treated, but by 1992 to 1993, nearly 80% received some form of systemic therapy. The proportion receiving hormonal therapy alone increased from 20% to 58% over the period, and the proportion receiving chemotherapy increased from 4% to 20%. Overall, younger women were more likely to receive adjuvant therapy than were older women. As reported previously [11], the type of adjuvant treatment received was associated with several of the characteristics known at baseline. Compared with other women, patients at higher risk of recurrence (ie, premenopausal status, large tumor size, higher grade, and ER- or PgR-negative tumors), were more likely to receive chemotherapy, and patients with ER- or PgR-positive tumors were more likely to receive hormonal therapy. Adjuvant chemotherapy was nearly always given as cyclophosphamide, methotrexate, and fluorouracil.

In the remainder of this report, we present data for the 543 patients in whom *neu/erbB-2* amplification and *p53* mutation status were determined. The distributions of patient and tumor characteristics in the group in which both molecular results were obtained ( $n = 543$ ) did not differ significantly from the group without one or the other ( $n = 92$ ).

***p53* mutation and *neu/erbB-2* amplification.** *p53* was mutated in 24.5% of the specimens and *neu/erbB-2* was amplified in 19.9% of the specimens. *p53* mutations were more frequent in the specimens with *neu/erbB-2* amplification than in those without (38.9% of 108 v 20.9% of 435;  $P = .001$  by  $\chi^2$  test). The four groups defined by *p53* mutation and *neu/erbB-2* amplification status did not vary significantly by menopausal status, tumor size, age, or multi-

**Table 1.** Patient and Tumor Characteristics According to p53 Mutation Status and neu/erbB-2 Amplification Status

Characteristic	Amplified				Not Amplified			
	Mutation		Wild Type		Mutation		Wild Type	
	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%
No. of patients, n = 543	42	7.7	66	12.2	91	16.8	344	63.3
No. of relapses, n = 96	17	17.7	9	9.4	15	15.6	55	57.3
No. of deaths, n = 104	17	16.4	12	11.5	20	19.2	55	52.9
From or with disease, n = 71	15	21.1	7	9.9	15	21.1	34	47.9
Without disease, n = 33	2	6.1	5	15.2	5	15.2	21	63.6
Lost to follow-up, n = 7	1		1		1		4	
Menopausal status								
Premenopause	15	35.7	25	37.9	40	44.0	106	30.8
Perimenopause	2	4.8	2	3.0	4	4.4	20	5.8
Postmenopause	25	59.5	39	59.1	47	51.6	218	63.4
Maximum size of tumor, cm								
< 0.5	0	0.0	1	1.5	0	0.0	5	1.5
≥ 0.5– < 1.0	1	2.4	5	7.5	3	3.3	18	5.2
≥ 1.0– < 2.0	14	33.3	19	28.8	22	24.2	133	38.6
≥ 2.0– < 5.0	23	54.8	37	56.1	62	68.1	164	47.7
≥ 5.0	4	9.5	4	6.1	4	4.4	24	7.0
ER status*								
Positive	21	50.0	50	75.8	42	46.2	296	86.0
Negative	20	47.6	14	21.2	41	45.0	32	9.3
Equivocal	1	2.4	1	1.5	6	6.6	11	3.2
Not known or not done	0	0.0	1	1.5	2	2.2	5	1.5
PgR status*								
Positive	14	33.3	41	62.1	28	30.8	245	71.2
Negative	27	64.3	18	27.3	51	56.0	74	21.5
Equivocal	1	2.4	6	9.1	10	11.0	20	5.8
Not known or not done	0	0.0	1	1.5	2	2.2	5	1.5
Age, years								
Mean		54.3		55.4		52.6		56.6
SD		11.0		10.7		12.4		11.2
Minimum		31.8		35.7		25.8		29.6
Maximum		75.1		75.8		74.1		75.8
Adjuvant therapy received*								
None	19	45.2	33	50.0	46	50.5	160	46.5
Hormonal	13	31.0	24	36.4	25	27.5	150	43.6
Chemotherapy	9	21.4	7	10.6	18	19.8	27	7.9
Both	1	2.4	2	3.0	2	2.2	7	2.0

Abbreviations: ER, estrogen receptor; PgR, progesterone receptor; SD, standard deviation.

\*P = .001 and P = .02 for receptors and treatment, respectively. Statistical comparisons of other proportions were not significant at the 5% level.

centricity (Tables 1 and 2). However, the groups with p53 mutations, both with and without neu/erbB-2 amplification, were less likely to be positive for the hormone receptors, more likely to be grade 3 for both histologic and nuclear grade, and less likely to have lobular subtype (Tables 1 and 2). The groups with p53 mutations were also more likely to have received chemotherapy (Table 1), presumably through the association of p53 status with known prognostic factors that influenced treatment decisions. The neu/erbB-2–amplified group with p53 mutations was more likely to have lymphatic invasion than the other three groups, and the two groups with neu/erbB-2 amplification were more likely to have a comedo-type intraductal component (Table 2).

### **Prognostic Value of p53 Mutation and neu/erbB-2 Amplification for DFS and OS**

To examine the association of p53 mutation with the risk of recurrence and death in node-negative breast cancer, we considered p53 mutation status alone, and then in combination with neu/erbB-2 amplification status and other prognostic factors. Menopausal status, tumor size category, ER and PgR status, and age were treated as the primary set of traditional prognostic factors and these were evaluated one at a time and as a group, with and without the treatment variables. The histopathologic markers (histologic grade, tumor subtype, lymphatic invasion, intraductal type) were added to the full multivariate models as secondary prognostic factors. The additional and independent contribution of

**Table 2.** Histopathologic Tumor Characteristics According to *p53* Mutation and *neu/erbB-2* Amplification Status

Characteristic	Amplified				Not Amplified			
	Mutation		Wild Type		Mutation		Wild Type	
	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%
No. of patients, N = 543	42	7.7	66	12.2	91	16.8	344	63.3
Histologic grade*								
1	1	2.4	8	12.2	1	1.1	29	8.4
2	12	28.6	23	34.9	15	16.5	117	34.0
3	21	50.0	16	24.2	51	56.0	71	20.6
Subtype	0	0.0	9	13.6	11	12.1	71	20.7
Not known	1	2.4	1	1.5	2	2.2	5	1.5
Not done	7	16.6	9	13.6	11	12.1	51	14.8
Nuclear grade†								
1	1	2.4	3	4.5	3	3.3	14	4.0
2	7	16.7	21	31.8	12	13.1	96	28.0
3	17	40.4	10	15.2	39	42.9	54	15.7
Not known	1	2.4	1	1.5	4	4.4	16	4.6
Not done	16	38.1	31	47.0	33	36.3	164	47.7
Lymphatic invasion‡								
Yes	12	28.6	7	10.6	11	12.1	40	11.6
No	29	69.0	59	89.4	80	87.9	303	88.1
Not known	1	2.4	0	0.0	0	0.0	1	0.3
Multicentricity†								
Yes	5	11.9	5	7.6	3	3.3	35	10.2
No	37	88.1	61	92.4	86	94.5	307	89.2
Not known or missing	0	0.0	0	0.0	2	2.2	2	0.6
Subtype§								
Lobular	0	0.0	6	9.1	2	2.2	35	10.2
Mucinous	0	0.0	1	1.5	1	1.1	17	4.9
Medullary	0	0.0	0	0.0	5	5.4	5	1.5
Papillary	0	0.0	2	1.5	1	1.1	1	0.3
Tubular	0	0.0	1	1.5	2	2.2	7	2.0
No subtype	41	97.6	57	86.4	80	88.0	272	79.1
Missing or not done	1	2.4	0	0.0	0	0.0	5	1.5
In situ type‡								
Comedo	13	31.0	19	28.8	17	18.7	42	12.2
Lobular	1	2.4	2	3.0	1	1.1	20	5.8
Noncomedo ductal	14	33.3	25	37.9	33	36.2	136	39.5
None	13	31.0	20	30.3	40	44.0	145	42.2
Missing	1	2.4	0	0.0	0	0.0	1	0.3

\**P* = .001.  
†Statistical comparison was not significant at the conventional 5% level.  
‡*P* = .006.  
§*P* = .03.

*p53* mutation was evaluated by adding it to the primary and full multivariate models with and without *neu/erbB-2* amplification status, comparing the goodness of fit of the models with and without *p53* mutation, and examining changes in RR estimates.

**Univariate analysis.** When *p53* mutation status was considered alone throughout the entire follow-up period (median time, 85 months), we found a 1.7-fold increase in the risk of recurrence associated with the presence of a mutation (Table 3; Fig 1). The risk of recurrence depended on the duration of follow-up (*P* = .03), with an RR of 2.89 (95% CI, 1.70 to 4.91) for recurrence within the first 36 months. Similarly, when *neu/erbB-2* amplification status

was considered alone, we found less than a two-fold increase in the risk of disease recurrence over the entire follow-up period (Table 3), but the RR during the first 36 months was 2.34 (95% CI, 1.34 to 4.09), with evidence that the risk of recurrence decreased with duration of follow-up (*P* = .02). This largely explains the reduction in RR compared with the value of 2.4 found previously in the same cohort when the median duration of follow-up was 36 months [11].

Remarkably, when *p53* mutation and *neu/erbB-2* amplification were considered jointly, we found a greater than three-fold elevated risk of disease recurrence when the tumor specimen had both *p53* mutation and *neu/erbB-2* amplification, compared with those having only one or

**Table 3.** Results of Disease-Free and Overall Survival Analysis by Univariate Cox Proportional Hazards Models (N = 543 with 96 recurrences and 104 deaths)

Prognostic Variable	Recurrence			Mortality		
	RR	95% CI	P	RR	95% CI	P
p53 status, mutation v wild type	1.69	1.10 to 2.58	.02	1.97	1.32 to 2.94	.001
neu amplification status, - 22 v 1	1.68	1.07 to 2.63	.02	1.72	1.12 to 2.65	.01
p53 mutation and neu amplification						
Mutation only v neither	1.07	0.60 to 2.89	.82	1.54	0.92 to 2.57	.10
Amplification only v neither	0.90	0.44 to 1.81	.76	1.20	0.64 to 2.25	.56
Interaction of p53 and neu	3.38	1.26 to 9.10	.02	1.74	0.71 to 4.28	.23
Both mutation and amplification, v other three groups combined	3.24	1.92 to 5.48	.0001	2.89	1.72 to 4.87	.0001
Menopausal status, pre- and perimenopausal v postmenopausal	1.45	0.97 to 2.16	.07	0.95	0.64 to 1.43	.82
Tumor size cm						
2-5 v < 2	1.50	0.96 to 2.33	.07	1.54	1.00 to 2.38	.05
≥ 5 v 2-5	1.81	0.86 to 3.82	.12	1.42	0.76 to 2.64	.27
ER status*	2.03	1.34 to 3.09	.001	2.19	1.47 to 3.27	.0001
PgR status*	1.82	1.22 to 2.71	.004	2.08	1.42 to 3.06	.0002
Age at diagnosis, years†						
35 v 55	1.54	0.85 to 2.77	.15	1.37	0.75 to 2.48	.31
45 v 55	1.23	1.01 to 1.51	.04	1.04	0.85 to 1.27	.73
65 v 55	0.82	0.64 to 1.06	.13	1.23	1.00 to 1.50	.04
75 v 55	0.69	0.33 to 1.42	.31	1.91	1.06 to 3.45	.03
Adjuvant treatment						
Hormonal v none	0.54	0.34 to 0.86	.01	0.72	0.48 to 1.09	.12
Chemotherapy v none	1.48	0.88 to 2.50	.14	1.76	1.08 to 2.93	.02
Histologic grade						
2 v 1	5.33	1.29 to 22.00	.02	2.03	0.72 to 5.69	.18
3 v 1	4.36	1.05 to 18.18	.04	2.70	0.97 to 7.53	.06
Not done v 1	2.32	0.52 to 10.48	.27	1.13	0.36 to 3.94	.84
Any subtype v 1	1.23	0.25 to 6.10	.80	0.96	0.30 to 3.05	.94
Lymphatic invasion	2.07	1.28 to 3.36	.003	1.80	1.12 to 2.88	.02
Intraductal component type						
Comedo v ductal	1.73	1.00 to 3.01	.05	1.45	0.86 to 2.47	.17
Lobular v ductal	0.93	0.28 to 3.03	.90	0.72	0.22 to 2.33	.58
None or not known v ductal	1.21	0.76 to 1.93	.42	1.00	0.64 to 1.55	.99

Abbreviations: RR, relative risk; ER, estrogen receptor; PgR, progesterone receptor.

\*Negative or equivocal v positive or not done. The small number of patients without receptor data was combined with the low-risk positive category. Receptor levels were categorized according to the standards of the laboratory in which they were determined.

†The relationship of age to the risk of recurrence was modeled using a continuous age variable with linear and quadratic terms.

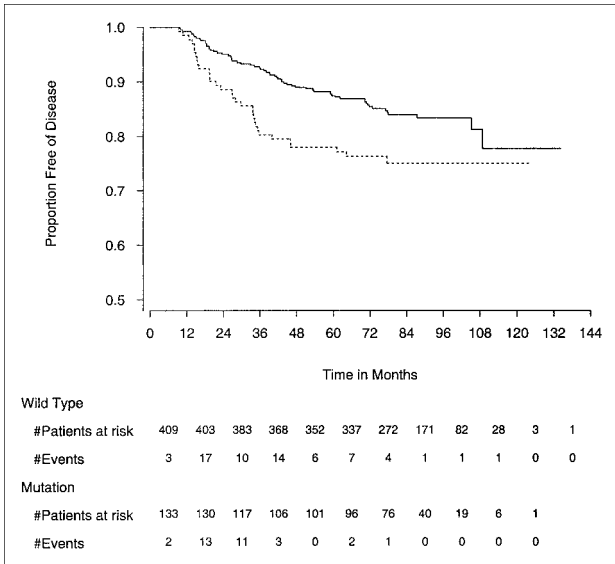
neither of the alterations (Fig 2). This elevated risk was detectable in a Cox model as a significant interaction between p53 mutation and neu/erbB-2 amplification status that did not appear to be strongly time dependent ( $P = .17$ ), and as a contrast between tumors with both p53 mutation and neu/erbB-2 amplification versus all others (Table 3).

In univariate analyses of the other prognostic factors, there was some evidence that each of the factors was related to DFS when considered one at a time (Table 3). The elevated risks of recurrence for ER-negative status and PgR-negative status were also found to decrease with the duration of follow-up ( $P = .001$  and  $0.001$ , respectively, for the tests of time dependence).

Univariate analyses of overall survival yielded results similar to those for DFS (Table 3 and Fig 3). The RR for each of p53 mutation, neu/erbB-2 amplification, the combina-

tion of p53 mutation and neu/erbB-2 amplification, larger tumor size, ER-negative status, PgR-negative status, and lymphatic invasion was significantly elevated. For each of these factors, there was also evidence that the RR attenuated with increasing duration of follow-up.

**Multivariate analysis.** The magnitude and the significance of the association of p53 mutation with DFS persisted with adjustment for most of the primary and histopathologic factors when these were considered one at a time, with the exception of ER and PgR status and the adjuvant treatment indicators. In these exceptions, the RR was reduced from 1.69 to between 1.34 and 1.48 because of the correlation of p53 mutation status with the hormonal and the treatment factors, and became nonsignificant at the 5% significance level (data not shown). When all prognostic factors examined in univariate analysis (Table 3) were in-



**Fig 1.** Kaplan-Meier disease-free survival curves stratified by *p53* mutation status (n = 542). (—) corresponds to patients without a *p53* tumor mutation (wild type); (· · ·) corresponds to patients with a *p53* tumor mutation.

cluded in a multivariate model (data not shown), the RR for *p53* mutation was reduced further to 1.11 (95% CI, 0.68 to 1.81; *P* = .69), and only lymphatic invasion retained a significant independent association with disease recurrence (RR, 1.82; 95% CI, 1.09 to 3.02; *P* = .02).

In striking contrast, the RR associated with having both *p53* mutation and *neu/erbB-2* amplification, as measured by their interaction, persisted with adjustment for adjuvant treatment and all prognostic factors in a full multivariate model (Table 4). Similar results were obtained for the full multivariate model (data not shown), using a binary variable for tumors with both *p53* mutation and *neu/erbB2* amplification versus those with only one or neither alteration (RR, 2.23; 95% CI, 1.28 to 3.88; *P* = .004;  $-2\log L = 1,107.99$ ). This combined effect also persisted in a summary multivariate model that was not significantly different from the full multivariate model (Table 5).

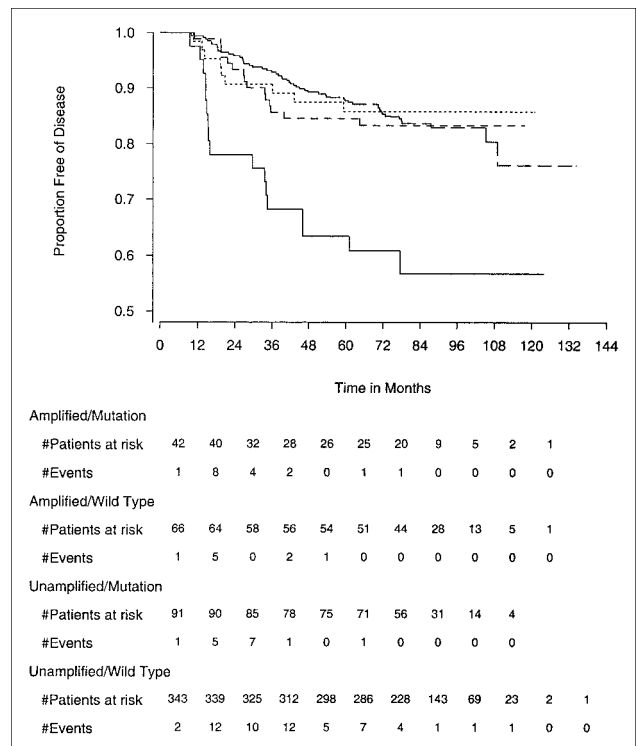
To assess whether adjuvant treatment modified the observed association of disease recurrence with *p53* mutation and *neu/erbB2* amplification, we conducted a test for interaction in the summary Cox model by adding product terms with the two treatment variables, and also examined summary models within each of the three corresponding patient subgroups: those who received no systemic adjuvant treatment, those who received hormonal therapy only, or those who received systemic chemotherapy with or without hormonal therapy. The RR of recurrence did not vary significantly across the subgroups (likelihood ratio test, *P* = .86), and in particular, the elevated RR persisted in the untreated subgroup (RR,

2.14), although with a wider confidence interval because of the smaller sample size.

Application of the same model-fitting strategy to the analysis of OS yielded results similar to those for DFS (Tables 4 and 5). In a parsimonious summary model for OS (Table 5) that was not significantly different from the full multivariate model for OS that included all of the prognostic factors (likelihood ratio test [9 df], 2.83; *P* = .97; Table 4, the combination of *p53* mutation and *neu/erbB-2* amplification, ER-negative status, and higher histologic grade were independently associated with an elevated risk of death as a result of any cause.

## DISCUSSION

The identification of patients with ANN breast cancer who are likely to develop systemic disease and might benefit from adjuvant therapy remains a challenge. Molecular alterations such as *neu/erbB-2* amplification and *p53* mutations have been shown to be associated with poor outcome in a number of studies (reviewed in [8-10,12-14]). However, the question of whether these markers, when evaluated in the same cohort, provide independent prognostic information has not been addressed. Therefore, we analyzed mutation of *p53* as the primary prognostic variable



**Fig 2.** Kaplan-Meier disease-free survival curves stratified by *p53* mutation status and by *neu/erbB-2* amplification status (n = 542) of patients with neither alteration (— — —), with a mutation and without amplification (— — —), without a mutation and with amplification (· · ·), and with both (— — —).

**Table 4.** Results of Disease-Free and Overall Survival Analysis by Full Multivariate Cox Proportional Hazards Model (N = 543)

Prognostic Variable	Recurrence			Mortality		
	RR	95% CI	P	RR	95% CI	P
<i>p53</i> mutation & <i>neu</i> amplification						
Mutation only v neither	0.69	0.36 to 1.31	.26	1.04	0.58 to 1.86	.91
Amplification only v neither	0.76	0.37 to 1.58	.46	1.04	0.54 to 1.99	.91
Interaction of <i>p53</i> and <i>neu</i>	3.68	1.32 to 10.27	.01	1.97	0.78 to 5.00	.15
Menopausal status, pre- or perimenopause v postmenopause	0.92	0.46 to 1.82	.81	1.24	0.61 to 2.54	.56
Tumor size, cm						
2-5 v < 2	1.45	0.90 to 2.32	.12	1.41	0.89 to 2.22	.14
≥ 5 v 2-5	1.34	0.61 to 2.97	.47	1.08	0.56 to 2.07	.81
ER status*	1.70	0.90 to 3.21	.10	1.49	0.81 to 2.77	.20
PgR status*	1.14	0.66 to 1.98	.63	1.38	0.82 to 2.33	.22
Age at diagnosis, year†						
35 v 55	1.65	0.72 to 3.77	.23	0.94	0.41 to 2.17	.89
45 v 55	1.24	0.89 to 1.72	.20	0.86	0.62 to 1.19	.36
65 v 55	0.87	0.62 to 1.21	.40	1.50	1.12 to 2.00	.006
75 v 55	0.81	0.35 to 1.87	.62	2.89	1.43 to 5.83	.003
Adjuvant treatment						
Hormonal v none	0.63	0.38 to 1.05	.07	0.91	0.57 to 1.46	.69
Chemotherapy v none	0.81	0.43 to 1.52	.51	1.21	0.64 to 2.27	.56
Histologic grade						
2 v 1	3.79	0.91 to 15.87	.07	1.59	0.55 to 4.55	.39
3 v 1	2.28	0.52 to 9.95	.27	1.51	0.51 to 4.40	.45
Not done v 1	1.34	0.29 to 6.21	.71	0.74	0.23 to 2.36	.61
Any subtype v 1	0.68	0.13 to 3.61	.65	0.71	0.21 to 2.39	.57
Lymphatic invasion	1.67	1.00 to 2.80	.05	1.49	0.90 to 2.46	.12
Intraductal component type						
Comedo v ductal	1.20	0.67 to 2.15	.55	1.16	0.66 to 2.04	.61
Lobular v ductal	2.68	0.72 to 9.96	.14	1.19	0.33 to 4.27	.79
None or not known v ductal	1.24	0.77 to 1.98	.38	0.96	0.62 to 1.50	.87
-2LogL	1,106.38			1,183.19		

Abbreviations: RR, relative risk; ER, estrogen receptor; PgR, progesterone receptor.  
 \*Negative or equivocal v positive or not done. The small number of patients without receptor data was combined with the low-risk positive category. Receptor levels were categorized according to the standards of the laboratory in which they were determined.  
 †The relationship of age to the risk of recurrence was modeled using a continuous age variable with linear and quadratic terms.

with DFS as the outcome in a prospective cohort of consecutive newly diagnosed node-negative patients in which *neu/erbB-2* amplification had been shown to be associated with outcome [11]. The sample size was designed to have high power to evaluate the prognostic value of *p53* mutation in the presence of an independent association of *neu/erbB-2* amplification with disease recurrence. In addition, we attempted to avoid the problems of incomplete detection of the *p53* mutation status by using SSCP analysis instead of immunohistochemistry and by examining exons 4 through 10. For the 543 patients in whom copy number and mutation status were determined, *p53* was mutated in 24.5% of the specimens; 83% of the mutations occurred in exons 5 to 9 and the remainder occurred in exons 4 and 10 (Ozcelik et al, manuscript in preparation). This frequency is higher than that reported when only exons 5 to 9 have been examined [12]) and the distribution is similar to that observed in studies that include exons 4 and 10 [21]. Our data support the importance of examination of exons 4 through 10 for

complete mutational analysis of *p53* [21]. As previously reported, *neu/erbB-2* was amplified in 19.9% of tumors in this group [11].

In univariate analyses, we found that *p53* mutation and *neu/erbB-2* amplification were each associated with the risk of recurrence. When traditional prognostic factors, such as tumor size, ER and PgR status, and histopathologic characteristics, including lymphatic invasion and histologic grade, were considered in a univariate analysis, each of these factors was also associated with a significant risk of recurrence. The results of the univariate analyses are similar to those in other studies that have examined the risk associated with *p53* mutation. In multivariate analysis that did not consider *p53* mutation status, the role of *neu/erbB-2* amplification, hormone receptors, and lymphatic invasion found previously [11] was confirmed in the same cohort with longer follow-up. For *neu/erbB-2* amplification and ER status, however, there was evidence that the risk of recurrence was higher during the first 36 months of follow-up, and attenu-

**Table 5.** Results of Disease-Free and Overall Survival Analysis for All Patients (N = 543) by Summary Multivariate Cox Proportional Hazards Model

Prognostic Variable	Recurrence			Mortality		
	RR	95% CI	P	RR	95% CI	P
Both mutation and amplification in the other three groups	2.32	1.35 to 3.97	.002	2.22	1.29 to 3.81	.004
Tumor size > 2 cm	1.01	0.67 to 1.54	.95	1.44	0.93 to 2.23	.10
ER status*	1.48	0.92 to 2.39	.10	1.82	1.14 to 2.90	.01
Age at diagnosis, years †						
35 v 55	1.32	0.71 to 2.44	.39	1.03	0.55 to 1.93	.93
45 v 55	1.14	0.92 to 1.42	.24	0.90	0.72 to 1.13	.38
65 v 55	0.88	0.68 to 1.14	.35	1.39	1.12 to 1.71	.002
75 v 55	0.79	0.38 to 1.63	.52	2.42	1.34 to 4.36	.003
Adjuvant treatment						
Hormonal v none	0.58	0.36 to 0.94	.03	0.89	0.57 to 1.40	.62
Chemotherapy v none	0.78	0.43 to 1.44	.43	1.21	0.65 to 2.25	.54
Histologic grade ‡						
2 or 3 v 1	2.68	1.60 to 4.49	.0002	2.06	1.3 to 2.23	.002
Lymphatic invasion	1.73	1.06 to 2.84	.03	1.58	0.97 to 2.57	.07
-2LogL	1,117.99			1,186.04		

Abbreviation: ER, estrogen receptor.  
 \*Negative or equivocal v positive or not done. The small number of patients without receptor data was combined with the low-risk positive category. Receptor levels were categorized according to the standards of the laboratory in which they were determined.  
 †The relationship of age to the risk of recurrence was modeled using a continuous age variable with linear and quadratic terms.  
 ‡In the summary model only, grade not done and any subtype categories were combined with the grade 1 category.

ated with time. This observation would be consistent with *neu/erbB2* amplification and receptor status being more important in determining early recurrences, or with selection in which patients at higher risk are removed from the cohort, leaving patients at lower risk in those remaining.

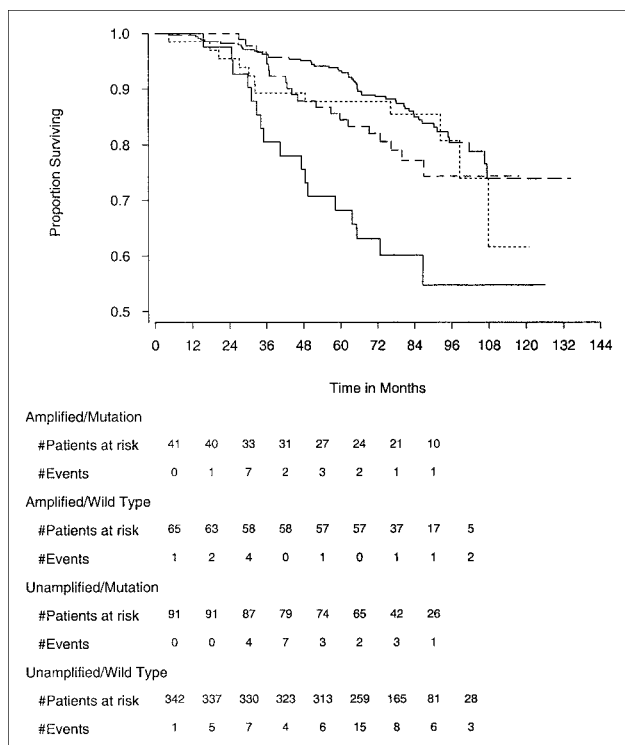
However, in multivariate analyses of *p53* mutation status, when we included *neu/erbB-2* amplification and adjusted for all prognostic variables, the elevated RR associated with *p53* mutation was reduced dramatically overall, and during the first 36 months, whereas the RR for *neu/erbB-2* amplification during the first 36 months remained significantly elevated (RR, 1.86; 95% CI, 1.05 to 3.28;  $P = .03$ ). In fact, we found that *p53* mutation was not an independent predictor of recurrence in the multivariate analyses.

In contrast, the RR associated with the subgroup of patients having both *p53* mutation and *neu/erbB-2* amplification was unchanged by adjustment for the same set of factors. In a summary multivariate model adjusting for age, tumor size, ER status, and adjuvant treatment, we found the joint occurrence of *p53* mutation and *neu/erbB-2* amplification, together with histologic grade and lymphatic invasion, to be significantly and independently important. Furthermore, stratification according to adjuvant treatment received revealed that the RR associated with the joint occurrence of both factors was consistently elevated in both treated and untreated patients. Finally, we observed comparable RRs for OS and DFS.

Our results differ from those of other studies of *p53* mutation in breast cancer. Even though the prognostic im-

portance of either *p53* mutation or *neu/erbB-2* amplification has been evaluated in a number of studies, the combination of these molecular markers (detected as DNA alterations) with traditional clinical factors has not been routinely examined. The nature of the interaction we detected between *p53* mutation and *neu/erbB-2* amplification is such that the observed prognostic importance of *p53* mutation alone will depend on the frequency of *neu/erbB-2* amplification status in the particular study sample, the length of followup, and the other prognostic factors taken into account in the analysis. This may partially explain the inconsistent results reported for *p53* mutation in the literature. Similarly, studies of *neu/erbB-2* amplification are likely to yield stronger associations when follow-up duration is less than 5 years and the sample includes tumors in which *p53* mutations are more common. As more molecular alterations are identified in breast cancer, it will become increasingly important to determine the value of these new factors in the context of previously validated markers and to identify excess risk associated with particular combinations of them.

Our finding of a significant and persistent risk of recurrence and overall mortality associated with having a *p53* mutation in combination with *neu/erbB-2* amplification, and no elevated risk associated with having only one of the alterations, was not expected. It suggests that the presence of a *p53* mutation alone does not identify additional patients at higher or prolonged risk of recurrence beyond those identified by *neu/erbB-2* amplification. Instead, it appears that the presence of *p53* mutation may be useful to



**Fig 3.** Kaplan-Meier overall survival curves stratified by p53 mutation status and by neu/erbB-2 amplification status (n = 542) of patients with neither alteration (— — —), with a mutation and without amplification (---), without a mutation and with amplification (· · ·), and with both (— · —).

select a subset of patients with neu/erbB-2 amplification who are at higher risk of disease recurrence and death.

It is interesting to note relevant findings recently reported in a clinical trial of response to dose-intensive chemotherapy (cyclophosphamide, doxorubicin, and flu-

orouracil) in node-positive breast cancer patients [22]. In this trial, the largest dose effect was found in the subgroup of 100 patients with p53 alteration and high erbB-2 expression, both detected by immunohistochemistry, indicating that this group may be more responsive to high-dose chemotherapy. Their data for 5-year DFS in the low-dose group (the group that is most similar to the adjuvant untreated and treated individuals in our cohort) indicated 39% survival in 33 patients with both p53 alteration and erbB-2 overexpression compared with 56% to 61% survival in patients with elevated expression for only one or no alteration or overexpression (total 289 patients). This pattern is consistent with our observation in ANN patients of an interaction between p53 mutation and neu/erbB-2 amplification, in which the combination is associated with increased risk.

This observation should be interpreted cautiously, however, and needs to be evaluated in the context of clinical trials evaluating the benefits of therapy (particularly chemotherapy) in ANN breast cancer patients before recommendations can be made regarding routine p53 mutation testing. Confirmation of our findings would support the inclusion of diagnostic testing for p53 mutations in patients with neu/erbB-2-amplified tumors.

### Acknowledgment

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

### Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

### REFERENCES

1. Fisher B, Redmond C, Dimitrov NV, et al: A randomized clinical trial evaluating sequential methotrexate and fluorouracil in the treatment of patients with node-negative breast cancer who have estrogen-receptor-negative tumors. *N Engl J Med* 320:473-478, 1989
2. Fisher B, Costantino J, Redmond C, et al: A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen-receptor-positive tumors. *N Engl J Med* 320:479-484, 1989
3. Mansour EG, Gray R, Shatila AH, et al: Efficacy of adjuvant chemotherapy in high-risk node-negative breast cancer: An intergroup study. *N Engl J Med* 320:485-490, 1989
4. The Ludwig Breast Cancer Study Group: Prolonged disease-free survival after one course of perioperative adjuvant chemotherapy for node-negative breast cancer. *N Engl J Med* 320:491-496, 1989

5. Early Breast Cancer Trialists' Collaborative Group: Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy—133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. *Lancet* 339:1-15, 1992
6. Slamon DJ, Clark GM, Wong SG, et al: Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 235:177-182, 1987
7. Slamon DJ, Godolphin W, Jones LA, et al: Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 244:707-712, 1989
8. Allred DC, Harvey JM, Berardo M, et al: Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol* 11:155-168, 1998
9. Fitzgibbons PL, Page DL, Weaver D, et al: Prognostic factors in breast cancer: College of American Pathologists Consensus Statement 1999. *Arch Pathol Lab Med* 124:966-978, 2000
10. Henderson IC, Patek AJ: The relationship between prognostic and predictive factors in the

management of breast cancer. *Breast Cancer Res Treat* 52:261-288, 1998

11. Andrulis IL, Bull SB, Blackstein ME, et al: Neu/erbB-2 amplification identifies a poor-prognosis group of women with node-negative breast cancer: Toronto Breast Cancer Study Group. *J Clin Oncol* 16:1340-1349, 1998
12. Hartmann A, Blaszyk H, Kovach JS, et al: The molecular epidemiology of p53 gene mutations in human breast cancer. *Trends Genet* 13:27-33, 1997
13. Elledge RM, Allred DC: Prognostic and predictive value of p53 and p21 in breast cancer. *Breast Cancer Res Treat* 52:79-98, 1998
14. Pharoah P, Day NE, Caldas C: Somatic mutations in the p53 gene and prognosis in breast cancer: A meta-analysis. *Br J Cancer* 80:968-973, 1999
15. Maniatis TFE, Sambrook J: *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press, 1989
16. Ozcelik H, Andrulis IL: Multiplex PCR-SSCP for simultaneous screening for mutations in several exons of p53. *Biotechniques* 18:742-744, 1995

17. Freedman LS: Tables of the number of patients required in clinical trials using the logrank test. *Stat Med* 1:121-129, 1982

18. Phillips AN, Pocock SJ: Sample size requirements for prospective studies, with examples for coronary heart disease. *J Clin Epidemiol* 42:639-648, 1989

19. Cox DR: Regression models and life tables (with discussion). *J R Stat Soc B* 187-220, 1972

20. Therneau T, Grambsch PN: *Modeling Survival Data: Extending the Cox Model*. New York, NY, Springer-Verlag, 2000

21. Hartmann A, Blaszyk H, McGovern RM, et al: p53 gene mutations inside and outside

of exons 5-8: The patterns differ in breast and other cancers. *Oncogene* 10:681-688, 1995

22. Thor AD, Berry DA, Budman DR, et al: ErbB-2, p53, and efficacy of adjuvant therapy in lymph node-positive breast cancer. *J Natl Cancer Inst* 90:1346-1360, 1998