Enzyme-Linked Immunoabsorbent Assay–Detected p53 Protein Accumulation: A Prognostic Factor in a Large Breast Cancer Cohort

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Purpose: **This study was designed to evaluate whether patients with an unfavorable breast cancer prognosis could be identified by p53 protein overexpression detected by a quantitative enzyme-linked immunoabsorbent assay (ELISA).**

Patients and Methods: **Extracts from 998 breast carcinomas were assayed for p53 protein by an ELISA that used both DO-1 monoclonal and CM-1 polyclonal antibodies. Relative risks (RRs) for cancer relapse and death after 6 years of follow-up for patients with p53-positive tumors based on different dichotomization criteria were determined by multivariate Cox regression, adjusted for patient age, tumor size, S-phase fraction, estrogen (ER) and progesterone (PR) receptor concentrations, DNA ploidy, and lymph node metastases. Disease-free (DFS) and overall (OS) survival probabilities of p53-positive and p53-negative groups, using a median cutoff, were also estimated by the Kaplan-Meier method and the log-rank test. These analyses were performed for all patients and for subgroups defined by ER status, node status, and primary postoperative treatment.**

Results: **Univariate analysis showed that p53 concentrations that exceeded the median indicated significantly increased risks for relapse ($P < .01$) and death ($P = .02$). Multivariate analyses confirmed these observations (RR = 1.40; $P = .02$ for DFS and RR = 1.50; $P < .01$ for OS) and showed trends for increasing risks for relapse ($P = .02$) and death ($P = .06$) when p53 was considered as a four-level categoric variable, and identified p53 positivity as a significant predictor of outcome in node-positive patients (RR = 1.67; $P < .01$ and RR = 2.10; $P < .01$ for DFS and OS, respectively), ER-positive patients (RR = 1.45; $P = .02$ and RR = 1.50; $P = .01$ for DFS and OS, respectively), and in patients treated with chemotherapy (RR = 1.73; $P = .04$ for relapse and RR = 2.04; $P = .03$ for death).**

Conclusion: **Assessment of p53 overexpression by ELISA, easily incorporated into the routine biochemical work-up of breast tumors, may be an independent predictor of reduced survival of breast cancer patients.**

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RATIONAL TREATMENT DECISIONS for breast cancer patients have traditionally been guided by the presence or absence of axillary lymph node metastases, tumor size, steroid hormone receptor expression, and histologic type. It has become the clinical consensus in recent years that a more reliable prediction of breast cancer outcome and of response to adjuvant therapies may be facilitated by the integration of the traditional prognostic factors into multiparametric schemes, together with additional markers of biologic relevance.[1] Although the mutational status of the p53 tumor-suppressor gene has been the most extensively studied of these newer markers, conflicting evidence has emerged regarding its ability to identify patients at increased risk for unfavorable outcomes independently of the more established clinicopathologic features of breast cancer.[2-7]

Mutation of the p53 gene has been reported to occur in 20% to 50% of sporadic breast carcinomas among women in Western countries[8,9] and may occur at higher frequencies or with a different pattern in other populations.[10,11] A shared consequence of these predominantly missense mutations, which are usually accompanied by loss of heterozygosity, is the disruption of the normal, pleiotropic function of the p53 protein. p53 is believed to be a critical determinant in the induction of cell-cycle arrest,[12] programmed cell death,[13] and possibly DNA repair[14] in response to cellular stresses that may lead to DNA damage. Therefore, it is not surprising that abrogation of p53 function in breast carcinoma has been associated with genomic instability,[15] higher proliferative rates,[16] and resistance to conventional antineoplastic agents of which cytotoxic effects are mediated by p53.[17] A second consequence of p53 mutation is usually, but not always, a conformational change in the expressed protein, which results in its accumulation in tumor cell nuclei and enables its detection immunohistochemically.[8] Findings that both p53 protein overexpression and mutation of the p53 gene

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often present contemporaneously with a number of clinical, cytologic, and molecular indicators of aggressive tumor phenotypes[18,19] are consistent with studies that show that p53 abnormalities are predictive of reduced survival of breast cancer patients.[2-4,7] Other studies, however, have not found p53 status to be of independent prognostic value in this disease.[5,6,16] At the heart of such discordant conclusions are likely methodologic differences between studies, particularly with respect to techniques used to determine p53 mutational status. Whereas direct sequencing of all 11 exons of the p53 gene may provide the most accurate assessment,[7] its use, even when confined to screening the most frequently mutated exons 5 to 8, may be inappropriate in some settings. Far more suitable for routine determination of p53 status are immunochemical methods, which detect p53 protein in tumor tissue and most often take the form of immunohistochemical staining procedures. However, the variable combinations of immunoreagents, specimen-processing details, and subjective immunostaining scoring systems reported have fueled disagreement between studies for which immunohistochemical methods were used to detect p53.[20]

The purpose of this study was to evaluate the prognostic value of p53 accumulation in breast carcinoma by means of enzyme-linked immunoabsorbent assay (ELISA), another immunochemical approach that has been less commonly used. Quantitative immunoassays of p53 have been applied to soluble extracts derived from various tumor types[21] and have recently shown utility for breast cancer prognosis by measuring p53 protein in cytosolic extracts prepared for steroid hormone–receptor analyses,[22,23] which thereby obviates the need for additional tumor tissue to perform these studies. A larger North American patient population and a well-characterized immunofluorometric assay distinguished our study, which also related p53 accumulation to survival in patients stratified into groups based on important prognostic categories: estrogen receptor (ER) status, lymph node status, and receipt of adjuvant chemotherapy, endocrine therapy, or no postoperative treatment.

PATIENTS AND METHODS

Study Population

Tumor specimens from 1,000 women who underwent surgery for primary invasive breast carcinoma were obtained from the Breast Cancer Tissue Resource (BCTR), a collaborative project between the University of Texas Health Science Center at San Antonio (San Antonio, TX) and Nichols Institute Research Laboratories (NIRL; San Capistrano, CA). BCTR maintains a large collection of breast tumor specimens at –70°C that had been sent from health care institutions throughout the United States to NIRL for routine quantification of ERs and progesterone receptors (PRs) and for flow cytometric analyses (see below). Tumor tissue had been frozen in liquid nitrogen or on dry ice immediately after surgery for shipment to NIRL. Criteria for the selection of specimens from the BCTR archive included the availability of at least 0.25 g of tumor tissue for p53 protein analysis (see below) and information regarding the status of other clinical and pathologic variables of potential prognostic significance, determined at NIRL (ER and PR concentrations, DNA ploidy, and S-phase fraction). The status of one or more of the demographic or clinical factors (patient age at surgery, number of lymph nodes positive for malignancy, tumor size, and postoperative therapy) provided by the institutions from which the specimens originated was unknown for 124 patients.

The patient cohort was drawn from 165 hospitals that were widely distributed geographically and in which surgery had been performed from August 1985 to October 1991. Age at surgery was known for all but one patient and ranged from 22 to 94 years, with a median age of 61 years. Lymph node metastases had been detected in 530 (55%) of the 959 patients for whom the presence or absence of this disease feature had been reported. The mean and median number of affected lymph nodes were 2.3 and 0, respectively, and the number ranged from 0 to 46. Tumor size ranged from 1 to 145 mm, with a median size of 23 mm. The percentages of malignant cells in 797 of the tumor specimens were estimated histopathologically by a single pathologist blinded to the results of the p53 protein, steroid hormone receptor, and flow cytometric analyses and to patient survival status; 4% of the specimens had tumor cellularities from 0% to 10%; 21% from 11% to 30%; 46% from 31% to 70%; and 29% had tumor cellularities that exceeded 70%. Data regarding tumor grade and histologic classification of the specimens were unavailable.

All patients had not been previously treated for breast cancer. Primary surgical therapy consisted of modified radical mastectomy with axillary lymph node dissection (97%), incisional biopsy (2%), or lumpectomy without axillary lymph node dissection (1%). Histopathologic examination of the resected tumor tissues confirmed the diagnosis of primary breast cancer in all patients. Of the 948 patients for whom the modalities of postoperative treatment were reported, 39% received no additional treatment, 9% received locoregional radiotherapy alone, 16% received only adjuvant chemotherapy, 17% received endocrine therapy alone, 4% received endocrine therapy and radiotherapy, 6% received both systemic adjuvant therapies, 7% received chemotherapy and radiotherapy, and 2% received all three treatment modalities. Information regarding lymph node status or other disease features of patients who received each treatment modality was unavailable.

Patient follow-up information, which included survival status (alive or deceased) and disease status (disease free or recurrence/metastasis) at last follow-up, together with the dates and circumstances of relapse and death, if applicable, was available for 997 patients and was updated annually by the institutions that submitted the specimens to NIRL. Treatment failure, defined as the first documented evidence of local recurrence, regional axillary relapse, distant metastasis, or new ipsilateral or contralateral breast cancer, as revealed by clinical, radiologic, or histologic evaluations, occurred in 213 (21%) patients. One hundred ninety-nine (20%) patients died during their respective follow-up periods. The distribution of follow-up times for patients still alive at the time of analysis ranged from 28 to 112 months, with a median of 77 months; only 33 and nine patients had been followed up less than 48 and 36 months, respectively.

This study had been approved by the Ethics and Research Committee at the University of Toronto and by the Institutional Review Board at the University of Texas Health Science Center at San Antonio, which assured patient confidentiality at every stage of the investigation.

Flow Cytometric and Steroid Hormone–Receptor Analyses

All 1,000 breast tumor specimens were subjected to DNA flow cytometry as described elsewhere.[24] Briefly, tumor tissue was gently

homogenized, filtered, centrifuged through a double cushion of sucrose, and the cells were resuspended and counted before being simultaneously lysed and stained with propidium iodide. Nuclei were collected and 50,000 were analyzed on an Epics V flow cytometer (Coulter Electronics, Hialeah, FL). DNA content and S-phase fraction were determined from the DNA histograms, in which diploid populations were defined as those with a DNA index of 1.0, and the percentage of cells in S-phase considered a favorable prognostic indicator was less than 6.7%. The optimal cutoff value for S-phase fraction had been previously determined by cutpoint analysis.[25]

Tumor specimens (n = 1,000) were pulverized in liquid nitrogen and homogenized in buffer, and the cytosolic fractions were obtained by ultracentrifugation and quantified for steroid hormone receptors as described by Dressler et al.[24] The results of the dual ligand-binding assay, in which dextran-coated charcoal was used to separate bound from free ligand, were interpreted by Scatchard analysis.[26] Protein concentrations of the cytosols were determined by the Lowry method.[27] Cutoff levels for positivity were 3 fmol/mg or greater and 5 fmol/mg or greater for ER and PR, respectively, as optimized previously.[28,29]

Quantitative p53 Protein Analysis

Frozen breast tumor tissues (~0.2 g) were pulverized on dry ice to a fine powder, which was suspended in 1 mL of lysis buffer (50 mmol/L of Tris, pH 8.0; 150 mmol/L of NaCl; 5 mmol/L of EDTA; 10 mL/L of NP-40 surfactant; 10 mg/L of phenylmethylsulfonylfluoride; and 1 mg/L each of aprotinin and leupeptin) and incubated for 30 minutes on ice before centrifugation at 14,000*g* for 30 minutes at 4°C to collect the supernates. The crude cell lysates were immediately assayed both for p53 protein by immunofluorometry and for total-protein content by a kit based on the bicinchoninic acid method (Pierce Chemical, Rockford, IL).

A sandwich-type ELISA, described in detail elsewhere,[30] was used to measure the p53 protein concentrations in 998 of the breast tumor extracts. Soluble p53 protein, present in extracts and calibrators diluted twofold in buffer A (50 mmol/L of Tris, pH 7.80; 60 g/L of bovine serum albumin; and 0.5 g/L of NaN_3) supplemented with 0.5 mol/L of KCl, 10 mL/L of mouse serum, and 5 mL/L of Tween-20 detergent, was first immobilized in microtiter wells coated with monoclonal DO-1 antibody (gift of Dr David Lane, University of Dundee, UK). After this initial 3-hour incubation step at 37°C, bound p53 protein was then detected by sequential 1-hour incubations at room temperature with polyclonal CM-1 antiserum (Novocastra, Newcastle upon Tyne, UK) raised in a rabbit host against recombinant wild-type human p53 and diluted 5,000-fold in buffer A, and then with alkaline phosphatase-conjugated goat antirabbit immunoglobulin (Jackson ImmunoResearch, West Grove, PA) diluted to 120 mg/L in buffer A that contained 0.5 mol/L of KCl and 100 mL/L of goat serum. Hydrolysis of the enzyme substrate (0.01 mol/L of diflunisal phosphate in 0.1 mol/L of NaOH, diluted 10-fold in 0.1 mol/L of Tris, pH 9.10, that contained 0.15 mol/L of NaCl, 1 mmol/L of $MgCl_2$, and 0.5 g/L of NaN_3), added for 10 minutes at room temperature, yielded a product that entered into a fluorescent complex when the developing solution (1 mol/L of Tris, 0.4 mol/L of NaOH, 2 mmol/L of $TbCl_3$, and 3 mmol/L of EDTA) was also added. Fluorescence at 615 nm was measured after 1 minute by a Cyberfluor-615 Immunoanalyzer (Cyberfluor, Toronto, Canada) in a time-resolved mode, which greatly reduces the background fluorescence signal.[31] All reagents were added to wells in 100-µL volumes. Concentrations of p53 were interpolated from a calibration curve generated by the simultaneous assay of a dilution series of an extract of Sf9 insect cells infected with a p53-expressing baculovirus (gift of Dr Thierry Soussi, Institut Curie, Paris, France), as described previously.[30] Values of these calibrators, which ranged from 0 to 75 µg/L, were established based on the assay of reconstituted preparations of premeasured, lyophilized recombinant human p53 protein (Oncogene Science, Uniondale, NY). All calibrators and samples were assayed in duplicate. Analytic characteristics of the ELISA include a sensitivity of ~0.04 µg/L and a linear response range from 0.15 to 75 µg/L. Concentrations of p53 protein in the breast tumor extracts were divided by the total-protein contents to adjust for differences in tissue masses and extraction efficiencies. Because the epitope recognized by DO-1 antibody is within an amino terminal domain shared by all conformations of p53 protein,[32] the ELISA is able to detect both mutant and wild-type p53 protein. Clinical specimens were assayed for p53 protein without knowledge of the corresponding patient clinicopathologic or survival information.

Statistical Analysis

The statistical analysis, performed with SAS version 6.03 software (SAS Institute, Cary, NC), examined associations between the total-protein–adjusted p53 ELISA results and clinical outcome, as well as between the p53 concentrations and other measurements or characteristics of the breast tumor population. Monotonic relationships between p53 protein as a continuous variable and patient age, tumor size, S-phase fraction, ER, PR, and number of involved lymph nodes were shown by the calculation of the Spearman correlation coefficient (r_s) in each case, appropriate given the non-Gaussian distribution of p53 concentrations. All other statistical procedures were similarly nonparametric and based on two-tailed tests of significance. To further investigate associations between p53 and the other clinicopathologic factors, χ^2 tests were applied to contingency tables after dichotomization of all variables: p53 (negative *v* positive by means of a cutoff point equal to the 50th percentile of the ELISA results distribution), age (< 50 years *v* ≥ 50 years), tumor size (≤ 2 cm *v* > 2 cm), nodal status (no lymph nodes involved *v* at least one node with histologic evidence of metastatic spread), S-phase fraction (< 6.7% *v* ≥ 6.7% of tumor cells in S-phase), ER status (negative *v* positive using 3 fmol/mg as the cutoff point), PR status (negative *v* positive using a 5-fmol/mg cutoff), DNA ploidy (diploid *v* aneuploid), endocrine therapy (not treated with tamoxifen *v* treated with tamoxifen alone or in combination with other therapies), chemotherapy (not treated with chemotherapy *v* treated with chemotherapy alone or in combination with other therapies), and radiotherapy (not treated with radiotherapy *v* treated with radiotherapy alone or together with other therapies).

Disease-free (DFS) and overall (OS) survival times were calculated from the dates of surgical resection of the tumors to the dates of occurrence of the two endpoints of interest, and the earliest diagnosis of recurrence or metastasis and patient death, respectively. Deaths without evidence of disease were considered censored for both DFS and OS. The prognostic roles of p53, singly or in conjunction with the other clinicopathologic factors, in determining DFS and OS were evaluated by fitting Cox proportional hazards regression models.[33] The hazards ratios and their 95% confidence intervals (CIs) were calculated from the models that used the best category, p53 negativity, as the reference in each case. In the multivariate analysis, the regression models were adjusted for age, tumor size, nodal status, S-phase fraction, DNA ploidy, and status for the steroid hormone receptors, all of which were considered dichotomous variables by using the classification criteria given previously. In the univariate and multivariate models, p53 was examined separately as a continuous variable after transformation into ascending ranks and as a dichotomous variable categorized by the median percentile cutoff point. In addition, the dose-response relationships between p53 and DFS and OS were evaluated by using the quartiles of the p53 distribution. These dose-response effects were also

examined by incorporating p53 into univariate and multivariate models as a quartile-divided, four-level continuous variable. Similar analyses were also performed in subgroups of patients stratified by the dichotomous status of three potentially confounding variables; namely, lymph node involvement, ER positivity, and receipt of principal postoperative breast cancer treatments. Univariate and multivariate Cox models that assessed median-dichotomized p53 were then constructed in each stratum. The p53 by ER, p53 by nodes, p53 by chemotherapy, and p53 by endocrine therapy interaction terms were also determined from univariate Cox models. The effects of p53 by nodes interaction on the risks for relapse and death conferred by p53 positivity were determined among the patients who received chemotherapy. The estimated power to uncover differences in survival between p53-negative and p53-positive patients in each subgroup was calculated by using STPLAN (shareware from Dr Barry W. Brown, University of Texas M.D. Anderson Cancer Center, Houston, TX), based on a one-sided test at the 5% level of significance and assuming that patients were accrued for 1 month and followed up for 77 months thereafter. The median p53 cutoff point was also used in survival curves, which examined the relapse and death rates of all p53-positive and p53-negative patients and for those within each of the above mentioned strata, which were plotted by the method of Kaplan and Meier[34] and compared by means of log-rank statistics.[35]

RESULTS

Distribution of p53 Protein Concentrations and Relationships to Other Clinicopathologic Variables

The distribution of the p53 concentrations in the 998 breast tumor extracts, which ranged from 0 to 110 μg/L, was positively skewed and had a mean, SD, and median of 1.89 μg/L, 7.78 μg/L, and 0.19 μg/L, respectively. Twelve percent of the extracts had p53 concentrations less than the assay detection limit. Expressed relative to the protein content of the extracts, the p53 concentrations considered in statistical analysis were similarly distributed (Fig 1), with a mean of 1.25 μg/g, an SD of 4.46 μg/g, a median of 0.16 μg/g, and a range from 0 to 58 μg/g. The 25th, 50th, and 75th percentiles of this distribution were 0.06, 0.16, and 0.41 μg/g, respectively. The other numeric variables followed somewhat less skewed distributions: ER (mean, 142 fmol/mg; SD, 186 fmol/mg; median, 73 fmol/mg; range, 0 to 1,786 fmol/mg), PR (mean, 218 fmol/mg; SD, 348 fmol/mg; median, 71 fmol/mg; range, 0 to 3,090 fmol/mg), and S-phase fraction (mean, 7.9%; SD, 6.48%; median, 5.8%; range, 0.2% to 65%). The distributions of patient age, tumor size, and number of lymph nodes have been previously described.

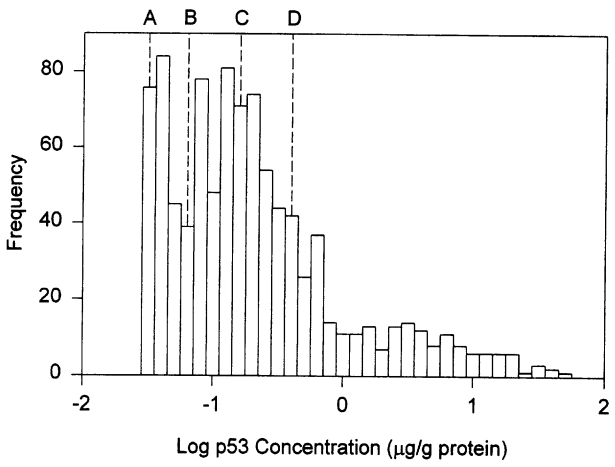

Fig 1. Frequency distribution of logarithmically transformed p53 protein concentrations in the 876 (of 998) breast tumor extracts that had p53 levels that exceeded the assay detection limit. The dashed lines indicate (A) the detection limit, (B) the 25th, (C) 50th, and (D) 75th percentiles of the frequency distribution.

Because the associations between p53 accumulation and patient survival times may have been affected by interactions between p53 and the other predictor variables, it was of interest to determine the relationships between other measurements or characteristics of the study population and the levels of p53 protein. Breast tumor extracts with p53 concentrations greater than the median cutoff point were more frequently less than the cutoff points for ER positivity and PR positivity (Table 1), which are well-established relationships[36] accompanied by significant, but very weak, negative correlations between p53 and ER ($r_s = -0.09$; $P < .01$) and PR ($r_s = -0.06$; $P = .04$). Table 1 also indicates that specimens positive for p53 protein were also associated with elevated S-phase fraction ($r_s = 0.18$; $P < .01$) and DNA aneuploidy, but that significant relationships were not shown between p53 and tumor size or nodal status, findings confirmed by correlation analysis (data not shown), or between p53 status and whether the patients received endocrine therapy, chemotherapy, or radiotherapy as part of their postsurgical management. Trends were evident, however, that suggested p53-positive malignancies were more likely to have been treated with radiation or tamoxifen. Whereas patient age was not associated with p53 accumulation status in the contingency table, the two variables were weakly correlated ($r_s = -0.12$; $P < .01$). Contingency tables that compared the proportions of p53-negative and p53-positive tumors, defined by 25th, 50th, or 75th percentile cutoffs, between four groups of specimens with tumor cellularities of 0% to 10%, 11% to 30%, 31% to 70%, or 71% to 100%, showed no significant association, consistent with the findings of other investigators[37] and possibly because of variable proportions of malignant cells that overexpressed p53 protein within the specimens.[38]

ELISA-Detected p53 Protein as a Predictor of Breast Cancer Patient Survival

Several approaches were used in the survival analysis to show associations between p53 and patient prognosis, which included the use of the Cox proportional hazards regression method in which p53 was expressed continuously or categori-

Table 1. Associations Between p53 Protein Status and Other Clinicopathologic Variables

Factor*	p53-Negative Tumors No.	%	p53-Positive Tumors No.	%	P
Age, years					
< 50	127	45.9	150	54.1	
≥ 50	369	51.2	351	48.8	.13
Tumor size, cm					
≤ 2	234	52.0	216	48.0	
> 2	242	47.7	265	52.3	.19
Nodal status					
Negative	260	49.2	269	50.8	
Positive	217	50.7	211	49.3	.63
S-phase fraction, %					
< 6.7	357	52.0	330	48.0	
≥ 6.7	140	45.0	171	55.0	.04
DNA ploidy					
Diploid	274	58.6	194	41.4	
Aneuploid	223	42.1	307	57.9	< .01
ER status					
Negative	54	39.4	83	60.6	
Positive	443	51.5	418	48.5	< .01
PR status					
Negative	126	44.8	155	55.2	
Positive	371	51.7	346	48.3	.05
Endocrine therapy					
Not treated	344	51.3	327	48.7	
Treated†	128	46.6	147	53.4	.19
Chemotherapy					
Not treated	329	50.8	319	49.2	
Treated‡	143	48.0	155	52.0	.43
Radiotherapy					
Not treated	381	51.4	361	48.6	
Treated§	91	44.6	113	55.4	.09

NOTE. *P* determined from χ^2 tests.

*See Patients and Methods section for details of patient dichotomization by p53, age, tumor size, nodal status, S-phase fraction, DNA ploidy, ER status, and PR status.

†Patients treated with endocrine therapy alone or in combination with chemotherapy and/or radiation.

‡Patients treated with chemotherapy alone or in combination with endocrine therapy and/or radiation.

§Patients treated with radiotherapy alone or in combination with endocrine therapy and/or chemotherapy.

cally and its contributions to DFS and OS were considered first singly, and then jointly, with the other predictive factors (Table 2). p53 protein levels expressed as their ranks were able to generate statistically significant hazards ratios, but the incremental risk differences were very small. The relative risks (RRs) for both relapse and death were significantly increased for p53-positive patients when p53 was classified into two groups based on the median. The use of the median cutoff point also indicated 40% and 50% increased risks for relapse and death, respectively, of p53-positive patients in multivariate analysis adjusted for all of the other variables listed in Table 2. Also predictive of patient outcome in multivariate analysis were lymph node positivity, associated with a twofold higher risk for relapse (95% CI, 1.51 to 2.71; $P < .01$) and 92% higher risk for death (95% CI, 1.42 to 2.60; $P < .01$), and tumor size larger than 2 cm, which yielded RRs of 1.51 for both relapse (95% CI, 1.12 to 2.03; $P < .01$) and death (95% CI, 1.11 to 2.06; $P < .01$). The S-phase fraction, DNA ploidy, ER, and PR variables were not significant prognostic factors in our series of breast cancer patients. In addition to simply dichotomizing patients on the basis of p53 negativity or p53 positivity, they were also classified into four groups based on the quartiles of the p53 distribution, by which patients in the second, third, and fourth quartiles were shown to have

Table 2. Associations Between p53 Protein and DFS and OS

p53 Status	Disease-Free Survival RR	95% CI	P	Overall Survival RR	95% CI	P
Univariate analysis						
Expressed as continuous variable*						
Rank	1.00			1.00		
Rank + 1	1.00	1.00-1.00	< .01	1.00	1.00-1.00	.02
Based on median cutoff point						
Negative	1.00			1.00		
Positive	1.47	1.12-1.93	< .01	1.41	1.06-1.87	.02
Based on quartiles†						
First quartile	1.00			1.00		
Second quartile	1.09	0.71-1.68		0.97	0.62-1.52	
Third quartile	1.35	0.89-2.05		1.39	0.91-2.13	
Fourth quartile	1.57	1.05-2.35		1.45	0.96-2.20	
P for trend			.01			.07
Multivariate analysis‡						
Expressed as continuous variable*						
Rank	1.00			1.00		
Rank + 1	1.00	1.00-1.00	.02	1.00	1.00-1.00	< .01
Based on median cutoff point						
Negative	1.00			1.00		
Positive	1.40	1.06-1.86	.02	1.50	1.11-2.02	< .01
Based on quartiles†						
First quartile	1.00			1.00		
Second quartile	1.18	0.03-1.35		1.13	0.98-1.30	
Third quartile	1.39	0.06-1.82		1.28	0.96-1.69	
Fourth quartile	1.64	1.09-2.46		1.44	0.94-2.20	
P for trend			.02			.08

NOTE. The number of patients included in univariate and multivariate survival analyses was 997 and 920, respectively. *P* are two-sided. Relative risk was estimated by the Cox proportional hazards regression model.

*p53 concentrations were ranked in ascending order.

†Estimated RR for second, third, and fourth quartiles compared with the first quartile are given. *P* are based on 1 degree of freedom tests of monotonic association.

‡Multivariate analysis adjusted for age, tumor size, nodal status, S-phase fraction, DNA ploidy, ER status, and PR status.

successively increasing risks for relapse compared with patients in the first quartile. In the corresponding multivariate models, this dose-response trend remained significant. Similar trends were also observed in the analysis of OS, but the *P* for the trends did not reach statistical significance.

It was also of interest to determine the prognostic value of p53 within subgroups of patients who had different prognostic or therapeutic implications. Stratification of patients by nodal status, ER status, and type of postoperative treatment would also have served to eliminate possible confounding effects on outcome between p53 and these other variables. Axillary node-positive patients, but not those without lymph node involvement, had significantly increased risks of 66% and 105%, respectively, for relapse and death if their tumors had p53 concentrations greater than the median cutoff level (Table 3). Similarly, patients whose tumors expressed ERs had higher risk for both endpoints when their tumors were p53 positive. Formal tests for interaction showed significant p53 by ER interaction for DFS (RR = 1.18; $P = .02$) and p53 by node interaction for OS (RR = 1.22; $P = .01$). Trends somewhat suggestive of p53 by ER interaction for OS (RR = 1.12; $P = .11$), and p53 by node interactions for DFS (RR = 1.12; $P = .13$) were also shown by this analysis. For patients who received postoperative treatment, p53 also initially appeared to have value in predicting the response to chemotherapy. p53 positivity was a predictor of poor prognosis in patients to whom chemotherapy was administered with or without accompanying radiotherapy, but not in those treated with endocrine therapy alone or in combination with radiation, or in patients who received only palliative care after surgery to remove their breast tumors. Within the node-positive, ER-positive, and chemotherapy-treated subgroups, p53 status retained its ability to indicate significantly elevated hazards ratios in multivariate analysis (Table 3).

Table 3. Associations Between p53 Protein and DFS and OS in Subgroups of Patients Defined by Nodal Status, ER Status, and Postoperative Treatment

p53 Status	Disease-Free Survival			Overall Survival		
	RR	95% CI	*P*	RR	95% CI	*P*
Univariate analysis						
Node-negative patients (n = 505)						
Negative	1.00			1.00		
Positive	1.15	0.74-1.81	.54	0.95	0.63-1.55	.95
Node-positive patients (n = 415)						
Negative	1.00			1.00		
Positive	1.66	1.15-2.39	< .01	2.05	1.37-3.06	< .01
ER-negative patients (n = 129)						
Negative	1.00			1.00		
Positive	1.11	0.54-2.29	.77	1.51	0.67-3.40	.32
ER-positive patients (n = 791)						
Negative	1.00			1.00		
Positive	1.44	1.05-1.95	.02	1.45	1.05-2.00	.02
Patients not treated postoperatively (n = 274)						
Negative	1.00			1.00		
Positive	1.85	0.93-3.70	.08	0.94	0.52-1.69	.83
Patients treated with endocrine therapy ± radiation (n = 168)						
Negative	1.00			1.00		
Positive	1.44	0.72-2.88	.30	1.45	0.77-2.76	.25
Patients treated with chemotherapy ± radiation (n = 184)						
Negative	1.00			1.00		
Positive	1.74	1.03-2.93	.04	2.04	1.08-3.86	.03
Multivariate analysis*						
Node-positive patients (n = 415)						
Negative	1.00			1.00		
Positive	1.67	1.16-2.41	< .01	2.10	1.40-3.13	< .01
ER-positive patients (n = 791)						
Negative	1.00			1.00		
Positive	1.45	1.06-2.00	.02	1.50	1.09-2.07	.01
Patients treated with chemotherapy ± radiation (n = 184)						
Negative	1.00			1.00		
Positive	1.73	1.03-2.92	.04	2.04	1.08-3.86	.03

NOTE. Relative risk was estimated by the Cox proportional hazards regression model. *P* are two-sided.

*Multivariate analysis adjusted for age, tumor size, S-phase fraction, DNA ploidy, PR status, and either ER status (for estimating RR in node-positive patients) or nodal status (for estimating RR in ER-positive patients), or both (for estimating RR in patients treated with chemotherapy ± radiation).

Consistent with the results of the Cox regression analysis, Kaplan-Meier plots also showed that p53-positive status was an indicator of poorer survival in patients with node-positive or ER-positive breast cancer, or in patients who received postoperative chemotherapy (Fig 2). However, with respect to the implied ability of p53 to predict outcome only in chemotherapy-treated patients, the lack of significant p53 by chemotherapy interaction (RR = 1.01; P = .87 for DFS and RR = 1.11; P = .17 for OS) suggested that the differences observed between treatment groups might have been because of the relatively few outcome events in the endocrine therapy–treated and untreated cohorts of patients. Furthermore, after multivariate adjustment of the effects of p53 on survival of patients who received chemotherapy by including a p53 by node interaction term (RR = 1.28; P = .03 for DFS and RR = 1.33; P = .04 for OS), the impact of p53 became insignificant (RR = 1.24; P = .37 for DFS and RR = 1.49; P = .17 for OS). Together, these data indicate that the assessment of the prognostic value of p53 within subgroups given different postoperative treatments, but not controlled for node status, must be interpreted cautiously.

DISCUSSION

The relationship between p53 abnormality and breast cancer prognosis remains unclear, despite the large body of literature focused on the topic. Studies that investigated the

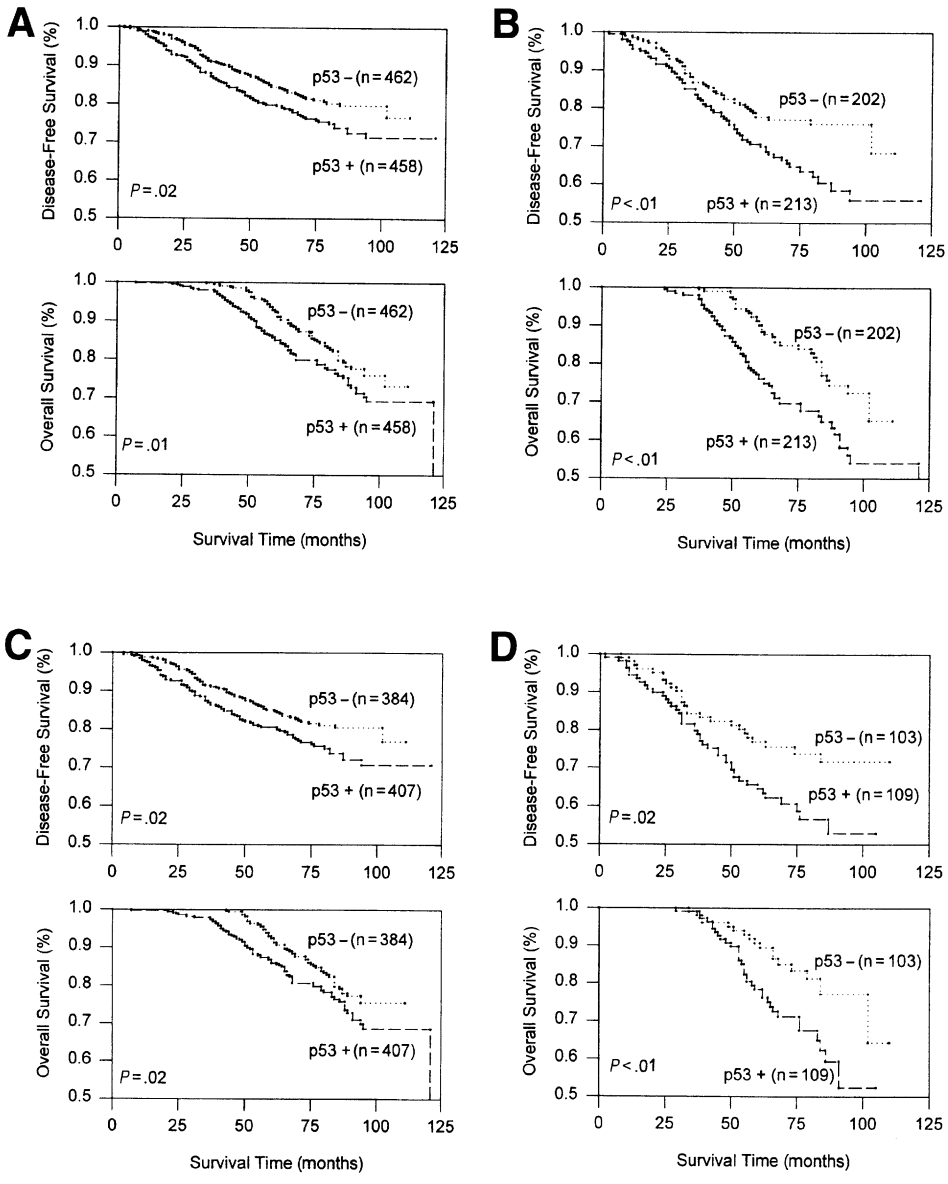

Fig 2. Kaplan-Meier DFS and OS plots of all p53-negative and p53-positive patients in the cohort (A) and within subgroups that were axillary lymph node–positive (B), ER-positive (C), and treated with chemotherapy alone or in combination with radiotherapy (D). p53 status was based on the median cutoff point. Differences between curves were determined by the log-rank test, the *P* of which are shown.

prognostic value of p53 protein accumulation assessed by immunohistochemical techniques comprise the bulk of the research, but often differ substantially with respect to procedural details and scoring criteria and may report prognostic information inferior to that obtainable by DNA sequence-based methods.[7,39] Quantitative analysis of p53 protein accumulation, implemented by densitometric image analysis of immunostained tissue[40] and by ELISA-type assays of tissue extracts[21,22,30] may offer improved reproducibility and might therefore serve as alternatives to conventional immunohistochemistry. However, the ability to assay cytosolic extracts already prepared for ER and PR assays may make ELISAs particularly suitable for p53 protein measurement in breast tumor tissues. Using a commercially available luminometric immunoassay, Borg et al[22] and de Witte et al[23] have shown the prognostic value of tumoral p53 concentration on the survival of 205 and 142 breast cancer patients, respectively. The results of our study confirm their findings in a larger patient population and validate an immunofluorometric assay developed in our laboratory[30] for clinical application.

Compared with studies in which p53 status is represented by a small number of groups (usually two), our use of a quantitative assay permitted more flexible data manipulation. In one approach that enabled full use of the data, the p53 assay results were used as continuous variables in the regression analysis. In another approach, p53 was considered a categoric variable divided into four levels by the quartiles of the p53 distribution. RRs for developing each outcome event in patients in the second, third, and fourth quartiles compared with the risks in the first quartile were also determined. Associated with the greatest information loss was the final approach, in which p53 was used as a dichotomous variable by using an arbitrarily selected cutoff. Because no a priori assumptions were made for selecting a particular cutpoint, and a "minimum P value approach"[41] was not used, the median p53 value (0.16 μg/g) was adopted in the multivariate analysis, in all regression analyses within subgroups of patients, and in the Kaplan-Meier analyses. Interestingly, this median value was very close to the optimal cutoff of 0.15 μg/g that defined 30% of the specimens to be p53 positive in the series of Borg et al.[22] Whereas de Witte et al[23] similarly classified 28% of specimens as p53 positive, the cutoff concentration used was more than 15-fold higher. Together, our results showed modest dose-response effects between p53 protein concentration and risks for relapse and death. Furthermore, multivariate analysis that used median-dichotomized p53 showed that these effects were not dependent on any other factor for which the tumors had been characterized.

Major objectives in breast cancer research in recent years have been the identification of factors capable of distinguishing node-negative patients at reduced risk for relapse who might be spared adjuvant treatment, as well as of factors predictive of adjuvant therapeutic success. In agreement with some investigators[5,6,16] but not others,[4,42,43] p53 was not a significant prognostic indicator in our series of 505 node-negative patients. p53 protein accumulation was, however, independently associated with poor outcome in the 415 node-positive patients in our study, an observation that had been reported previously.[44] The significant interaction for OS and a trend for DFS between p53 and nodes, as well as the demonstration of adequate power in the node-negative subgroup (86%), supports our observation that p53 had prognostic value only in the node-positive patients in our series. When patients were stratified by ER status, indicative of endocrine therapy responsiveness under many circumstances, p53 positivity was associated with increased risks for relapse and death only in ER-positive patients, ie, in women who might otherwise be expected to have favorable outcomes.[2,3] This finding is in contrast to that of Caleffi et al,[36] who earlier reported that p53 mutation was not of prognostic value in ER-negative or ER-positive patients. Our results, which suggest that the impact of p53 on survival may have been mediated by ER positivity, are consistent with evidence for interaction between p53 and ER, especially with respect to DFS. The relatively low relapse rate, however, among the 129 ER-negative patients made it unlikely that statistically significant differences in survival between p53-negative and ER-positive patients would have been found; the power in this subgroup was only 38%. Stratification of our study population into three treatment groups that had received endocrine treatment alone or in combination with radiation, chemotherapy with or without additional radiation, or no postsurgical medical intervention showed the assessment of p53 status to be of no apparent prognostic value in patients who received endocrine treatment or who did not receive adjuvant therapy, but to be highly significant in the survival analysis of patients treated with chemotherapeutic drugs. Because significant p53 by chemotherapy interaction could not be shown, and, although this analysis probably lacked in power, the most likely explanation for the apparent differences in effect of p53 between treatment groups lies in the small number of patients in the untreated or endocrine therapy–treated cohorts who underwent relapse. The majority of these patients would have been expected to be node negative, in contrast to patients in the subgroup who received chemotherapy. Our data suggest that it was the relationship between p53 and node status within the chemotherapy-treated subgroup that

led to the apparent prognostic value of p53 overexpression. Given that the patients in our study were not randomized to the treatments they received, we were unable to unambiguously determine whether the prognostic value of p53 protein accumulation is dependent on any particular treatment administered. Other studies, however, have provided in vitro and clinical evidence that implicated p53 as a mediator of apoptosis induced by cancer chemotherapeutic agents and radiation.[45,46] It has been proposed that tumors that lack functional p53 may be unable to activate the apoptotic cascade, which leads to treatment failure and earlier patient death. The responsiveness of breast tumors to cancer chemotherapeutic agents might, therefore, be predicted by p53 functional status, reflected by accumulation of p53 protein. Although studies of the association between p53 alteration and chemosensitivity have not yielded consistent findings,[17,47-49] sequencing of the entire p53 coding region has recently shown systemic therapy and radiotherapy to be of less therapeutic value for p53-mutated breast tumors.[50] Additional work must certainly be performed to establish p53 status as a predictive factor for adjuvant therapy in breast cancer.

The study of p53 alterations in relation to breast cancer survival and treatment response probabilities has been greatly facilitated by the close correlation between p53 gene mutational changes and accumulation of mutant p53 protein, detected in the majority of studies by immunohistochemistry. The results of this study indicate that a simple and sensitive ELISA for p53 protein may also provide prognostic information for breast cancer patients. Furthermore, we have identified the subgroups of patients with lymph node–positive and possibly ER-positive disease for whom the prognostic significance of p53 may be particularly relevant.

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