[CANCER RESEARCH 60, 2155–2162, April 15, 2000]

Complete Sequencing of *TP53* Predicts Poor Response to Systemic Therapy of Advanced Breast Cancer[1]

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ABSTRACT

***TP53* has been implicated in regulation of the cell cycle, DNA repair, and apoptosis. We studied, in primary breast tumors through direct cDNA sequencing of exons 2–11, whether *TP53* gene mutations can predict response in patients with advanced disease to either first-line tamoxifen therapy (202 patients, of whom 55% responded) or up-front (poly)chemotherapy (41 patients, of whom 46% responded). *TP53* mutations were detected in 90 of 243 (37%) tumors, and one-fourth of these mutations resulted in a premature termination of the protein. The mutations were observed in 32% (65 of 202) of the primary tumors of tamoxifen-treated patients and in 61% (25 of 41) of the primary tumors of the chemotherapy patients. *TP53* mutation was significantly associated with a poor response to tamoxifen [31% *versus* 66%; odds ratio (OR), 0.22; 95% confidence interval (CI), 0.12–0.42; $P < 0.0001$]. Patients with *TP53* gene mutations in codons that directly contact DNA or with mutations in the zinc-binding domain loop L3 showed the lowest response to tamoxifen (18% and 15% response rates, respectively). *TP53* mutations were related, although not significantly, to a poor response to up-front chemotherapy (36% *versus* 63%; OR, 0.34; 95% CI, 0.09–1.24). In multivariate analysis for response including the classical parameters age and menopausal status, disease-free interval, dominant site of relapse, and levels of estrogen receptor and progesterone receptor, *TP53* mutation was a significant predictor of poor response in the tamoxifen-treated group (OR, 0.29; 95% CI, 0.13–0.63; $P = 0.0014$). *TP53*-mutated and estrogen receptor-negative (<10 fmol/mg protein) tumors appeared to be the most resistant phenotype. Interestingly, the response of patients with *TP53* mutations to chemotherapy after tamoxifen was not worse than that of patients without these mutations (50% *versus* 42%; OR, 1.35, nonsignificant). The median progression-free survival after systemic treatment was shorter for patients with a *TP53* mutation than for patients with wild-type *TP53* (6.6 and 0.6 months less for tamoxifen and up-front chemotherapy, respectively). In conclusion, *TP53* gene mutation of the primary tumor is helpful in predicting the response of patients with metastatic breast disease to tamoxifen therapy. The type of mutation and its biological function should be considered in the analyses of the predictive value of TP53.**

INTRODUCTION

TP53, a tumor suppressor gene, has been shown to have prognostic value in patients with breast cancer. A few studies with conflicting results on its predictive value have been published. Endocrine treatment and chemotherapy improve the survival of women with (primary or advanced) breast cancer. The antiestrogen tamoxifen is one of the major compounds used for the endocrine treatment of breast cancer. It is effective in about one-third of all patients with metastatic disease and in about 50% of those breast cancer patients that are ER[3] positive. Like chemotherapeutic agents, tamoxifen may cause DNA damage (1). TP53, by regulating response to DNA damage, is a key element in DNA repair. This tumor suppressor gene has also been implicated in the regulation of normal cell growth and division, gene transcription, genomic stability, apoptosis, and senescence. Mutations in the *TP53* gene (also known as P53) are the most frequent genetic changes found in human breast cancer. Mutation is often accompanied by deletion of the second allele, resulting in the elimination of wild-type TP53 activity. P53 protein accumulation, which often results from *TP53* gene mutation, can be measured with immunological techniques, *i.e.*, IHC or LIA/ELISAs on tumor extracts.

In two studies on patients with metastatic breast cancer (including 92 and 205 patients, respectively), no significant relation between immunohistochemically assessed P53 expression and response to endocrine treatment was observed (2, 3), whereas in another study (on 17 patients), P53 overexpression was associated with a poor response to endocrine therapy (4). Recently, we have shown a significant relation between P53 accumulation, as measured by a LIA, and poor response to tamoxifen therapy in a series of 401 patients (5). Moreover, the existing data on TP53 do not clarify its capability to predict resistance to chemotherapy for advanced or recurrent breast cancer (6–9). Furthermore, the data on its predictive role in the adjuvant setting are conflicting as well (10–16). Studies on the predictive value of TP53 are hampered by a number of methodological issues including immunological *versus* molecular biological analyses, use of different cutoff levels in the immunohistochemical analyses, or selection of patient groups.

About 20% of the *TP53* gene mutations do not result in p53 protein accumulation, whereas, on the other hand, p53 accumulation may also occur without a gene mutation. Therefore we have studied *TP53* gene mutations in a relatively large series of 243 patients with advanced disease. Analysis of the various mutations allowed us to investigate the biological significance of particular mutations, which may eventually aid in selecting those aberrations that could be of predictive value in breast cancer. We show that *TP53* gene mutations predict a poor response to first-line tamoxifen therapy and probably to chemotherapy in advanced breast cancer. In an exploratory subset analysis, we also observed that mutations in one zinc-binding domain, *i.e.*, loop L3, or in codons that directly contact DNA were related with an even poorer response to tamoxifen.

Received 10/12/99; accepted 2/17/00.

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[1] Supported in part by Grant DDHK 96-1234 from the Dutch Cancer Society.

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[3] The abbreviations used are: ER, estrogen receptor; PgR, progesterone receptor; OR, odds ratio, CI, confidence interval; LIA, luminometric assay; CMF, cyclophosphamide, methotrexate, and 5-fluorouracil; CAF, cyclophosphamide, Adriamycin, and 5-fluorouracil; CR, complete remission; PR, partial remission; PFS, progression-free survival; IHC, immunohistochemistry.

PATIENTS AND METHODS

Assays of Cell Biological Factors

Sequence-based Analysis of *TP53*. cDNA-based sequencing was used essentially as described by Bergh *et al.* (14), Sjögren *et al.* (17), Kressner *et al.* (19) and Falette *et al.* (20). Frozen untreated primary tumor samples were verified histologically for the presence of tumor cells, and only those samples containing more than 20% tumor cells were included in our study. mRNA was prepared from the frozen samples under stringent conditions to avoid degradation and contamination, and cDNA was generated. TP53 was amplified from the tumor cDNA by PCR using four sets of overlapping primers (one primer in each pair was biotin-labeled) to cover the complete protein coding region (exons 2–11) of the *TP53* cDNA. Solid-phase sequencing was carried out using Autoload solid-phase sequencing combs and T7 DNA polymerase (Amersham Pharmacia Biotech). Samples were analyzed on an automated laser fluorescence DNA sequencer (Amersham Pharmacia Biotech). Sequence gels and sequences were evaluated with Sequence Evaluator and Mutation Analyzer software (Amersham Pharmacia Biotech). All mutations were confirmed by reamplifying the relevant cDNAs and sequencing the new PCR products.

Assays of ER and PgR. ER and PgR levels were determined in cytoplasmic extracts (cytosols) prepared routinely according to procedures recommended by the European Organization for Research and Treatment of Cancer Breast Cancer Cooperative Group with ligand binding assays or enzyme immunoassays (ER-EIA and PgR-EIA; Abbott Laboratories, IL), as described previously (20).

Tumor Samples and Patients

To evaluate the clinical significance of *TP53* gene alterations in advanced disease, 265 primary breast tumor specimens were analyzed. Sequence data (protein coding exons 2–11 of the *TP53* gene) were successfully obtained from 243 tumors (92%; Table 1). In five cases, the sequence analysis was incomplete, although a mutation was observed; these samples were included. For nine cases, the sequence analysis was incomplete, but no gene mutation was observed; these nine samples were not included because mutations could not be excluded. In 13 cases (5%), no PCR product could be generated (in two separate experiments). Consequently, the analysis with respect to *TP53* mutation and response to systemic therapy was performed on 243 breast cancer patients who developed recurrent disease. The patient group in the present study is not identical to the population described previously, *i.e.*, 165 patients were described previously (5). A total of 202 patients (83%) received first-line tamoxifen therapy, whereas 41 (17%) patients, especially younger women with ER-negative tumors, received first-line chemotherapy for advanced disease.

First-Line Tamoxifen Treatment. Between 1979 and 1991, 202 patients underwent resection of their primary tumors (breast-conserving therapy, 67 patients; modified mastectomy, 132 patients; and biopsy, 3 patients) and were selected according to the criteria described previously (21). In the first-line tamoxifen-treated group, 20 patients were stage I, 110 patients were stage II, 45 patients were stage III, and 22 patients were stage IV. With respect to stage, data on five patients are unknown. Twenty-seven patients (13%) received systemic adjuvant chemotherapy (CMF, 18 patients; CAF, 9 patients). Twenty-three patients (11%) were diagnosed with metastatic disease (M_1) at the time of primary surgery. All patients were treated with 40 mg of tamoxifen daily as a first-line hormonal therapy after relapse. All patients were tamoxifen naïve and had not received prior chemotherapy for advanced disease. The median age of these 202 patients at the start of first-line tamoxifen therapy was 62 years (range, 28–85 years). Additional characteristics are listed in Table 2. The median follow-up of patients who are still alive is 81 months (range, 51–123 months) from primary surgery and 44 months (range, 8–80 months) from the start of tamoxifen treatment. A total of 163 patients have died (median survival time, 20 months). Tumor progression occurred in most patients (190 of 202 patients, 94%) during follow-up. Median time to progression was 7.5 months. After tumor progression on first-line tamoxifen treatment, 70% of the patients were treated with one or more additional endocrine agents (mostly high-dose progestins), whereas 117 patients were subsequently treated with one or more regimens of chemotherapy after development of hormone resistance.

First-Line (Up-Front) Chemotherapy. Forty-one patients underwent resection of their primary tumors (breast-conserving lumpectomy, 22 patients; modified mastectomy, 19 patients) between 1983 and 1991. In the up-front chemotherapy group, 6 patients were stage I, 22 patients were stage II, 9 patients were stage III, and 4 patients were stage IV. After primary surgery, six patients received adjuvant chemotherapy, and six patients received adjuvant hormonal therapy. Four patients (10%) were diagnosed with metastasis at the time of surgery for their primary tumor. As a first-line treatment for advanced disease, 22 of these 41 patients (54%) received CMF, 16 of 41 patients (39%) received CAF, 1 of 41 patients received Adriamycin weekly, and 2 of 41 patients received a platinum-containing chemotherapy. The median age at start of chemotherapy was 50 years (range, 29–74 years). Additional characteristics are listed in Table 4. Median follow-up was 33 months (range, 5–107 months) from the date of primary surgery and 12 months (range, 1–75 months) from the start of chemotherapy for recurrent disease. Tumor progression occurred in all but one of the patients (98%) during follow-up after the start of chemotherapy. Median time to progression was 4 months. After 12 months, 19% of the patients were alive without progression. Response to systemic treatment was defined by standard Union Internationale Contre le Cancer criteria as a patient having either CR or PR or prolonged stable disease of more than 6 months (21, 22).

Statistical Methods

The associations of TP53 with continuous variables (ER, PgR, age, and disease-free interval) were studied with a nonparametric test. Mutation subsets were analyzed with a Wilcoxon rank-sum test. Two-sided *P*s < 0.05 were considered statistically significant. Logistic regression analysis was used for the analysis of response to treatment. Cox regression analysis was used for the analysis of time to treatment failure and overall survival after start of treatment. Survival curves were generated using the method of Kaplan and Meier (23). *TP53* gene status and the following factors were evaluated in the multivariate regression analysis: (*a*) age; (*b*) menopausal status; (*c*) adjuvant therapy; (*d*) disease-free interval; (*e*) the dominant site of relapse (in case of multiple sites, the site with the worst prognosis was considered dominant); (*f*) ER; and (*g*) PgR. TP53 was added to this model.

Table 1 *Patient and tumor characteristics and TP53 mutation*

	TP53 gene status		
	No. of patients	% mutated	*P*
All patients	243	37	
Menopausal status[a]			
Premenopausal	70	41	
Postmenopausal	173	35	0.37
Age (yr)[a]			
<40	25	64	
40–55	79	39	
55–65	58	29	
>65	81	32	0.01[c]
Dominant site of relapse			
Soft tissue	34	50	
Bone	115	26	
Visceral	94	46	0.003
Disease-free interval (mo)			
<12	90	49	
>12	153	30	0.003
Prior adjuvant therapy			
No	204	35	
Yes	39	46	0.20
ER levels (fmol/mg protein)[b]			
<10	63	67	
10–75	58	36	
>75	121	22	<0.0001[c]
PgR levels (fmol/mg protein)[b]			
<10	82	57	
10–75	68	35	
>75	90	19	0.0001[c]

[a] Age and menopausal status at start therapy.
[b] ER data are missing for one patient, but PgR data are present. PgR data are missing on three patients for whom ER data are available.
[c] Test for trend.

Table 2 *Patient and tumor characteristics and response rate to first-line tamoxifen therapy of recurrent disease*

	No. of patients	Response rate (%)	Univariate			Multivariate			Survival after start of therapy (median)
			P	OR	CI	*P*	OR	CI	
All patients	202	55							24 mo
Menopausal status									
Premenopausal	47	45		1			1		30 mo
Postmenopausal	155	58	0.11	1.71	0.89–3.31	0.67	1.28	0.41–4.01	23 mo
Age (yr)[a]									
<40	15	33		1			1		18 mo
40–55	61	51		2.07	0.63–6.76		1.33	0.31–5.68	29 mo
55–65	51	57		2.64	0.79–8.82		1.44	0.24–8.78	25 mo
>65	75	61	0.21	3.17	0.98–10.22	0.55	2.47	0.41–15.01	20 mo
Dominant site of relapse									
Soft tissue	24	79		1			1		28 mo
Bone	105	53		0.30	0.10–0.87		0.12	0.03–0.44	28 mo
Visceral	73	49	**0.03**	0.26	0.09–0.76	**0.001**	0.11	0.03–0.43	19 mo
Disease-free interval (mo)									
<12	70	37		1			1		18 mo
>12	132	64	**0.0002**	3.06	1.68–5.59	**0.0027**	2.95	1.44–6.07	29 mo
Prior adjuvant therapy									
No	175	54		1			1		24 mo
Yes	27	59	0.63	1.22	0.54–2.79	0.23	1.95	0.65–5.84	25 mo
ER levels (fmol/mg protein)[b]									
<10	37	30		1	1		1	1	16 mo
10–75	47	47		2.08	0.84–5.16		1.84	0.57–5.92	23 mo
>75	117	66	**0.00025**	4.55	2.04–10.14	**0.03**	3.82	1.27–11.51	30 mo
PgR levels (fmol/mg protein)[b]									
<10	53	43		1	1		1	1	19 mo
10–75	61	48		1.18	0.56–2.48		0.70	0.27–1.82	21 mo
>75	85	68	**0.0054**	2.80	1.38–5.70	0.28	1.33	0.52–3.41	36 mo
TP53 gene status									
Wild-type	137	66		1			1		29 mo
Mutated	65	31	**<0.0001**	0.22	0.12–0.42	**0.0014**	0.29[c]	0.13–0.63	20 mo

[a] Age (years) at the time of the primary tumor.
[b] ER information on one patient and PgR information for three patients are missing.
[c] The increment in χ^2 is 10.2. Significant data are shown in bold.

RESULTS

***TP53* Gene Mutations.** We have sequenced the entire open reading frame of the *TP53* gene (exons 2–11). Mutations were detected in 90 of the 243 (37%) samples, 9 of which (10%) were observed outside the sequence-specific DNA binding domain (codons 102–292). Fifty of these 90 mutations (56%) were restricted to the conserved region. Within the zinc-binding domain regions L2 and L3 (residues 163–195 and 236–251, respectively), 31 (34%) mutations were found. Moreover, we observed 15 mutations in four of the seven amino acids important in direct DNA binding (*i.e.*, codons 241, 248, 273, and 280). As expected, the majority of the mutations were transitions, and the amino acid arginine was altered most frequently. Of all 90 mutations, 62 (69%) were missense mutations, including 3 complex mutations and 1 tandem mutation. In total, these amount to 42 different changes. The remaining 28 mutations (referred to as non-missense mutations) were 5 in-frame deletions/insertions, 11 nonsense mutations leading to a stop codon, and 12 out-of-frame deletions/insertions causing a premature stop codon. These latter "null mutations" were localized mainly in exon 6. These results imply that one-fourth of the mutations detected in this study would not result in an increased expression of the TP53 protein, *i.e.*, false negatives.

Patient and tumor characteristics are summarized in Table 1. The prevalence of *TP53* gene mutations is highest in tumors from younger women (<40 years of age), in patients who experienced a shorter disease-free interval, and in ER- or PgR-negative (<10 fmol/mg protein) tumors. The median ER levels are about 10 times lower in the tumors with mutations than in those without a *TP53* gene mutation (15 *versus* 144 fmol/mg protein, respectively). Menopausal status and prior adjuvant therapy were not significantly related to *TP53* gene mutation. Interestingly, if the primary tumor had a *TP53* mutation, the metastases more often developed in soft tissue (50%) or visceral tissues (46%) than in bone (26%) as the dominant site of disease. In an exploratory analysis, we observed a relatively low ratio between missense and nonmissense mutations in the primary tumor of patients who developed a relapse in soft tissue (ratio, 1.4) when compared with the ratios in patients who relapsed to bone or visceral tissues (ratios, 3 and 3.5, respectively) as the first site of relapse.

***TP53* Gene Mutations and Response to Tamoxifen.** We observed a mutation in 65 of 202 (32%) primary tumor samples, and this resulted in a premature termination of the protein in 13 of these 65 samples (20%). Of the 202 patients, 55% responded to first-line tamoxifen therapy (10 CRs, 25 PRs, and 76 cases of stable disease). Very young patients (<40 years) tended to have a lower response rate (33%; Table 2). Furthermore, the presence of bone or visceral metastasis, a short disease-free interval after primary surgery, and low ER and PgR levels were significantly associated with low response rates to tamoxifen, whereas prior adjuvant therapy showed no relation with response (Table 2). Sixty-six percent of the patients without a TP53 mutation responded to tamoxifen, whereas only 31% of the patients with a TP53 mutation responded to tamoxifen therapy (OR, 0.22; $P < 0.0001$; Table 2). There was no significant difference between the response percentages of either missense or nonmissense mutations (29% and 38%, respectively). The *TP53* gene mutations were stratified according to the type of mutation, *i.e.*, mutations in the zinc-binding domains L2 and L3 of the protein, or by residues that directly contact DNA in an exploratory analysis. Five of 12 (42%) patients with mutations in L2 but only 2 of 13 (15%) patients with mutations in L3 or 2 of 11 (18%) patients with mutations in codons that directly contact DNA showed a response compared to a response of 66% in patients with a wild-type *TP53* gene.

Multivariate Analysis for Response to Tamoxifen. The independent relationship of the variables with response to tamoxifen therapy for advanced disease was studied using multivariate logistic

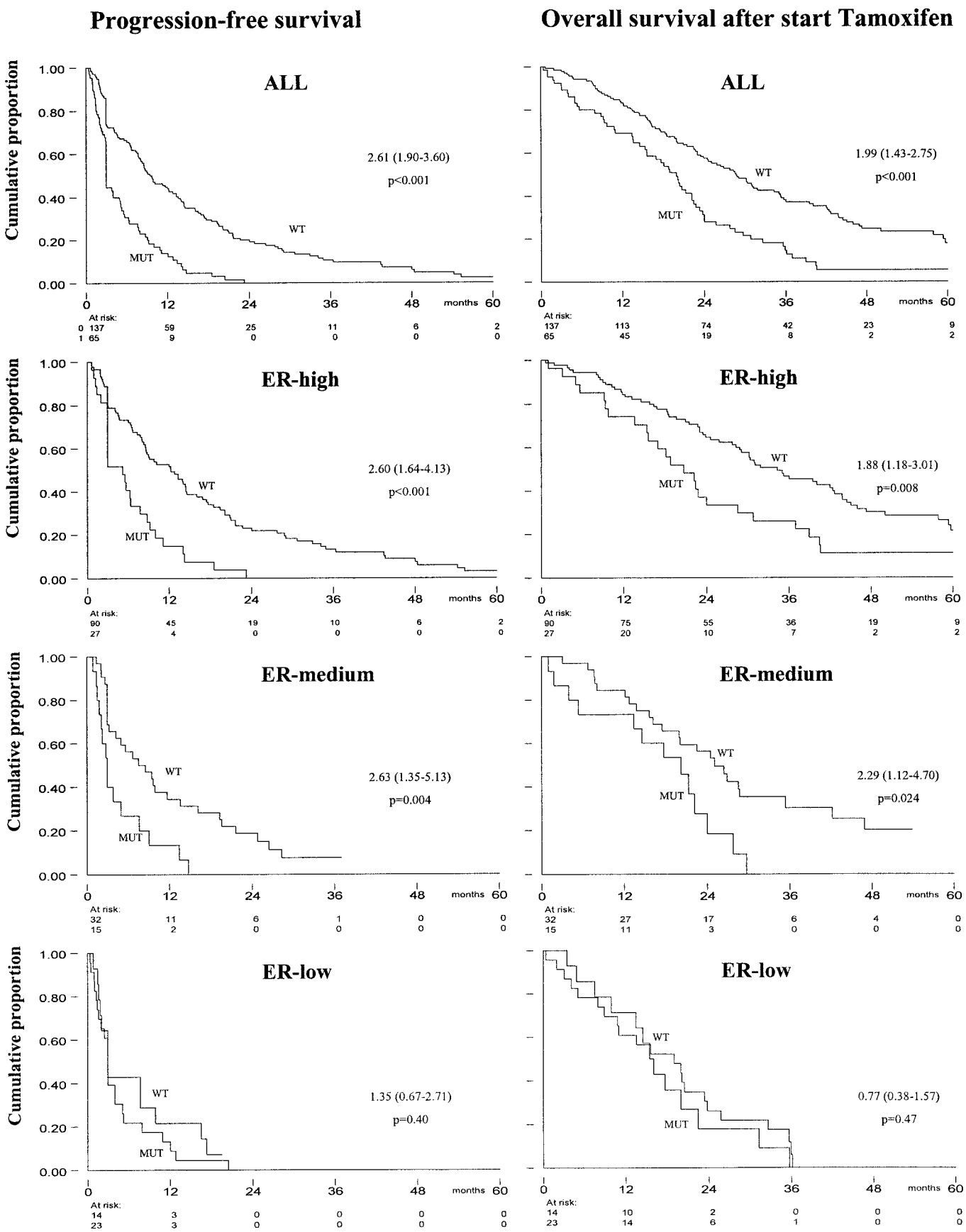

Fig. 1. PFS (*left*) and survival after the start of tamoxifen treatment (*right*) as a function of *TP53* gene status in all patients (*ALL*), patients with high ER (>75 fmol/mg protein; *ER-high*), patients with intermediate ER (10 > ER <75 fmol/mg protein; *ER-medium*), and patients with low ER (<10 fmol/mg protein; *ER-low*). The number of patients below the *X* axis represents the number at risk over total patients in the wild-type and mutated subgroups, respectively.

Table 3 *Patient and tumor characteristics and response to chemotherapy of patients with recurrent disease*

	Up-front chemotherapy					Chemotherapy after tamoxifen				
		Response		Univariate			Response		Univariate	
	No. of patients	(%)	*P*	OR	95% CI	No. of patients	(%)	*P*	OR	95% CI
All patients	41	46				113	45			
Menopausal status										
Premenopausal	23	61		1		29	48		1	
Postmenopausal	18	28	**0.03**	0.25	0.07–0.93	84	44	0.69	0.84	0.36–1.97
Age (yr)										
<40	10	50		1		10	50		1	
40–55	18	56		1.25	0.27–5.89	39	49		0.95	0.24–3.81
55–65	7	43		0.75	0.11–5.24	34	47		0.89	0.22–3.64
>65	6	17	0.38	0.20	0.02–2.39	30	37	0.75	0.58	0.14–2.46
Dominant site of relapse										
Soft tissue	10	40		1		4[a]	50		1	
Bone	10	60		2.25	0.38–13.47	46	52		1.09	0.14–8.42
Visceral	21	43	0.60	1.13	0.24–5.21	63	40	0.42	0.66	0.09–4.98
Disease-free interval (mo)										
<12	20	55		1		39	44		1	
>12	21	38	0.28	0.50	0.14–1.75	74	46	0.81	1.10	0.50–2.40
Prior adjuvant therapy										
No	29	48		1		96	45		1	
Yes	12	42	0.70	0.77	0.20–2.98	17	47	0.86	1.10	0.39–3.08
ER levels (fmol/mg protein)										
<10	26	46		1		21	43		1	
10–75	11	55		1.40	0.34–5.76	31	45		1.10	0.36–3.35
>75	4	25	0.59	0.39	0.04–4.25	60	47	0.95	1.17	0.43–3.18
PgR levels (fmol/mg protein)										
<10	29	48		1		29	41		1	
10–75	7	43		0.80	0.15–4.25	40	43		1.05	0.40–2.76
>75	5	40	0.92	0.71	0.10–4.93	42	50	0.71	1.42	0.55–3.68
TP53 gene status										
Wild type	16	63		1		73	42		1	
Mutated	25	36	0.10	0.34	0.09–1.24	40	50	0.44	1.35	0.62–2.94

[a] Dominant site of relapse at start of chemotherapy after tamoxifen treatment. Significant data are shown in bold.

regression analysis. *TP53* gene mutation status (as a dichotomized variable) was added to the classical variables (see Table 2). Disease-free interval, dominant site of relapse, and ER and TP53 status were significant in this multivariate analysis. There was no statistically significant interaction between TP53 mutation and ER levels.

Response in Clinically Relevant Subsets of Patients Stratified by ER and PgR Status. We explored the association of *TP53* gene status with response to tamoxifen therapy in clinically relevant subgroups. Subsets of tumors from patients with low ER levels (<10 fmol/mg protein, median survival of the patients = 16 months; see Table 2), intermediate ER levels (≥10 fmol/mg protein but <75 fmol/mg protein, median survival of the patients = 23 months), and high ER levels (>75 fmol/mg protein, median survival of the patients = 30 months) were created. In all three subgroups, according to ER status, the response rate was better in patients with wild-type TP53 in their primary tumors. The worst overall response was observed in patients with ER-negative and TP53-mutated tumors ($n = 23$), of whom only 22% responded. The best response was observed in the patients with high ER levels and wild-type TP53 in their primary tumors ($n = 90$), of whom 73% responded.

Relationship Between P53 Mutation and PFS after the Start of Treatment. Duration of response is perhaps as important as time to treatment failure because the duration of response contributes directly to the quality and duration of life. Using Cox univariate regression analysis, we observed that the presence of a *TP53* gene mutation was significantly associated with a shorter duration of response (16 *versus* 11 months; $P = 0.0006$) in 111 patients who responded to tamoxifen. However, *TP53* mutation was not significantly associated with survival after the start of treatment (40 *versus* 31 months) in the responders.

The median PFS was shorter for patients with a TP53 mutation as compared with those with wild-type TP53 (3.0 *versus* 9.6 months). Patients with *TP53* gene mutation in their primary tumors experienced a shorter PFS and an earlier death (relative hazard rate, 2.61 and 1.99, respectively, both $P < 0.001$) than those with TP53 wild-type tumors (see Fig. 1, *ALL*). In addition (see Table 2), younger patients (age < 40 years) and those with visceral metastasis, a short disease-free interval, low ER or PgR levels, or TP53 mutation showed a shorter median overall postrelapse survival after the start of tamoxifen treatment. In the multivariate analysis for survival after the start of tamoxifen treatment, the presence of a TP53 mutation was associated with decreased survival (RHR, 1.39; 95% CI, 0.94–2.07; $P = 0.10$).

We next explored the predictive value of TP53 mutations in subgroups with low, intermediate, and high ER levels. The relationship between TP53 status and PFS and overall survival after the start of treatment was most apparent in the high ER and intermediate ER (Fig. 1, *ER-medium*) subsets of patients but absent in the ER-negative subset (Fig. 1, *bottom panels*).

***TP53* Gene Mutations and Response to Chemotherapy.** Of the 113 patients that were evaluable for chemotherapy after tamoxifen therapy in this study, 51 (45%) responded to chemotherapy (1 CR, 19 PRs, and 31 cases of stable disease). When compared with bone metastasis and soft tissue, patients with visceral metastasis had the worst response to chemotherapy after tamoxifen treatment (OR, 0.66). As shown in Table 3, patients with TP53 mutations did not respond differently to chemotherapy after tamoxifen therapy than did patients without mutations. Stratification according to domain L3 of the protein or by residues that directly contact DNA (see above) revealed that three of five patients with mutations in L3 and two of seven patients with mutations in codons that directly contact DNA showed a response when compared with an overall response of 42% of patients with the wild-type *TP53* gene.

Of the 41 patients studied with respect to up-front (first-line) chemotherapy, 19 (46%) responded (4 CRs, 11 PRs, and 4 cases of stable disease). A relatively high prevalence (61%) of *TP53* gene mutation was observed, *i.e.*, *TP53* gene mutation was observed in 25

Table 4 *Predictive relevance of TP53 in breast cancer*

Author	Year	No.	Stage of disease	Technique	% abnormal	Treatment	Predictive
Endocrine therapy		1435					
Bergh	1995	298	Primary	cDNA sequencing	22%	Adjuvant TAM[a] and RT	Resistant
Archer	1995	92	Advanced	IHC	58%	Hormonal	No
Silvestrini	1996	240	Primary	IHC	14%	Adjuvant TAM	Resistant
Elledge	1997	202	Metastatic	IHC	20%	TAM	No
Berns	1998	401	Metastatic	LIA	Median	TAM	Resistant
Berns	2000 (this study)	202	Metastatic	cDNA sequencing	32%	TAM	Resistant
Chemoendocrine therapy		341					
Elledge	1995	261	Primary	IHC	38%	Adjuvant chemo (CMF & prednisone)	No
Markris	1997	80	Primary	IHC	39%	Neoadjuvant chemo and TAM	No
Chemo		3428					
Jacquemier	1994	81	Primary	IHC	39%	Adjuvant chemo	No
Faille	1994	38	Loc advanced	PCR-SSCP sequencing	36%	Chemo	Resistant
Muss	1994	394	Primary	IHC	35%	Adjuvant chemo	No
Stål	1995	139	Primary	IHC	14%	Adjuvant chemo	Sensitive
Aas	1996	63	Loc advanced	IHC	???	Doxorubicin monotherapy	No
Aas	1996	63	Loc advanced	CDGE/cDNA sequencing	29%	Doxorubicin monotherapy	Resistant
MacGrogan	1996	125	Primary	IHC	27%	Neoadjuvant chemo	No
Linn	1997	70	Primary	IHC	65%/30%	Neoadjuvant chemo high dose & GM-CSF	No
Dublin	1997	277	Primary	IHC	21%	Adjuvant CMF	No
Niskanen	1997	103	Metastatic	IHC	16%	FEC	No
Bonetti	1998	43	Metastatic	IHC	51%	CMF/CA-EF	No
Rozan	1998	329	Primary	IHC	38%	Neoadjuvant chemo	No
Degeorges	1998	277	Primary	IHC	24%	Adjuvant chemo	No
Clahsen	1998	440	Primary	IHC	18%	FAC, one course perioperative	Resistant
Järvinen	1998	55	Advanced	IHC	33%	Epirubicin monotherapy	No
Thor	1998	994	Primary	IHC	33%	Adjuvant CAF dose intensive	Sensitive
Radiotherapy		781					
Jansson	1995	168	Primary	cDNA-based sequence	18%	Adjuvant locoregional RT	Sensitive
Silvestrini	1997	613	Primary	IHC	18%	Adjuvant RT	Sensitive

[a] TAM, tamoxifen; RT, radiotherapy; chemo, chemotherapy; GM-CSF, granulocyte macrophage colony-stimulating factor; FEC, 5-fluorouracil, epirubicin, and cyclophosphamide; CA-EF, cyclophosphamide Adriamycin-epirubicin 5-fluorouracil; FAC, 5-fluorouracil, doxorubicin, and cyclophosphamide; Loc, local; SSCP, single strand conformational polymorphism; CDGE, constant denaturing gel electrophoresis.

of 41 primary tumors. In 10 of these 25 samples (40%), this resulted in a premature termination of the protein. As shown in Table 3, only 36% percent of the 25 patients with a TP53 mutation responded to first-line chemotherapy, in contrast to 63% of the 16 patients without a TP53 mutation (OR, 0.34). In this exploratory study on a small number of patients, only menopausal status was significantly correlated with response to up-front chemotherapy; none of the other patient and tumor characteristics studied were significantly correlated with response to up-front chemotherapy. The median PFS was 3.0 months for those patients with TP53-mutated tumors *versus* 3.6 months for patients without TP53 mutation.

DISCUSSION

The onset and progression of breast cancer is accompanied by multiple genetic alterations that result in quantitative and qualitative changes in the expression of these genes. Mutations in the *TP53* gene are the most frequent genetic changes in human cancer, and, depending on the method of detection, the frequencies of *TP53* mutations reported in invasive breast cancer range from 12–46% (24). In recent years, numerous reports have appeared on the relation between TP53 status and (disease-free) survival, and conflicting conclusions were reached on the prognostic value of TP53 in breast cancer. The lack of unanimity between authors may be explained by: (*a*) differences in techniques used for the analyses of p53 status (*e.g.*, immunohistochemical analyses with different antibodies on frozen or paraffin-embedded tissues using different cutoff levels, ELISA/LIA, PCR-single-strand conformational polymorphism/constant denaturing gel electrophoresis analyses of primarily exons 5–8, or cDNA sequencing of the entire gene); (*b*) patient sample size; (*c*) subset analyses; (*d*) retrospective nature of the studies; (*e*) different (adjuvant) treatments of the patient population; (*f*) different (modern) prognostic covariates used in the multivariate analyses; (*g*) the subjectivity inherent to some approaches; and (*h*) publication bias.

Moreover, the relapse tumor could be dissimilar to the primary tumor because it was shown that *TP53* mutations found in the primary tumors can be absent in the metastasis.

In the present study, we evaluated the predictive value of *TP53* gene mutations, as estimated through cDNA sequencing of the entire coding sequence of the *TP53* gene, in a relatively large series of breast cancer patients who were treated with tamoxifen or chemotherapy for advanced disease. We observed *TP53* gene mutations in 32% of the 202 primary tumors of tamoxifen-treated patients and in 61% of the 41 patients (with mainly ER-negative tumors) treated with up-front chemotherapy. The observed prevalence is slightly higher than the mean percentage of 25% [range, 15–71%; examined in 1425 breast tumor samples worldwide; reviewed by Hartmann *et al.* (25)] and a prevalence of 29% [range, 15–71%; examined in 16 populations by Soussi (26)]. This difference can be explained by the fact that the entire coding sequence of *TP53* was investigated and that only those patients who developed advanced disease were included in the present study. *TP53* gene mutation was related with a poor response to up-front chemotherapy in our small series of patients (OR, 0.34) and was similarly but significantly related with a poor response to first-line tamoxifen treatment in both univariate analysis (OR, 0.22; $P < 0.0001$) and multivariate analysis. In contrast, patients with mutated *TP53* in their primary tumors may respond better to chemotherapy (50% *versus* 42%) after tamoxifen treatment, but they did show a shorter PFS and duration of response.

When evaluating only those reports that have covered TP53 alterations in relationship with the efficacy of various treatments in breast cancer, we noticed that there is no agreement on the significance of the predictive value of TP53. The summary of these 26 studies on almost 6000 breast cancer patients is listed in Table 4. In 15 reports, there was no significant relation between TP53 status and type of response (2, 3, 6–12, 15, 28, 30–33), 7 reports described resistance (Refs. 5, 6, 14, 16, 27 and 29 and this study), whereas the other 4

studies reported sensitivity (13, 34–36). However, this survey indicates that the TP53 genotype, not the immunohistochemical results, is predictive of response in breast cancer patients: all 5 studies that used DNA analysis showed a predictive value of *TP53*, whereas this was only true in 6 of the 21 immunohistochemical studies, *i.e.*, 2 studies showed sensitivity, and 4 showed resistance. Both studies on radiotherapy showed sensitivity to this form of treatment (34, 36).

The mechanisms that underlie the relationship of p53 alterations and a poor response to tamoxifen are not clear. Tamoxifen acts as an antiestrogen via the ER, but some of its effects are thought to be mediated through the activation of transforming growth factor β and by decreasing plasma insulin-like growth factor I levels. Previous data suggest that the mutant forms of TP53 inhibit the antiproliferative effect of transforming growth factor β by interfering with its signaling pathway. In addition, wild-type TP53 can repress the insulin receptor and the insulin-like growth factor I receptor promotor in other cell types. Although the exact mechanisms are unclear, this complex interplay provides a link between the *TP53* gene and signaling pathways in breast cancer cells (discussed in Ref. 5). Our finding on the relationship between *TP53* gene mutation and poor response to tamoxifen treatment of advanced disease is well in line with our previous study on p53 protein levels by LIA in 401 patients with advanced disease (5) but disagrees with the studies of Archer *et al.* (2) and Elledge *et al.* (3), who assessed p53 expression immunohistochemically and found no relationship with response to endocrine treatment. Bergh *et al.* (14) and Silvestrini *et al.* (32) showed that tamoxifen also appears to be of less benefit in patients with *TP53* gene mutations or overexpression, respectively, in the adjuvant setting (see Table 4). The relationship of *TP53* gene status with PFS is most apparent in the high ER (>10 fmol/mg protein) subgroups of our patients. In contrast to our findings, Elledge *et al.* (3), who studied the accumulation of p53 using IHC in mainly ER-positive metastatic breast cancer, did not observe a relation between p53 status and response to tamoxifen therapy, although they stated that breast tumors with altered p53 protein are inherently more aggressive, even after they have metastasized. These conflicting outcomes may again be explained by the different techniques and study designs.

It is known that some chemotherapeutic agents and ionizing radiation act by inducing apoptosis in tumor cells. Lowe *et al.* (37) showed that cells expressing mutant TP53 were totally resistant to apoptosis on treatment, whereas cells expressing the wild-type gene were sensitive to these therapeutic agents. In the present study, *TP53* gene mutations were related, although not significantly, to a poor response to up-front chemotherapy (36% *versus* 63%; OR, 0.34). However, the number of patients receiving first-line chemotherapy was small, the regimen is heterogeneous, and the follow-up was relatively short. Based on this, it is not possible to draw firm conclusions. Furthermore, we suspect a better response to chemotherapy after tamoxifen treatment.

Three reported molecular analyses also revealed that *TP53* gene mutations predict a response to chemotherapy or adjuvant radiotherapy in breast cancer patients (6, 33, 35). In contrast, all 4 reports on metastatic disease and 7 of 10 reports in the adjuvant setting revealed no predictive value of TP53 when using immunohistochemical analyses.

Various mutations can alter the TP53 protein distinctly and lead to different biological characteristics and tumorigenic potential (38, 39). L2 and L3 loops of the *TP53* gene contain residues involved in direct DNA contact as well as protein stabilization. In the present study, those patients with *TP53* gene mutations in codons that directly contact DNA or with mutations in the zinc-binding domain loop L3 showed the lowest response to tamoxifen (18% and 15% response rates, respectively). Survival analyses showed a significantly reduced survival rate for patients with mutations affecting the zinc-binding domains L2 and L3 compared with patients with mutations outside these regions or with no mutations (40) or for patients with mutations affecting the direct DNA contact (41). Moreover, Aas *et al.* (6) showed that mutants affecting the L3 loop were significantly associated with *de novo* resistance to doxorubicin monotherapy in small subsets of patients. Extending the analysis of these various mutations allows us to focus attention on the biological significance of particular mutations that may assist the selection of residues that could be of predictive value in breast cancer.

IHC is not able to detect every TP53 alteration. In the present study, we have shown that one-fourth of *TP53* mutations found are "null mutations" that do not lead to TP53 accumulation. Furthermore, IHC does not us allow to distinguish the heterogeneity of TP53 mutants. Based on the data from the literature and the present results, we conclude that the presence of mutation and the type of mutation have predictive value for response to tamoxifen treatment. Therefore, we reason that, if tissue resources permit, the TP53 mutation status should preferably be included in the daily practice of treatment of metastatic breast cancer patients.

The main conclusion of this study is that *TP53* mutation is significantly associated with a poor response to tamoxifen treatment in patients with advanced breast cancer. Based on the present results and the data reviewed, it is tempting to hypothesize that the *TP53* genotype in particular and, to a lesser degree, the immunohistochemical analysis are predictive of response to therapy in breast cancer. Prospective studies should be performed to support this conclusion. Besides mutation analyses, a direct functional assay (42) or the measurement of downstream components of the TP53 pathway such as p21 or MDM2 may confirm the TP53 integrity.

ACKNOWLEDGMENTS

We thank Esfir Lôfman and Nigel Tooke (Amersham Pharmacia Biotech) and J. Bolt-de Vries, S. E. Binnendijk-Noordegraaf, and H. Peters (Division of Endocrine Oncology, University Hospital Rotterdam, Rotterdam, the Netherlands) for excellent technical assistance.

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Complete Sequencing of *TP53* Predicts Poor Response to Systemic Therapy of Advanced Breast Cancer

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Cancer Res 2000;60:2155-2162.