[CANCER RESEARCH 61, 2505–2512, March 15, 2001]

Influence of *TP53* Gene Alterations and c-erbB-2 Expression on the Response to Treatment with Doxorubicin in Locally Advanced Breast Cancer[1]

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ABSTRACT

***TP53* status [mutations, immunostaining, and loss of heterozygosity (LOH)], expression of c-erbB-2, bcl-2, and histological grading were correlated to the response to doxorubicin monotherapy (14 mg/m^2) administered weekly to 90 patients with locally advanced breast cancer. Mutations in the *TP53* gene, in particular those affecting or disrupting the loop domains L2 or L3 of the p53 protein, were associated with lack of response to chemotherapy ($P = 0.063$ for all mutations and $P = 0.008$ for mutations affecting L2/L3, respectively). Similarly, expression of c-erbB-2 ($P = 0.041$), a high histological grade ($P = 0.023$), and lack of expression of bcl-2 ($P = 0.018$) all predicted chemoresistance. No statistically significant association between either p53 immunostaining or *TP53* LOH and response to therapy was recorded, despite the finding that both were associated with *TP53* mutation status (p53 immunostaining, $P < 0.001$; LOH, $P = 0.021$). Lack of immunostaining for p53 despite mutation of the *TP53* gene was particularly seen in tumors harboring nonsense mutations or deletions/splices (7 of 10 negative for staining compared with 4 of 16 with missense mutations). *TP53* mutations (total/affecting L2/L3 domains) were associated with expression of c-erbB-2 ($P < 0.001$ for both), high histological grade ($P = 0.001$ and $P = 0.025$), and bcl-2 negativity ($P = 0.003$ and $P = 0.002$). *TP53* mutations, histological grade, and expression of bcl-2 (but not LOH or c-erbB-2 expression) all predicted for relapse-free as well as breast cancer-specific survival in univariate analysis (*P*s between <0.0001 and 0.0155), but only tumor grade was found to be predictive in multivariate analysis ($P = 0.01$ and $P = 0.0007$, respectively). Our data are consistent with the hypothesis that certain *TP53* mutations predict for resistance to doxorubicin in breast cancer patients. However, the observation that the majority of patients with *TP53* mutations affecting or disrupting the L2/L3 domains with LOH in addition ($n = 12$) obtained a partial response ($n = 4$) or stabilization of disease ($n = 5$) during chemotherapy suggests redundant mechanisms to compensate for loss of p53 function. Our findings are consistent with the hypothesis that other defects may act in concert with loss of p53 function, causing resistance to doxorubicin in breast cancers.**

INTRODUCTION

Chemoresistance is the main obstacle to successful therapy in cancer patients. Although many prognostic factors have been studied in different malignancies and the predictive value of steroid receptors with respect to endocrine therapy in breast cancer has been known for more than two decades (1), we are still at the beginning of learning the mechanisms of chemoresistance in human malignancies.

The merging understanding of the key role of apoptosis to the effects of chemotherapy (2) has led to a focus on defects in the apoptotic machinery as a cause of chemoresistance. Thus, *in vitro* (3) and animal *in vivo* (4) studies have found defects in the *TP53* function to predict for resistance to chemotherapy. Several studies have evaluated alterations in p53 (the protein encoded by the *TP53* gene) in relation to chemosensitivity in breast cancer as well as in other malignancies. Studies determining p53 status by immunostaining have, with a few exceptions (5, 6), failed to show a predictive value for response to different chemotherapy regimens in breast cancer patients. This is the case both in the adjuvant as well as in the "neoadjuvant" or the metastatic setting (7–15). However, not all *TP53* mutations cause increased expression of a p53 protein detectable by immunostaining (16, 17). In addition, it has been shown that detection of *TP53* alterations at the gene level is superior to immunostaining as a prognostic factor when both analyses were performed in parallel on the same group of breast cancer patients (18).

Studies evaluating the predictive value of p53 immunostaining to chemotherapy sensitivity in different solid tumors have reported conflicting results (19–24). In contrast, studies detecting *TP53* gene defects by DNA sequencing have reported mutations to predict for chemoresistance in hematological malignancies (25–28).

Several other factors, such as expression of P-glycoprotein, alterations in glutathione status, or expression of topoisomerase II (29–32), have been proposed to be involved in chemoresistance in breast cancer. However, mutations in the *TP53* gene and overexpression of c-erbB-2 (also known as *HER-2*), the human homologue of the murine *neu* oncogene (33), are the only two parameters thus far with substantial evidence of being linked to chemoresistance (15, 17, 34). Although some authors have claimed expression of c-erbB-2 to be correlated to p53 alterations (35, 36), a recent large study reported expression of c-erbB-2 to be independent of p53 expression (15).

We reported in 1996 the preliminary results from a neoadjuvant study revealing mutations in the *TP53* gene and, in particular, those affecting or disrupting the L2/L3 loop domains of the protein (codons 163–195 and 236–251, respectively), to predict for resistance to doxorubicin monotherapy in 63 patients treated for locally advanced breast cancer (17). This protocol is now closed. We here give the mature data reporting the predictive value of *TP53* mutations, p53 immunostaining, and *TP53* LOH[3] together with protein expression data on c-erbB-2, bcl-2, and histological grading to the response to doxorubicin monotherapy in 90 patients. We also evaluated the prognostic impact of each of these parameters with respect to relapse-free and breast cancer-specific survival with a median follow-up time from enrollment in the study of 62.5 months.

PATIENTS AND METHODS

Patients. Ninety-four patients suffering from locally advanced breast cancer (T_3/T_4 and/or N_2 tumors) were enrolled in a prospective study evaluating predictive factors for response to doxorubicin monotherapy. Their median age was 64 years (range, 32–88 years). One patient received treatment with cyclophosphamide in addition to doxorubicin therapy and was omitted from further analysis. Twelve patients, in addition to their locally advanced tumor, also revealed minor distant metastasis at the time of diagnosis.

Tumor Type. Of the 93 tumor samples, two tumors consisted mainly of ductal carcinoma *in situ* with minor invasive components. These two tumors

Received 1/26/00; accepted 1/31/01.

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[3] The abbreviations used are: LOH, loss of heterozygosity; CR, complete response; PR, partial response; PD, progressive disease; StbD, stable disease; CDGE, constant denaturant gel electrophoresis; TTGE, temporal temperature gel electrophoresis.

Table 1 *Characteristics of patients harboring TP53 mutations*

		Mutation						IHC				
Patient no.	Clinical response	Type	Codon	Nucleotid change	Affecting L2/L3 domain	p53 IHC[a]	LOH[b]	c-erbB-2[c]	bcl-2[d]	Histological grade	OS[e]	RFS[f]
11	PD	Stop	204	GAG-TAG	Yes	1	ND[g]	2	3	2	15.5	0
19	PD	Transition	249	AGG-GGG	Yes	1	Yes	0	0	3	20	0
26	PD	Deletion	217–221	14 bp	Yes	0	Yes	3	3	2	42.7	9.4[h]
57	PD	Transition	248	CGG-CAG	Yes	9	ND	3	3	3	9.5	0
95	PD	Splice/deletion	[i]	G-A	Yes	9	Yes	1	2	3	33.1	0
5	StbD	Splice/deletion	[i]	G-A	Yes	9	Yes	0	0	3	17.6	9.1
22	StbD	Transition	266	GGA-AGA	No	9	Yes	3	3	3	33.6	9.7
37	StbD	Transversion	176	TGC-TTC	Yes	9	Yes	0	0	3	22	12.1
51	StbD	Deletion	232–234	6 bp	Yes	6	Yes	0	6	3	60.3	60.3[j]
55	StbD	Transition	190	CCT-CTT	Yes	0	No	2	0	2	16.5	10.5
63	StbD	Transition	286	GAA-AAA	No	6	Yes	1	3	3	41.2	24.7
64	StbD	Transition	273	CGT-CAT	No	4	No	2	6	2	56.4	56.4[j]
80	StbD	Transition	175	CGC-CAC	Yes	6	Yes	3	0	2	11.3	8.7
92[k]	StbD	Transversion	168	CAC-CCC	Yes	9	Yes	2	0	3	29.4	0
98	StbD	Transition	277	TGT-TAT	No	6	Yes	0	3	2	31.9	31.9[j]
1	PR	Transition	151	CCC-TCC	No	6	Yes	0	9	3	24.1	15.8
7	PR	Transition	273	CGT-CAT	No	6	No	0	6	2	89	39.4
15	PR	Transition	163	TAC-TGC	Yes	9	Yes	0	3	3	79.1	79.1[j]
20[k]	PR	Splice/deletion	[i]	G-A	Yes	0	ND	0	6	1	18.6	0
39	PR	Transversion	237	ATG-ATT	Yes	6	Yes	0	3	2	66.8	66.8[j]
41[k]	PR	Transition	273	CGT-CAT	No	9	Yes	0	0	3	8.6	0
53	PR	Deletion	239–242	11 bp	Yes	1	No	3	2	2	34.1	22.9
75[k]	PR	Transversion	244	GGC-AGC	Yes	4	ND	2	3	1	40.2	0
100	PR	Stop	165	CAG-TAG	Yes	0	Yes	0	9	3	16.9	10.1
101	PR	Stop	136	CAA-TAA	Yes	0	Yes	3	2	2	32.9	19.9[j]
109	PR	Stop	213	CGA-TGA	Yes	0	Homozygote	0	2	3	25.5	25.5[j]

[a] p53 immunohistochemistry given as staining index (product of staining intensity and area).
[b] LOH at the *TP53* locus.
[c] Membraneous c-erbB-2 immunostaining given as staining intensity; 0, negative; 1, weak; 2, moderate; and 3, strong.
[d] bcl-2 immunostaining given as staining index.
[e] Breast cancer-specific survival.
[f] Recurrence-free survival (both given in months).
[g] ND, not determined because of lack of tissue.
[h] Patient 26 received palliative surgery after 12 weeks on first-line chemotherapy.
[i] Mutation located 1 bp upstream of exon 5, causing a 21-bp deletion of exon 5 on cDNA.
[j] Patients still alive and relapse free at the end of the observation period.
[k] Primary stage IV (limited distant metastasis).

were excluded from any further statistical analysis. Of the residual 91 invasive tumor samples, 76 were invasive ductal carcinomas, 8 were lobular carcinomas, and 7 were classified as other histological types (mucinous carcinoma, papillary carcinoma, and poorly differentiated carcinoma). Histological grading was performed using the criteria of Elston and Ellis (37).

Tissue Sampling. Prior to therapy, tissue was obtained by an open biopsy for snap-freezing (liquid nitrogen in the theater) in addition to formaldehyde fixation and paraffin embedding. Similarly, a second sample was snap-frozen in nitrogen in patients having surgery after their chemotherapy.

Treatment Protocol. The study protocol was approved by the local ethical committee, and the patients gave their informed consent. Primary treatment consisted of weekly doxorubicin (14 mg/m^2) scheduled for 16 weeks (17) with 4-weekly assessment of clinical response according to the Union International Contre Cancer criteria (38). Thus, responses were classified as a CR (complete disappearance of all tumor lesions), a PR (reduction $\geq$50% in the sum of all lesions, calculated for each as the product of the largest diameter and the one perpendicular to it), PD (increase in the diameter product of any individual tumor lesion by $\geq$25%), and StbD (anything between a PR and a PD). The terms StbD and a PR are pragmatic terms that describe a status of tumor "growth arrest" with or without a certain degree of macroscopic reduction in tumor size; the discrimination may often be arbitrary (several patients having a modest reduction in size of their tumors). On the other hand, tumors that are progressing lack sensitivity to treatment. The PD tumors are distinctive, and easily discriminated clinically from the other groups. To analyze for the predictive value of the different parameters, we compared tumors with PD to the combined group of tumors with StbD/PR/CR. Therapy was terminated immediately in case of PD.

The clinical response to doxorubicin treatment could not be classified in one patient harboring an invasive carcinoma for technical reasons (patient 25), leaving 90 patients for response evaluation to doxorubicin and 79 for follow-up analysis (excluding all patients with distant metastases at diagnosis but including patient 25).

Initially, the study protocol involved 12 weeks of postoperative chemotherapy in patients with a PR and no signs of distant metastasis (not any of the other patients). However, several patients refused such treatment because of side effects, and it was subsequently deleted from the protocol.

Median follow-up time was defined from inclusion in the study up to December 31, 1998. Deaths attributable to other causes than breast cancer were treated as censored observations. Patient identity numbers refer to all patients referred for locally advanced breast cancer (whether eligible for study or not).

Genetic Analysis. Mutations in the *TP53* gene were initially analyzed by CDGE as described elsewhere (17, 39, 40) with primers covering the evolutionary conserved regions of the gene covering exons 5–8. In addition, samples from all tumors expressing PD during chemotherapy were sequenced using cDNA. Because of the fact that one sample (tumor 26, Table 1; see also "Results") had a mutation detected by sequencing that could not be seen on CDGE, all samples were analyzed with an improved method (TTGE) as described elsewhere.[4] The method involves use of primers covering the regions (exons and introns) from exons 2 to 11. All samples with aberrantly migrating bands on TTGE were submitted to direct sequencing of PCR products with standard dideoxy sequencing reaction using the Dye Terminator Cycle Sequencing kit with AmpliTag FS on an ABI 373 sequencer (Perkin-Elmer). Samples with known *TP53* mutations in each of the exons were included in the analysis as positive controls. To further test the sensitivity of the TTGE method, direct sequencing using cDNA was also performed on tumor samples from 32 of our patients, including all of the 9 with a clinical PD (5 mutated and 4 without mutations) and an additional 23 tumors (5 of which had mutations detected by TTGE). Tumor mRNA was prepared by use of the Quick Prep Micro Purification kit (Pharmacia Biotech), followed by cDNA synthesis, with the use of the Gene Amp RNA-PCR Core kit (Perkin-Elmer) and sequencing.

[4] T. Sørlie, S. Lystad, H. Johnsen, P. Vu, A. L. Borresen-Dale, Protocol for mutation screening of the TP53 gene by Temporal Temperature Gradient Gel Electrophoresis (TTGE), submitted for publication.

Table 2 *Clinical response in relation to different parameters*

	Clinical response				
	PR (n = 34)	StbD (n = 47)	PD (n = 9)	PI[a]	PII[b]
TP53 wild-type (n = 64)	23 (36%)	37 (58%)	4 (6%)		
TP53 mutations (all, n = 26)	11 (42%)	10 (39%)	5 (19%)	0.098[c]	0.063[c]
TP53 mutations affecting L2/L3 (n = 19)	8 (42%)	6 (32%)	5 (26%)	0.014[d]	0.008[d]
TP53 mutations non-missense[e] (n = 10)	5 (50%)	2 (20%)	3 (30%)	0.028	0.025
TP53 mutations missense affecting L2/L3 (n = 9)	3 (33%)	4 (45%)	2 (22%)	0.436	0.198
Normal allele present (n = 22)	9 (41%)	11 (50%)	2 (9%)		
LOH[f] (n = 35)	12 (34%)	18 (52%)	5 (14%)	0.793	0.561
p53 IHC index <6[g] (n = 70)	27 (39%)	36 (51%)	7 (10%)		
p53 IHC index ≥6 (n = 19)	7 (37%)	10 (53%)	2 (10%)	0.990	0.946
c-erbB-2 IHC intensity, 0 or 1+[h] (n = 72)	27 (37%)	40 (56%)	5 (7%)		
c-erbB-2 IHC intensity, 2+ or 3+ (n = 17)	7 (41%)	6 (35%)	4 (24%)	0.087	0.041
bcl-2 IHC index <6[i] (n = 46)	17 (37%)	21 (46%)	8 (17%)		
bcl-2 IHC index ≥6 (n = 43)	17 (40%)	25 (58%)	1 (2%)	0.058	0.018
GI[j] (n = 23)	11 (48%)	11 (48%)	1 (4%)		
GII (n = 42)	15 (36%)	25 (59%)	2 (5%)	0.075[k]	0.023[k]
GIII (n = 25)	8 (32%)	11 (44%)	6 (24%)		

[a] *p*s in regard to clinical response comparing PR, StbD, PD.
[b] *p*s in regard to clinical response comparing PR + StbD *versus* PD.
[c] Wild-type *versus* all *TP53* mutations.
[d] *TP53* mutations affecting the L2/L3 domains *versus* wild-type plus *TP53* mutations not affecting the L2/L3 domains.
[e] *TP53* mutations including nonsense, splice, deletion, frame shift, and in-frame mutations.
[f] LOH, analysed for 60 patients; informative for 57 patients.
[g] p53 immunohistochemistry (IHC) given as staining index; ≥6 considered positive.
[h] Membraneous c-erbB-2 immunostaining given as staining intensity; 2+ or 3+ considered positive.
[i] bcl-2 immunohistochemistry given as staining index.
[j] Histological grade I–III.
[k] *P* comparing the distribution of histological grade in the three response group (left) and in PD versus PR + StbD (right).

Normal tissue to assess LOH of the *TP53* gene was available from 60 patients (blood samples from 51 patients and paraffin-embedded normal tissue from 9 patients). Of these, 57 were found to be informative (heterozygotes) for one or both of the two markers used, one variable number tandem repeat in intron 1 (41) and a CA repeat in the nontranslated 3′-end of the gene (42). Fluorescent end-labeled primers in the PCR were used, and the PCR products were analyzed on an ABI PRISM 310 Genetic Analyzer. The data were analyzed automatically by comparing normal and tumor tissue allele peak height ratios. A sample was scored as having LOH when a reduction in peak height of at least 25% occurred.

Immunohistochemistry. Immunohistochemical examination of p53 protein expression was performed on formaldehyde-fixed and paraffin-embedded material using the avidin-biotin complex method with the antibody DO-7 (Dako, Copenhagen, Denmark), dilution 1:100, with incubation at room temperature for 1 h after microwave pretreatment of the slides. Antigen localization was achieved by the peroxidase method. Negative controls were incubated as described, omitting the primary antibody, and no positive staining was observed. The examiner was blinded to the treatment outcome. Nuclear staining was recorded using a semiquantitative and subjective grading, considering both the intensity of staining and the proportion of tumor cells showing an unequivocal positive reaction. Intensity was recorded as 0 (no staining) to 3 (strong staining), and the amount of positive cells was recorded as: 0 (no tumor cells positive); 1 (positive staining in <10% of the tumor cells); 2 (positive staining in 10–50% of the tumor cells); or 3 (positive staining in >50% of the tumor cells). A staining index was calculated as the product of staining intensity and staining area.

The c-erbB-2 status was evaluated immunohistochemically on frozen tissue sections using the monoclonal antibody OP15 (Oncogene Science, Manhasset, NY) as described previously (43). In accordance with previous studies, membrane staining was graded by intensity (0–3), rating a membrane staining of 2 and 3 as positive.

bcl-2 immunohistochemical staining was performed on formaldehyde-fixed, paraffin-embedded tissue according to an indirect avidin-biotin complex procedure using the antibody 124 obtained from Dako in dilution 1:50. After microwave pretreatment, slides were incubated for 3 h at room temperature, followed by incubation overnight at 4°C. The complex was detected with the peroxidase method. Negative controls were incubated as described, omitting the primary antibody, and no staining was recorded. Normal lymphocytes in the peritumoral tissue were present in several cases and presented as a positive internal control for bcl-2 immunostaining. Cytoplasmic staining was recorded as described above for p53 nuclear staining.

Statistical Analysis. Mutations of the *TP53* gene, LOH for the *TP53* gene, histological grading, and the different immunohistochemical stainings were correlated to response to chemotherapy and to each other with use of the χ^2 method. Comparison between the fractions of patients with *TP53* missense and nonsense/splice/deletion mutations that did not show immunoreactivity was performed with the Fisher exact test (because of the small number). Because the L2 and L3 domains of the p53 protein play a critical role in DNA binding (44) and mutations affecting these domains predict a poor prognosis in breast cancer (39), we analyzed separately the predictive value of mutations affecting these domains (total and missense), as well as "non-missense" mutations (deletions/splice and stop codons) in the total material.

Relapse-free and breast cancer-specific survival were analyzed by the log-rank test. Factors found to predict outcome ($P < 0.05$) in univariate analysis (histological grade, *TP53* mutations, and expression of bcl-2) were further analyzed by the Cox multivariate proportional hazards model.

RESULTS

Twenty-six of 91 patients (28.6%) were found to harbor mutations of the *TP53* gene in their tumors using TTGE, followed by sequencing of genomic DNA (Table 1). The 32 samples sequenced from cDNA gave the same results.

Of the 26 mutations, 16 were missense mutations (4 transversions and 12 transitions), 6 were deletions/splices, and 4 were nonsense mutations. Nineteen of these mutations (9 of the missense and all of the nonsense and deletions/splice mutations) affected or disrupted the L2 and/or L3 domains of the p53 protein. The same *TP53* mutation profile was obtained after treatment in all but one patient (no. 75, Table 1), whose sample taken after treatment did not give any conclusive result for technical reasons.

The clinical response to therapy could not be determined in one patient. Table 2 shows the clinical responses to doxorubicin therapy classified according to the Union International Contre Cancer criteria (38) and stratified with respect to predictive factors in the remaining 90 patients. Detailed characteristics of patients with primary PD are presented in Table 3. Although *TP53* mutations in total were nonsignificantly associated with PD ($P = 0.063$), *TP53* mutations affecting or disrupting the L2/L3 domains (n = 19) and non-missense mutations (n = 10; including nonsense, splice, deletion, and frame shift

Table 3 *Characteristics of patients with primary PD*

Patient no.	*TP53* gene mutation	Affecting L2/L3 domain	LOH	p53 IHC[a]	IHC		Histological grade	Outcome[d]
					c-erbB-2[b]	bcl-2[c]		
11	Yes	Yes	ND[e]	1	2	3	2	D15
19	Yes	Yes	Yes	1	0	0	3	D20
26	Yes	Yes	Yes	0	3	3	2	D43
48	No	No	Yes	0	1	0	3	D7
57	Yes	Yes	ND	9	3	3	3	D10
65	No	No	No	0	0	1	3	D23
95	Yes	Yes	Yes	9	1	2	3	R33
104	No	No	No[f]	0	2	3	3	F31
112	No	No	Yes	1	0	9	1	F22

[a] p53 immunohistochemistry given as staining index.
[b] Membraneous c-erbB-2 immunostaining given as staining intensity.
[c] bcl-2 immunohistochemistry given as staining index.
[d] F "X", disease free after "X" months of follow-up; D "X", breast cancer death after "X" months; R "X", alive, but suffering a relapse after "X" months.
[e] ND, not determined.
[f] Patient 104 had LOH in the analyses done on tumor tissue after chemotherapy.

mutations) predicted for resistance to doxorubicin ($P = 0.008$ and $P = 0.025$, respectively). Although a significant statistical correlation between p53 immunostaining and *TP53* mutations was found (Table 4; $P < 0.001$), p53 immunostaining did not predict resistance to therapy (Table 2).

Similar to p53 immunostaining, *TP53* LOH correlated to *TP53* mutation status (Table 4; $P = 0.021$ and $P = 0.031$) but did not predict chemoresistance (Table 2). Among 14 patients harboring *TP53* mutations affecting the L2/L3 domains of the p53 protein for whom allelic imbalance could be determined, only one of five and one of six of the patients who obtained a PR or a StbD, respectively, and zero of three with PD had retained the normal *TP53* allele (Table 5).

TP53 mutation status and LOH were also evaluated in tumor tissue obtained after primary chemotherapy in 78 and 52 patients, respectively. The *TP53* status did not change in any of these tumors but was unavailable in one sample taken after treatment (no. 75, Table 1) because of technical reasons. Eight tumors expressing LOH in the first biopsy were found to express the normal allele in the second sample. One tumor expressing the normal allele in the first biopsy demonstrated LOH in the second sample.

In addition to *TP53* mutations, high histological grade ($P = 0.023$), expression of c-erbB-2 ($P = 0.041$), and lack of expression of bcl-2 ($P = 0.018$) all predicted for primary resistance to therapy.

Table 4 summarizes the correlations between the different parameters. As mentioned above, we observed a strong statistical correlation between *TP53* mutations and expression of the p53 protein evaluated by immunostaining. However, not all patients with *TP53* mutations expressed immunostaining of the p53 protein. Regarding a p53 staining index of 6 or above as positive, 11 of 26 mutated tumors did not stain (Table 1); of those, 9 had a staining index of 0 or 1. Lack of staining was preferentially seen in tumors harboring a nonsense mutation or a splice/deletion; thus, 7 of 10 of these tumors did not stain, compared with 4 of 16 tumors harboring a missense mutation ($P = 0.032$). Thus, three of the five tumors that progressed on therapy and harbored a *TP53* mutation were negative for p53 immunostaining (Table 1). Although a positive immunostaining for c-erbB-2 was associated with *TP53* mutations ($P < 0.001$), we found no statistical significant association between immunostaining for p53 and c-erbB-2. Interestingly, of the four patients with PD who also showed positive immunostaining for c-erbB-2, three were found to harbor a *TP53* mutation affecting the L2/L3 domains (Table 3). Expression of c-erbB-2 was also associated with lack of expression of bcl-2 ($P = 0.001$) but revealed no association to any of the other parameters examined. Lack of expression of bcl-2 was also associated with *TP53* mutations ($P = 0.003$).

Relapse-free and breast cancer-specific survival among patients with no evidence of distant metastasis at diagnosis ($n = 79$) is depicted in Figs. 1 and 2. *TP53* mutations (total and those affecting or disrupting the L2/L3 domains), a high histological grade, and lack of bcl-2 expression (but not *TP53* LOH) all predicted for a poor relapse-free and breast cancer-specific survival by univariate analysis. Among the patients without distant metastasis who obtained a PR on chemotherapy, no difference in relapse-free or overall survival between those receiving 12 weeks of chemotherapy ($n = 19$) *versus* fewer than 12 courses ($n = 3$) *versus* no chemotherapy ($n = 10$) after surgery was observed (data not shown). Multivariate analysis was performed including bcl-2 expression, histological grade, and *TP53* mutations. This revealed only histological grade to be predictive for relapse-free ($P = 0.01$) as well as breast cancer-specific ($P = 0.0007$) survival,

Table 4 *Correlations between different parameters evaluated given as Ps*

	LOH	p53 IHC[a]		bcl-2 IHC[b]		c-erbB-2 IHC[c] intensity	Tumor grade	Tumor stage
		Intensity	Index	Intensity	Index			
TP53 mutations[d]	0.021	<0.001	<0.001	0.003	0.003	<0.001	0.001	0.219
TP53 mutations[e]	0.056	<0.001	<0.001	0.005	0.005	0.001	0.010	0.446
TP53 mutations[f]	0.031	0.010	0.002	0.002	0.002	<0.001	0.025	0.552
LOH		0.072	0.016	0.230	0.230	0.524	0.133	0.073
p53 IHC intensity				0.111		0.053	0.070	0.090
p53 IHC index					0.111	0.706	0.001	0.146
bcl-2 IHC intensity						0.001	0.117	0.807
bcl-2 IHC index						0.001	0.117	0.807
c-erbB-2 intensity							0.326	0.181
Tumor grade								0.548

[a] p53 immunohistochemistry given as staining intensity and staining index.
[b] bcl-2 immunohistochemistry given as staining intensity and staining index.
[c] Membraneous c-erbB-2 immunostaining given as staining intensity.
[d] *TP53* gene mutations grouped as wild-type *versus* all mutations.
[e] *TP53* gene mutations grouped as mutations affecting the L2 or L3 domains *versus* mutations not affecting the L2 or L3 domains *versus* wild-type (three separate groups).
[f] *TP53* gene mutations grouped as mutations affecting L2 or L3 domains *versus* wild-type plus *TP53* gene mutations not affecting the L2 or L3 domains (two separate groups).

Table 5 *LOH in relation to response to therapy*

	Clinical response		
	PR (n = 21)	StbD (n = 29)	PD (n = 7)
TP53 gene mutations aff. L2/L3 and LOH[a]	4/5	5/6	3/3
TP53 gene mutations not aff. L2/L3 and LOH[b]	2/3	3/4	0/0
LOH without *TP53* gene mutation[c]	6/13	10/19	2/4

[a] *TP53* gene mutations affecting L2 or L3 domains with concomitant LOH.
[b] *TP53* mutations not affecting L2 or L3 domains with concomitant LOH.
[c] LOH without *TP53* mutation.

independent of whether *TP53* status was evaluated as mutated *versus* wild type or whether mutations affecting or disrupting the L2/L3 domains were handled as a separate group.

DISCUSSION

Recently, several papers focused on the sensitivity of different methods to detect *TP53* mutations (45, 46). In the previous study of a subset of samples (17), one mutation was overlooked by CDGE but detected by direct sequencing. This mutation was a deletion of codon 217–221 that could not be detected with the primers used at that time. As a consequence, the primers were redesigned, and an improved method, TTGE, was introduced covering all coding exons. By this improved method, the previously overlooked mutation, as well as each of the mutations found by CDGE, was detected. Sequencing cDNA from a subset of 32 tumors revealed no additional mutation not identified by the TTGE, confirming the validity of this method.

The literature regarding the predictive value of p53 alterations to chemoresistance in breast cancer is conflicting, with a substantial number of papers reporting negative findings (7–15, 47, 48). However, all these studies evaluated p53 status by immunohistochemistry. Many antibodies do not discriminate between normal and mutated proteins. In addition, ~30% of the tumors harboring *TP53* mutations are recorded as negative by immunostaining (16). In contrast, after our report in 1996 (17), we are aware of another four studies (49–52) reporting a correlation between *TP53* mutations and therapy resistance in breast cancer. Although the study of Berns *et al.* (53) reported a significant correlation between *TP53* mutations and lack of response to tamoxifen in 202 patients, each of these studies contained a smaller number of patients evaluable for resistance to chemotherapy compared with our preliminary report (63 patients). Although the results of these studies in general concurred with our original observations, the results are not directly comparable with ours. With the exception of one treatment arm in the study by Kandioler-Eckerberger *et al.* (49) who used paclitaxel monotherapy, these studies all used combined drug regimens, making a direct evaluation of possible mechanisms of resistance to individual drugs difficult. Two of the studies also differed with respect to treatment principles or statistical analysis. Formenti *et al.* (51) administered chemotherapy and radiotherapy in concert, thus making it difficult to interpret which treatment modality *TP53* status predicted response to. Lizard Nacol *et al.* (50) combined *TP53* mutation status and LOH in their statistical analysis stating that "alterations in the *TP53* gene" predict for chemoresistance. Although the study of Berns *et al.* (53) could not observe a statistically significant correlation between *TP53* mutation and response to combined chemotherapy, the number of patients evaluated in this subgroup was only 41.

This report extends and strengthens our previous finding regarding the predictive value of *TP53* mutations affecting or disrupting the L2 and/or L3 domains of the p53 protein with respect to primary resistance to doxorubicin therapy in breast cancer. To our knowledge, this is the largest study thus far directly relating response to chemotherapy to *TP53* alterations in breast cancer patients.

In the present study, we show that p53 immunostaining did not predict for chemoresistance, whereas *TP53* mutation status did. A large number of tumors harboring *TP53* mutations showed absent or minimal immunoreactivity, despite the fact that we used an antibody directed against epitopes located in the NH_2-terminal domain of the protein. Lack of immunostaining was particularly frequent in tumors with nonsense mutations and among those who progressed on therapy. This selectivity may explain the lack of predictive value of p53 evaluated by immunostaining to treatment outcome, despite a strong statistical correlation between p53 staining and *TP53* mutation status.

Mutations in the *TP53* gene may have different biological effects. Although mutations often are associated with loss of function, some mutated p53 proteins still bind to DNA to a variable degree (52). Other mutant proteins may exert a dominant-negative effect by driving normal p53 into a mutant conformation (54), exert a "gain-of-function" by binding to alternative DNA sites (55), or act by mechanisms possibly independent of DNA binding (56, 57). Although these biological effects have been observed for many mutants *in vitro*, their importance as mechanisms to chemoresistance *in vivo* is incom-

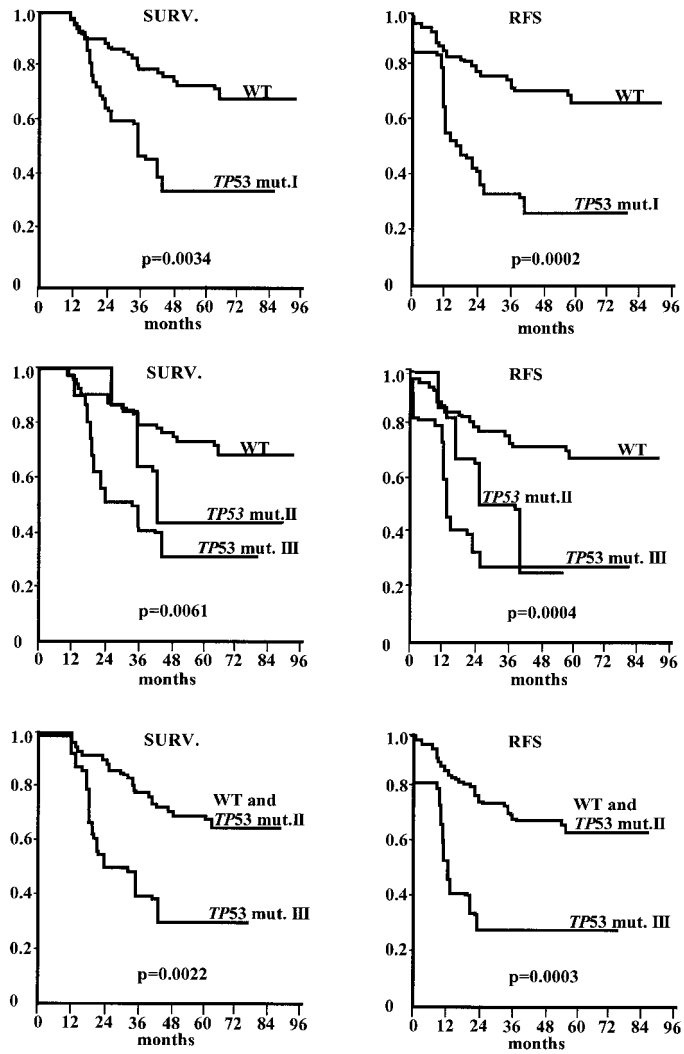

Fig. 1. Survival (*left*) and relapse-free survival (*right*) of patients without evidence of distant metastasis at diagnosis stratified according to *TP53* mutations. WT, wildtype; *TP53* mut.I, all *TP53* mutations, *TP53* mut.II, *TP53* mutations not affecting the L2/L3 domains, *TP53* mut.III, *TP53* mutations affecting the L2/L3 domains. Deaths due to causes other than breast cancer are treated as censored observations.

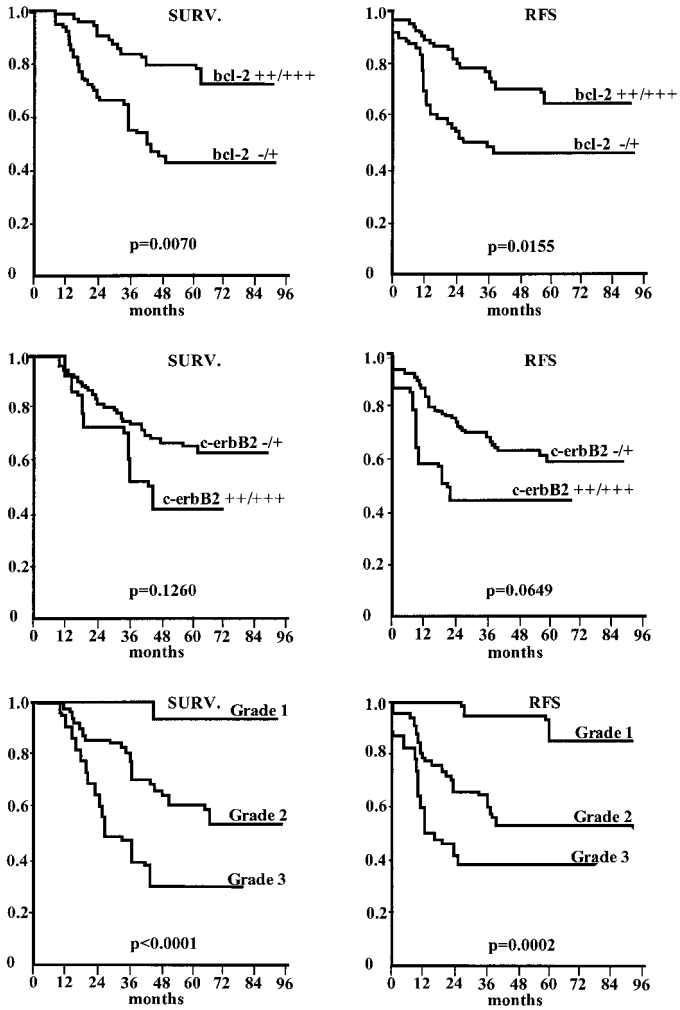

Fig. 2. Survival (*left*) and relapse-free survival (*right*) of patients without evidence of distant metastasis at diagnosis stratified according to bcl-2 expression, c-erBb-2 expression and histologic grade. bcl-2 −/+, bcl-2 staining index negative or less than 6, bcl-2 ++/+++, bcl-2 staining index 6 or more, c-erBb2 −/+, negative or weak membranous c-erBb2 staining intensity, c-erBb2 ++/+++, moderate to strong membranous c-erBb2 staining intensity. Deaths due to causes other than breast cancer are treated as censored observations.

pletely understood. The L2 and L3 domains together chelate a zinc atom and are critical to DNA binding (44). Interestingly, another study showed that mutations affecting these domains were associated with a particularly poor prognosis in breast cancer (39, 58).

Although our data suggest that mutations in these domains also may be of particular importance predicting resistance to doxorubicin therapy, this hypothesis is based on a limited number of observations and should be confirmed in other studies. The possibility also exists that another grouping of mutants based on functional status may further improve the predictive value of certain *TP53* mutations. Thus, in addition to demonstrating the importance of gene sequencing instead of immunostaining to evaluate the biological effects of *TP53* alterations, our data underline the importance of reporting individual mutations in detail for comparison between different studies and combined analysis.

Considering definitions of the L2 and L3 domains, there may be some inconsistency as to whether the border codons (L2 nos. 163 and 194; L3 nos. 236 and 251) should be included (44). Here, we used a conservative approach including these nucleotides in the domains. To exclude them would mean to change the classification of one tumor (no. 15, Table 1). This tumor harbored a point mutation at codon 163 and was classified as a mutation affecting or disrupting the L2/L3 domains. It responded to therapy (PR), and the patient is currently relapse free 79.1 months after diagnosis. To classify this tumor as one not affecting or disturbing the L2/L3 domains would strengthen our hypothesis with respect to response as well as survival.

Similar to what we observed here, a correlation between *TP53* mutations affecting the DNA-binding domains of the p53 protein and LOH has been reported in head and neck cancers (59). Thus, an interesting question is whether concomitant *TP53* LOH plays a key role in the mechanism of chemoresistance. Although our data should be interpreted with caution because of the limited number of events and the heterogeneity of the *TP53* mutation spectrum, it is tempting to speculate about how these mutations fit in with current hypotheses regarding their biological effects. All our patients harboring *TP53* mutations affecting or disrupting the L2/L3 domain with PD and for whom information about allelic imbalance was available also had *TP53* LOH ($n = 3$). However, this was also the case for 9 of 11 patients with L2/L3-affecting mutations who obtained a PR or StbD during doxorubicin therapy (Table 5). Although the possibility exists that preservation of a normal *TP53* allele could be a rescue mechanism in some patients with *TP53* mutations, it does not seem to be a major discriminator for response to therapy in this patient group. On the other hand, our findings are consistent with the hypothesis that redundant mechanisms may exist, because many patients with loss of *TP53* function (mutation with LOH) still may respond to initial chemotherapy. Although one patient (no. 11) with PD had a stop codon at 204, another three patients (nos. 100, 101, and 109) with stop codons in the 136–213 area (two with documented LOH) all obtained a PR to doxorubicin therapy, and one of them is still relapse free. Our results do not exclude the possibility that some of the missense mutations may cause a gain-of-function p53 protein, but the results are more consistent with the hypothesis that other gene alterations may operate in concert with *TP53* mutations and LOH to cause primary chemoresistance to doxorubicin.

Although the same *TP53* mutation profile was revealed in all but one tumor investigated before and after chemotherapy, LOH status changed in nine tumors. Interestingly, the one tumor showing LOH in the second sample but not in the first sample (no. 104, Table 3) had a particular clinical course. This tumor showed some initial regression but then started to regrow after 12 weeks on chemotherapy, suggesting that there could have been a selection of resistant clones during therapy. Considering the eight tumors that revealed LOH prior to chemotherapy but not afterward, one possible explanation could be a reduced number of tumor cells compared with normal tissue in some of these samples. However, this change occurred in patients with StbD ($n = 3$) and PD ($n = 1$) in addition to those having a PR ($n = 4$).

In addition to *TP53* mutations, we also found expression of c-erbB-2 to predict for chemoresistance. However, although we were able to reproduce the data of Thor *et al.* (15) showing lack of correlation between expression of c-erbB-2 and p53 detected by immunostaining, comparing c-erbB-2 expression with *TP53* mutational status revealed a different picture. Thus, our finding of a strong correlation between *TP*53 mutations and c-erbB-2 expression but not between p53 immunostaining and c-erbB-2 underlines the lack of specificity when using immunostaining as a surrogate marker for *TP53* mutations. Notably, among our patients with primary PD, there was only one tumor of four with positive c-erbB-2 immunostaining that did not harbor a *TP53* mutation affecting the L2/L3 domains. Although our observations do not allow any conclusive statement regarding the predictive value of *TP53* mutations *versus* c-erbB-2 staining, our data question the hypothesis of an independent role for *TP53* and c-erbB-2 with respect to chemoresistance (15). Thus, it is mandatory for future studies evaluating the predictive value of c-

erbB-2 expression to chemoresistance to also include proper sequence analysis of the *TP53* gene. Notably, response rates to herceptin (humanized specific antibody to c-erbB-2) in patients with high expression of c-erbB-2 are in the range of 10–25% only (60, 61); one explanation could be that *TP53* mutations may predict for resistance to this type of therapy as well.

Our findings that expression of bcl-2 was associated with a better response to chemotherapy may be somewhat surprising, associating this gene with antiapoptotic activity (62). The finding is consistent with the results of Makris *et al.* (13) but contrasts those of Bonetti *et al.* (9), who found bcl-2 expression to predict for resistance. However, these studies used different treatment regimens than the present. Other studies have shown that the biological effect of bcl-2 depends on its interaction with other proteins of this gene family, such as bax (63, 64). bcl-2 has been shown to be down-regulated by wild-type p53 in breast cancer cells (65) and probably had little effect on chemoresistance on its own in our patients.

Although *TP53* mutations affecting or disrupting the L2/L3 domains (but not p53 immunostaining), histological grading, and expression of c-erbB-2 all predicted for a poor relapse-free as well as breast cancer-specific survival in patients with no sign of distant metastasis at diagnosis in univariate analysis, histological grade was the only factor predicting relapse-free as well as breast cancer-specific survival in multivariate analysis. Notably, time to relapse and death not only depends on treatment effect but also on tumor growth rate and metastatic potential, which may be controlled by other factors than those regulating apoptosis.

In summary, our data confirm that *TP53* mutations affecting certain domains of the p53 protein are associated with primary resistance to doxorubicin therapy in breast cancer patients. They may further explain the conflicting results in the literature with respect to the predictive value of *TP53* mutations *versus* p53 protein expression and confirm a strong association between *TP53* mutations and expression of c-erbB-2. The finding that different *TP53* mutations concomitant with LOH were present in patients with PD but also in patients responding to drug therapy suggests that redundant mechanisms may compensate for loss of *TP53* function. This is consistent with the hypothesis that other gene defects in addition to loss of p53 function may occur in breast cancers resistant to doxorubicin therapy.

ACKNOWLEDGMENTS

We thank the technical assistance of D. Ekse, B. Nordanger, H. Berntsen, B. Leirvaag, L-M. Jørgensen, and P. Vu. We also thank R. Skjærven for the statistical support needed to perform the survival analysis.

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Cancer Res 2001;61:2505-2512.