

TP53 Mutation and p53 Overexpression for Prediction of Response to Neoadjuvant Treatment in Breast Cancer Patients¹

Daniela Kandioler-Eckersberger,² Carmen Ludwig, Margarethe Rudas, Sonja Kappel, Elisabeth Janschek, Catharina Wenzel, Hermine Schlagbauer-Wadl, Martina Mittlböck, Michael Gnant, Günter Steger, and Raimund Jakesz

Departments of Surgery [D. K.-E., C. L., S. K., E. J., M. G., R. J.], Pathology [M. R.], Internal Medicine [C. W., G. S.], Pharmacology [H. S.-W.], and Medical Computer Sciences [M. M.], University of Vienna Medical School, A-1090 Vienna, Austria

ABSTRACT

The value of p53 to predict the cytotoxic effect of two commonly used chemotherapy regimens was assessed in patients with advanced breast cancer. Response to a DNA-damaging combination therapy [fluorouracil, epirubicin, cyclophosphamide (FEC)] considered to induce p53-dependent apoptosis was compared with a microtubule stabilizing therapy (paclitaxel) expected to be independent of p53 function. The p53 status of the patients' breast tumors was assessed using both immunohistochemistry (IHC) and direct sequencing of the entire p53 gene. p53 findings were correlated with treatment response, and linkage between p53 function and cellular response was assessed by terminal deoxynucleotidyl transferase-mediated nick end labeling assay.

In a series of 67 breast tumors, 19% had TP53 gene mutations, 40% had a positive p53 IHC, and 12% had both. In the FEC group, treatment failure was related to both the presence of TP53 gene mutations ($P = 0.0029$) and a positive IHC ($P < 0.0001$). Apoptosis was almost exclusively found in tumors having normal p53 in both parameters ($P < 0.0001$). In the paclitaxel group, treatment response was neither related to apoptosis nor to normal p53. Combination of sequencing and IHC results revealed a significant association between abnormal p53 and response to paclitaxel ($P = 0.011$).

We found TP53 mutations, as well as p53 protein overexpression, to be associated with response to chemotherapy. Whereas clinical response to FEC was found to be dependent on normal p53, the cytotoxicity of paclitaxel was related to defective p53. The efficiency of paclitaxel during mitosis might be supported by lack of G₁ arrest due to p53 deficiency. Therefore, patients with p53-deficient tumors may benefit from paclitaxel.

INTRODUCTION

Neoadjuvant cytotoxic therapy has been introduced in breast cancer patients with advanced disease to reduce tumor volume preoperatively and to gain control of probably existing systemic disease. Data in the literature show that up to 50% of women who are given preoperative cytotoxic therapy do not respond to this treatment (1, 2). Thus, there is a need for methods that allow the identification of treatment responders to avoid the toxic effects of therapy in nonresponders.

The biological functions of the p53 tumor suppressor make it a potential predictive marker. p53 has been observed to function as a transcription factor that regulates normal cell growth by controlling genes that promote progression through the cycle and by controlling those that cause arrest in G₁, when the genome is damaged (3). Active p53 can further promote apoptosis in growth-arrested cells and has been related to the efficient execution of programmed cell death in response to DNA damage in the presence of oncogenic triggers (4). Intact p53 has been shown to induce apoptosis in response to ionizing radiation, whereas loss of p53 function has been reported to enhance cellular resistance to a number of chemotherapeutic agents (5, 6).

Because p53 has been recognized to influence response to chemotherapy, efforts have been undertaken to study its usefulness as a predictive factor in patients (7, 8). The majority of studies performed thus far comprised patients receiving adjuvant treatment regarding overall survival as the end point and using IHC³ for p53 analysis. Most of these studies reported a trend but no significant relationship between positive immunohistochemical staining and overall survival (7–10).

The combination therapy of FEC is widely used in the treatment of breast cancer (11). Alkylating substances (cyclophosphamide) as well as anthracyclines (epirubicin) induce DNA damage by cross-linking DNA strands. This results in apoptosis due to p53 activation (12). Another substance that exhibits significant activity in human tumors, including breast

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² To whom requests for reprints should be addressed, at Department of Surgery, University of Vienna, Medical School, Waehringer Guertel 18–20, A-1090 Vienna, Austria. Phone: 43-1-40400-5621; Fax: 43-1-40400-6777; E-mail: Daniela.Kandioler@akh-wien.ac.at.

³ The abbreviations used are: IHC, immunohistochemistry; FEC, fluorouracil, epirubicin, and cyclophosphamide; CR, complete remission; PR, partial remission; SD, stable disease; PD, progressive disease; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

cancer, is paclitaxel (13). It stabilizes tubulin polymerization, inhibits formation of the mitotic spindle, and leads to cell death (14). This mechanism is considered not to require the transcriptional activity of p53.

In our study, we analyzed the value of p53 for predicting response to neoadjuvant chemotherapy, using pathological response and apoptosis as end points. We compared a DNA-damaging therapy (FEC) to a microtubule stabilizing therapy (paclitaxel). p53 function was determined by immunohistochemical staining of p53 protein expression and direct DNA sequencing of the entire *p53* gene. The p53 findings were correlated with treatment response, and the linkage between p53 function and cellular response was corroborated by an apoptosis assay.

MATERIALS AND METHODS

Patients. Eighty-two patients with histologically proven advanced breast cancer (T₃ and T₄) received two different treatment regimens prior to surgery in accordance with Phase II study protocols at the Department of General Surgery at the University of Vienna. The protocols, both extending from 1993–1996, were approved by the local ethics committee. During this period, overall, 91 patients were registered with T₃/T₄ tumors at our department, and 82 of these patients were included in the two study protocols. Sixty-seven patients were available for our retrospective analysis.

Neoadjuvant Therapy. Thirty-five patients received anthracycline-based combination therapy (FEC) as first-line treatment (600 mg of 5-fluorouracil per square meter of body surface as a 15-min infusion, 60 mg of epirubicin per square meter as a 1-h infusion, 600 mg of cyclophosphamide per square meter as a 1-h infusion, repeated every 21 days for a total of three or four cycles). FEC was given in full dose for four cycles in 27 patients and for three cycles in 8 patients.

Thirty-two breast cancer patients were given monotherapy consisting of paclitaxel in escalating doses from 215–300 mg per square meter of body surface administered as a 3-h infusion, repeated every 21 days for a total of four cycles in 26 patients and three cycles in 6 patients. Chemotherapy was followed by local surgical treatment in all patients. The reason for surgery after three cycles was lack of compliance partly in connection with toxicity as well as progressive disease (in one patient in the paclitaxel group and in three patients in the FEC group).

Tumor Response. Tumor diameter was assessed at time of presentation using measurements from mammography, clinical examination, and ultrasound. Clinical assessment was recorded monthly, and complete tumor assessment was repeated before surgery. All patients had subsequent local surgical treatment. Pathohistological examination of surgical specimen was used to determine extent of tumor regression. According to Union International Contre Cancer criteria, CR corresponds to more than a 95% tumor reduction in the product of the two largest tumor diameters and PR corresponds to more than a 50% tumor reduction. Stable disease (SD) corresponds to a <50% reduction or less than a 25% increase, and PD to more than a 25% increase in tumor size. Patients with CR and PR were grouped together as responders and compared with patients with SD and PD grouped together as nonresponders.

Tumor Material. Needle biopsies were taken routinely from the tumors at the time of presentation. These formalin-fixed and paraffin-embedded biopsies were used for histological diagnosis, p53 IHC, and *p53* gene analysis.

For apoptosis assay, paraffin-embedded sections from tumors obtained at surgery were used.

DNA Isolation from Paraffin-embedded Tissue. Depending on the size of the biopsy, three to five unstained sections of 4- μ m thickness were used for DNA extraction. The sections were mounted on uncoated slides, and tumor tissue was dissected. Microdissection was not used because tumor content was already confirmed by pathohistological diagnosis derived from sections of the same biopsy. The dissected tumor material was subjected to 200 μ l of Xylo (Merck). The mixture was centrifuged at 12,000 *rpm* for 10 min, and the supernatant was discarded. The remaining pure tissue was digested with 20 μ g/ μ l proteinase K (Boehringer Mannheim) for 2 h at 65°C. The enzyme was inactivated by incubation at 95°C for 10 min. DNA was stored at –20°C.

PCR Amplification. The obtained DNA was usually diluted 1:5. The *p53* gene was amplified in eight different PCRs using DNA primers of 20 bases in length, placed in the adjacent intron regions of exons 2–3, 4, 5, 6, 7, 8–9, 10, and 11, as listed previously (15).

Amplification was performed in a volume of 50 μ l for 45 cycles using 2 μ l of diluted DNA and 20 pmol of each sense and antisense primer including, 1.25 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer Corp., Foster City, CA). For DNA Thermal Cycler 480 (Perkin-Elmer Corp.), the following thermal profiles were used: 10-min denaturation (and activation of AmpliTaq Gold) at 95°C, followed by a 1-min 18 s annealing at 64°C, and a 30-s extension at 74°C with slight modifications for exons 2, 3, and 11. The length of the amplification products ranged from 250–350 bases. The relative shortness of PCR products is probably the reason for having encountered no difficulties in obtaining PCR products from DNA extracted of 4- to 5-year-old paraffin-embedded tissue. PCR products were analyzed on precast 6% acrylamide/bis-acrylamide gels (Novex, San Diego, CA).

Sequencing. Enzymatic pretreatment of PCR products and subsequent sequencing was performed as described previously (16). We used the Thermo Sequinase radiolabeled terminator cycle sequencing kit (United States Biochemical, Cleveland, OH) for direct sequencing. The reaction mix was prepared following the instructions provided by the manufacturer, with some modifications: we added only 1 pmol of the unique primer and used the forward strand primer for exons 2–3, 4, 6, 7, 10, 11 and the reversed strand primers for exons 5 and 8–9. Every identified mutation was confirmed in a separate experiment.

p53 IHC. Histological sections of 4- μ m thickness from paraffin blocks were incubated overnight at 4°C with a 1:50 dilution of the monoclonal antibody p1801 (Oncogene Science, Inc., Uniondale, NY), which recognizes both wild-type and mutant forms of the p53 protein. The protocol used was described previously (17). As positive and negative controls, sections from breast cancers with known p53 mutation and immunoreactivity, as well as normal breast tissue, were included to confirm the consistency of the analysis. A specimen scored

Table 1 Characterization of TP53 mutations detected in 67 patients with breast cancer

Patient no.	Therapy	UICC	p53 IHC%	p53 Gene mutation	Exon	Codon	Normal sequence	Mutant sequence	AS-change
248	FEC	SD	40	T-A transversion	10	338	5' CGC TTC 3'	5' CGC ATC 3'	Phe-Ile
351	FEC	PD	20	C-A transversion	8	276	5' TGT GCC 3'	5' TGT GAC 3'	Ala-Asp
345	FEC	SD	20	Deletion	10			Complex	Frameshift
11	FEC	SD	80	C-T transition	7	248	5' CGG 3'	5' TGG 3'	Arg-Try
363	FEC	SD	80	C-T transition	8	278	5' GCC TGT CCT 3'	5' GCC TGT CTT 3'	Pro-Leu
361	FEC	PD	0	9b deletion	7	261-intron	5' TCC AGG tcaggagccactt 3'	5' TCC gccactt 3'	Splicing
357	FEC	SD	0	2b del	7	260-261	5' GAC TCC AG 3'	5' GAC TCT 3'	Frameshift
290	Paclitaxel	PR	0	1 b deletion	4	102	5' ACC TAC 3'	5' ACT ACC . . . 3'	Frameshift
507	Paclitaxel	PR	0	Deletion	10			Complex	Frameshift
458	Paclitaxel	PR	<10	g-t transversion	8	307-intron	5' CGA Ggt aac 3'	CGA Gtt aac 3'	Splicing
471	Paclitaxel	PR	60	A-G transition	6	214	5' CGA CAT 3'	5' CGA CGT 3'	His-Arg
X9	Paclitaxel	PR	10	G-A transition	7	245	5 GGC 3'	5' AGC 3'	Gly-Ser
608	Paclitaxel	SD	80	C-T transition	7	250	5' AGG CCC ATC 3'	5' AGG CTC ATC 3'	Pro-Val

negative when nuclear staining was rare (below 10%) or absent (18, 19).

Apoptosis Assay (TUNEL). The immunohistochemical detection of apoptosis in paraffin-embedded tumor tissue sections was performed by TUNEL staining (Boehringer Mannheim, Mannheim, Germany). Terminal deoxynucleotidyl transferase labeling with fluorescein-dUTP was done according to the manufacturer's recommendations. As negative control, all samples were incubated with a nucleotide mixture lacking terminal transferase. The tissue sections were counterstained with DAPI (4',6-diamidino-2'-phenylindole dihydrochloride; Boehringer Mannheim). Cells carrying fluorescent signals were counted, and the percentages were calculated.

Statistical Analyses. Patients who responded to chemotherapy (CR, PR) were compared with those who were resistant to chemotherapy (SD, PD). Associations between chemotherapy responders and p53 parameters coming from two analysis methods (p53 sequencing, p53 IHC) were tested with Fisher's exact test for each method separately and for the combined p53 parameters called p53 status. The p53 status was defined as negative in tumors with normal p53 sequence and negative IHC staining and defined as positive in tumors with either TP53 mutation or positive staining or both. The probability of recognizing responders and nonresponders correctly (sensitivity and specificity) was calculated for p53 sequencing, p53 IHC, and for the two combined parameters (p53 status). The accuracy of p53 status to predict response and resistance in patients with FEC and paclitaxel treatment is given.

For correlation of response and apoptosis, the percentage of apoptotic cells was dichotomized (negative value $\leq 1.5\%$ and positive $> 1.5\%$) and calculated with Fisher's exact test.

The mean cumulative dose per patient for paclitaxel (considering escalating doses and number of cycles) was 1020 mg/m² in responders and 1017 mg/m² in nonresponders. To exclude the actually delivered dose as confounding factor for the relation between p53 status and treatment response, the partial correlation of p53 status and response was calculated.

RESULTS

In 13 of 67 breast cancer patients (19%) receiving neoadjuvant treatment, TP53 mutations could be identified, including

eight point mutations, three minor deletions, and two complex deletions. Mutations were located in exons 4, 6, 7, 8, and 10 of the p53 gene, including two mutations in the intron region affecting the splice sites (Table 1). A positive p53 immunohistochemical staining (IHC) was seen in 27 of 67 patients (40%); seven patients carried a mutation and were IHC positive.

Anthracycline-based combination therapy (FEC) was administered in 35 patients; the response rate was 51%. Eighteen patients showed PR; no CR was seen. In 18 patients with response to treatment, none of the tumors had a TP53 mutation and only one stained positive. Of 17 patients with treatment failure (14 SDs, 3 PDs), 7 had a TP53 mutation and 14 had a positive IHC. Only 1 of 17 patients not responding to treatment had neither a mutation nor positive IHC. In the FEC group, treatment failure was related to the presence of both TP53 gene mutations ($P = 0.0029$) and a positive immunohistochemical staining ($P < 0.0001$; Table 2).

Resistance to FEC was correctly predicted by p53 sequencing in 100% and by p53 IHC in 94%. Combination of both p53 parameters defined as p53 status (see "Statistical Analyses") revealed the highest sensitivity and specificity in recognizing responders and nonresponders (Table 2). Presence of apoptosis was related to a normal p53 status and treatment response ($P < 0.0001$; Table 2). In patients with response, the mean percentage of apoptotic cells was seven (Fig. 1). Of 17 patients with treatment failure, 15 had 0% and two patients only 1% apoptotic cells (Fig. 2).

Paclitaxel monotherapy was administered in 32 patients; the overall response rate was 47%. Of 17 patients with treatment failure (16 SDs, 1 PD), 13 had a normal (negative) p53 status. Fourteen of 15 patients with treatment response had a PR (two had a residual tumor of only 3 mm), and 1 patient had a CR on pathohistological examination. Of 15 patients responding to paclitaxel, 5 had TP53 mutations in their tumors, 8 had a positive immunohistochemical staining, and 2 had both. A positive (abnormal) p53 status (see "Materials and Methods" for definition) was significantly associated with response to paclitaxel ($P = 0.011$; Table 2). The partial correlation between p53 status and response considering differences in dose and number of cycles was significant ($P < 0.01$). In responders, the mean percentage of apoptotic cells was 0.4% (Fig. 2). In contrast to

Table 2 Relation of clinical response to p53 sequencing and immunohistochemical results and to apoptosis in breast cancer patients with neoadjuvant treatment

Two treatment regimen with different cytotoxic mechanisms (FEC and paclitaxel) are compared. Sensitivity and specificity of p53 sequencing, p53 IHC, p53 status, and apoptosis to detect responders (CR, PR) and nonresponders (SD, PD) is presented and corresponding confidence intervals are added in brackets.

	FEC			Paclitaxel			Paclitaxel		
	CR, PR	SD, PD		CR, PR	SD, PD				
p53 sequencing			$P = 0.0029$					$P = 0.0755$	
Mutant	0	7	Sensitivity	100%	(84.67; 100)	5	1	Sensitivity	33.33% (11.8; 61.6)
Normal	18	10	Specificity	41.18%	(18.44; 67.08)	10	16	Specificity	94.10% (71.3; 99.9)
p53 IHC			$P < 0.0001$					$P = 0.1437$	
Positive	1	14	Sensitivity	94.44%	(72.71; 99.86)	8	4	Sensitivity	53.33% (26.6; 78.7)
Negative	17	3	Specificity	82.35%	(56.57; 96.20)	7	13	Specificity	76.47% (50.1; 93.2)
p53 status ^a			$P < 0.0001$					$P = 0.0118$	
Positive	1	16	Sensitivity	94.44%	(72.71; 99.86)	11	4	Sensitivity	73.33% (44.9; 92.2)
Negative	17	1	Specificity	94.12%	(71.31; 99.86)	4	13	Specificity	76.47% (50.1; 93.2)
Apoptosis ^b			$P < 0.0001$			^c	^d	$P = 0.0058$	
Positive	17	0	Sensitivity	94.44%	(72.71; 99.86)	0	7	Sensitivity	100% (79.4; 100)
Negative	1	17	Specificity	100%	(83.84; 100)	13	7	Specificity	50.00% (23.0; 77.0)

^a The p53 status represents the combination of results from p53 sequencing and IHC. The p53 status is positive in tumors with either p53 gene mutation or positive immunohistochemical staining or both. The p53 status is negative when both parameters are normal (normal p53 gene and negative staining).

^b Apoptosis results were dichotomized for correlation with clinical response (see "Statistical Analyses"). All P s were calculated with a two-sided Fisher's exact test. Apoptosis assay was done for a total of 27 of 32 patients of the paclitaxel group; five were missing. ^c two in the responder group and ^d three in the nonresponder group.

FEC treatment, response to paclitaxel was neither related to apoptosis nor to normal p53 status, but was significantly related to a positive p53 status.

DISCUSSION

DNA damage is a well characterized stimulus of p53-dependent induction of apoptosis (20). We demonstrated a strong relation between p53 status and response to neoadjuvant treatment with a combination of FEC in breast cancer patients. Alkylating substances such as cyclophosphamide, as well as anthracyclines like epirubicin, intercalate with DNA and induce DNA cross-links and strand breaks considered to produce a p53-dependent induction of apoptosis. The third component of the combination therapy, fluorouracil, has been shown to inhibit processing and function of RNA. Recently, the depletion of ribonucleotide triphosphate, necessary for RNA synthesis, by fluorouracil has been detected to be a stimulus for p53 induction (12). Hence, the three substances seem to synergistically trigger p53-dependent apoptosis. Our results clinically support the assumption that the response to FEC combination therapy is p53 related.

Microtubule-stabilizing paclitaxel is considered to have p53-independent effects. However, *in vivo* and *in vitro* data concerning the efficiency of paclitaxel and p53 status are controversial (21, 22). Response to paclitaxel has been observed in experiments with tumors known to have p53 mutations and has also been demonstrated to be compromised in cell lines lacking p53 (23–25). In our clinical analysis, a negative (normal) p53 status proved to be associated with resistance to paclitaxel, whereas response was supported by deficient p53. Functional p53 has been found to arrest cell cycle in G₁ phase to prevent transition into subsequent phases in the presence of DNA damage (26). For p53-deficient tumor cells it has been shown that

this cell cycle checkpoint is bypassed. In the presence of spindle inhibitors, it has been observed that tumor cells lacking p53 were able to repeatedly enter S phase and become polyploid, abrogating the requirement of mitosis before reinitiation of DNA replication (27). In summary, we speculate that p53-deficient cell populations have a high cycling fraction and that, therefore, paclitaxel, which preferentially acts during the mitosis phase, is highly efficient in cells without functional p53. However, there is an apparent discrepancy between the effects of p53 status on the response of cells to paclitaxel *in vivo versus in vitro*.

Apoptosis was rarely present in our patients responding to paclitaxel. We did not observe apoptosis in 87% of patients responding to paclitaxel, whereas apoptosis was present in 50% of patients with treatment failure. Thus, we found that treatment resistance was not associated with the absence of apoptosis and treatment response was not related to the presence of apoptosis.

The efficiency of paclitaxel has been attributed to cytostatic and apoptotic effects that seem to be independent (21). It has been reported that low concentrations of paclitaxel result in apoptosis (28) and that the maximum apoptotic effect can be observed at 10-fold lower drug concentrations as compared with the maximum cytostatic effect (29). These plasma concentrations have been reported to be at or below clinically achievable concentrations. As paclitaxel was applied in escalating doses in our study, high plasma concentrations might have promoted the cytostatic effect of paclitaxel. This might explain why we rarely observed apoptosis in patients responding to paclitaxel.

Overexpression of the p53 protein has been analyzed in various breast cancer studies. The use of different antibodies, staining standards, tumor material, scores for positivity, and the inclusion of variously selected groups of breast cancer patients might be the reason why the frequency of positive p53 staining

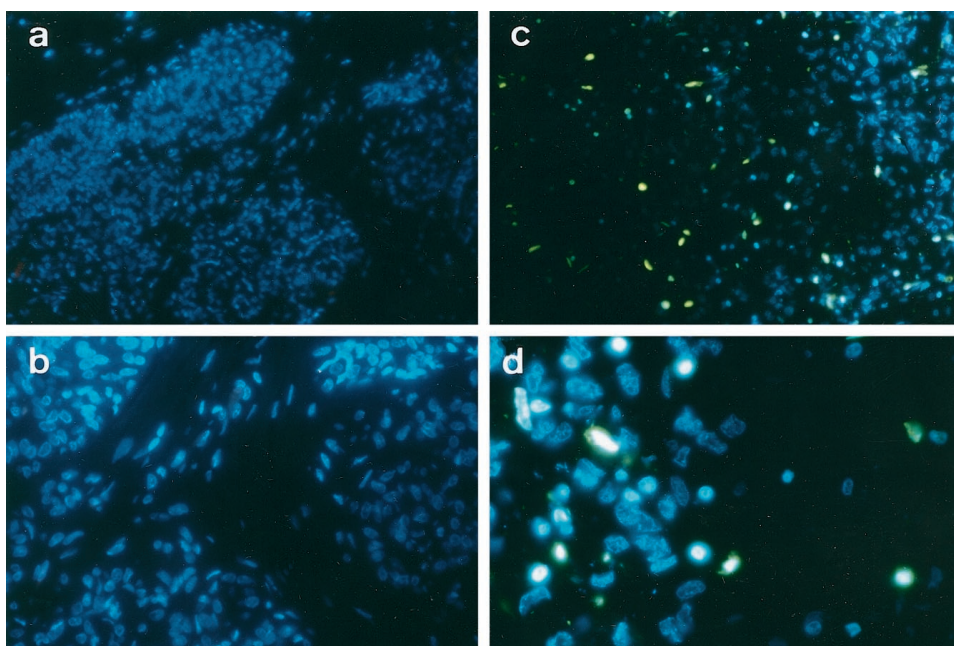


Fig. 1 Detection of apoptosis by TUNEL assay in two breast cancer patients after treatment with FEC. Tumors with clinical response and resistance are compared. Apoptotic cells appear *fluorescent green*, the counterstain is DAPI. TUNEL assay of the tumor of patient 361 showing 0% apoptotic cells [original magnification, $\times 20$ (*a*) and $\times 40$ (*b*)]. Patient 361 progressed during chemotherapy (FEC) and carried a nine-base deletion in the *p53* gene. Tumor of patient 4 showing 8% apoptotic cells $\times 20$ (*c*) and $\times 40$ (*d*); patient 4 achieved PR after chemotherapy (FEC), the *p53* gene of the tumor was normal, and the p53 IHC was negative (negative p53 status).

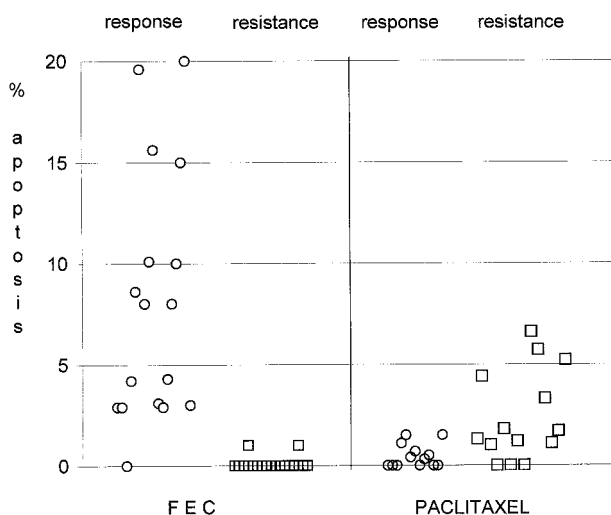


Fig. 2 Percentage of apoptotic tumor cells in breast cancers after neoadjuvant treatment with either FEC or paclitaxel. Comparison of percentage of apoptotic cells in patients who responded (○) or were resistant (□) to chemotherapy.

ranges from 20–50% in the literature (30–32). The rate of positive IHC was as high as 40% in our study. At our institution, the p53 IHC is routinely performed in operated breast cancers. In these unselected patients, we observe a positive p53 IHC in about 25%, which is in accordance with most published reports. Hence, excluding technical reasons, we tend to attribute the high rate of positive p53 staining in our study to the selection of patients with advanced stages of breast cancer (T_3 and T_4 tumors).

The lack of concordance between immunoreactivity and

TP53 gene mutation is a matter of discussion and growing interest because p53 is under evaluation as a predictive factor for response to chemotherapy (33, 34). In our study, the *TP53* mutation rate was 19%, which is in accordance with the published frequencies of breast cancer (35, 36). We found IHC to be positive in 16 patients (23% of the total) without evidence of genetic alterations. Originally, positive p53 staining was thought to indicate *TP53* gene mutations. Positive staining in tumors without gene mutations was attributed to stabilization of the protein by interruption of degradation and considered to be “false positive.” The correlation of the results of IHC to treatment response and tumor cell apoptosis in our study shows that positive IHC staining without gene mutation is of functional importance. However, the exact mechanism leading to a positive IHC staining is still unclear. On the other hand, IHC can be false negative. This finding is typically associated with insertion or deletion mutations, when the antibody binding site of the mutated protein is lost or no protein is produced (30). Our study supports this observations because 5 of 13 *TP53* gene alterations were not reflected by positive IHC staining. The mutations consisted of a one-base deletion, a two-base deletion, a nine-base deletion, a point mutation affecting the splice site, and a complex deletion.

Response assessment using mammography and clinical evaluation can lead to underestimation of therapeutic response due to interpretation of fibrous reaction or persisting calcification as residual tumor. Additionally overestimation of complete response can occur in case of minimal residual disease (37). Our retrospective analysis included patients from two neoadjuvant trials with subsequent surgical resection. Pathological examination was used to determine the extent of tumor regression to avoid pitfalls of confounding radiological and clinical features. The use of microscopic criteria to assess treatment response is reflected by the small

number of CRs that are twice as high in studies without the information from pathohistology.

We have demonstrated that p53 plays a distinct drug-specific role in chemoresistance. The response to a combination of FEC was directly related to normal p53 and tumor cell apoptosis in breast cancer patients. These results provide clinical evidence of a p53-dependent cytotoxic effect of these DNA-damaging agents. In contrast, response to paclitaxel seemed to be related to p53-deficient tumors. We suggest that loss of cell cycle control (lack of G₁ arrest) due to p53 deficiency possibly allows tumor cells to more effectively enter the phase of mitosis, thus supporting the tubulin-specific cytotoxicity of paclitaxel. Because the number of patients in our study was small, the results need to be confirmed in larger groups.

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