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

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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/m²) 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 ($P$ 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 32–32), 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 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 (T4/T4a and/or N2 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...
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 (liquid nitrogen in the theater) in addition to formaldehyde fixation and paraffin embedding. Similarly, a second sample was snap-frozen (liquid nitrogen in the theater).

**Mutation Analysis.** TP53 mutations were detected using TTGE as described elsewhere (17, 39, 40) with primers covering the evolutionarily 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. The method involves use of primers covering the regions (exons and introns) from exons 5 to 11. All samples with aberrantly migrating bands on TTGE were submitted for publication.

**Genetic Analysis.** Mutations in the TP53 gene were initially analyzed by CDGE as described elsewhere (17, 39, 40) with primers covering the evolutionarily 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. The method involves use of primers covering the regions (exons and introns) from exons 5 to 11. All samples with aberrantly migrating bands on TTGE were submitted for direct sequencing of PCR products with standard dideoxy sequencing reaction using the Dye Terminator Cycle Sequencing kit with AmpliTaq 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.
TP53 and Doxorubicin Resistance in Breast Cancer

Table 2: Clinical response in relation to different parameters

<table>
<thead>
<tr>
<th>Clinical response</th>
<th>PR (n = 34)</th>
<th>StbD (n = 47)</th>
<th>PD (n = 9)</th>
<th>PIa</th>
<th>PIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53 wild-type (n = 64)</td>
<td>23 (36%)</td>
<td>37 (58%)</td>
<td>4 (6%)</td>
<td>0.098</td>
<td>0.063</td>
</tr>
<tr>
<td>TP53 mutations (all, n = 26)</td>
<td>11 (42%)</td>
<td>10 (39%)</td>
<td>5 (19%)</td>
<td>0.014</td>
<td>0.008</td>
</tr>
<tr>
<td>TP53 mutations affecting L2/L3 (n = 19)</td>
<td>8 (42%)</td>
<td>6 (32%)</td>
<td>5 (26%)</td>
<td>0.028</td>
<td>0.025</td>
</tr>
<tr>
<td>TP53 mutations non-missense* (n = 10)</td>
<td>5 (50%)</td>
<td>2 (20%)</td>
<td>3 (30%)</td>
<td>0.793</td>
<td>0.561</td>
</tr>
<tr>
<td>TP53 mutations missense affecting L2/L3 (n = 9)</td>
<td>3 (33%)</td>
<td>4 (45%)</td>
<td>2 (22%)</td>
<td>0.436</td>
<td>0.198</td>
</tr>
<tr>
<td>Normal allele present (n = 22)</td>
<td>9 (41%)</td>
<td>11 (50%)</td>
<td>2 (9%)</td>
<td>0.990</td>
<td>0.946</td>
</tr>
<tr>
<td>LOH (n = 35)</td>
<td>12 (34%)</td>
<td>18 (52%)</td>
<td>5 (14%)</td>
<td>0.971</td>
<td>0.018</td>
</tr>
<tr>
<td>p53 IHC index &lt;6 (n = 70)</td>
<td>27 (39%)</td>
<td>36 (51%)</td>
<td>7 (10%)</td>
<td>0.075</td>
<td>0.023</td>
</tr>
<tr>
<td>p53 IHC index ≥6 (n = 19)</td>
<td>7 (37%)</td>
<td>10 (53%)</td>
<td>2 (10%)</td>
<td>0.436</td>
<td>0.198</td>
</tr>
<tr>
<td>c-erbB-2 IHC intensity, 0 or 1± (n = 72)</td>
<td>27 (37%)</td>
<td>40 (56%)</td>
<td>5 (7%)</td>
<td>0.436</td>
<td>0.198</td>
</tr>
<tr>
<td>c-erbB-2 IHC intensity, 2± or 3± (n = 17)</td>
<td>7 (41%)</td>
<td>6 (35%)</td>
<td>4 (24%)</td>
<td>0.436</td>
<td>0.198</td>
</tr>
<tr>
<td>bcl-2 IHC index &lt;6 (n = 46)</td>
<td>17 (37%)</td>
<td>21 (46%)</td>
<td>8 (17%)</td>
<td>0.436</td>
<td>0.198</td>
</tr>
<tr>
<td>bcl-2 IHC index ≥6 (n = 43)</td>
<td>17 (40%)</td>
<td>25 (58%)</td>
<td>1 (2%)</td>
<td>0.058</td>
<td>0.018</td>
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<tr>
<td>Gi (n = 23)</td>
<td>11 (48%)</td>
<td>11 (48%)</td>
<td>1 (4%)</td>
<td>0.436</td>
<td>0.198</td>
</tr>
<tr>
<td>GII (n = 42)</td>
<td>15 (36%)</td>
<td>25 (59%)</td>
<td>2 (5%)</td>
<td>0.436</td>
<td>0.198</td>
</tr>
<tr>
<td>GIII (n = 25)</td>
<td>8 (32%)</td>
<td>11 (44%)</td>
<td>6 (24%)</td>
<td>0.436</td>
<td>0.198</td>
</tr>
</tbody>
</table>

*P in regard to clinical response comparing PR, StbD, PD.
*P in regard to clinical response comparing PR + StbD versus PD.
*Wild-type versus all TP53 mutations.
*TP53 mutations affecting the L2/L3 domains versus wild-type plus TP53 mutations not affecting the L2/L3 domains.
*TP53 mutations including nonsense, splice, deletion, frame shift, and in-frame mutations.
*LOH, analysed for 60 patients; informative for 57 patients.
*Wild-type versus all TP53 mutations.
*Membranous c-erbB-2 immunostaining given as staining intensity; 2± or 3± considered positive.
*bcl-2 immunohistochemistry given as staining index.
*Histological grade I–III.
*P comparing the distribution of histological grade in the three response groups (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 (heterozygous) for one or both of the two markers used, one variable number tandem repeat in intron 1 (44) 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 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. Nuclear lymphocytes in the perinuclear area 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 χ2 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...
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 3 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 ($P = 0.032$). 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.

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### Table 3 Characteristics of patients with primary PD

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>TP53 gene mutation</th>
<th>Affecting L2/L3 domain</th>
<th>LOH</th>
<th>p53 IHC</th>
<th>LOH</th>
<th>Histological grade</th>
<th>Outcome</th>
</tr>
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<tbody>
<tr>
<td>11</td>
<td>Yes</td>
<td>Yes</td>
<td>ND</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>D15</td>
</tr>
<tr>
<td>19</td>
<td>Yes</td>
<td>Yes</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>3</td>
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<tr>
<td>26</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>0</td>
<td>3</td>
<td>3</td>
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<tr>
<td>48</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>0</td>
<td>1</td>
<td>3</td>
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<tr>
<td>57</td>
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<td>Yes</td>
<td>ND</td>
<td>9</td>
<td>3</td>
<td>3</td>
<td>D10</td>
</tr>
<tr>
<td>65</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>0</td>
<td>1</td>
<td>3</td>
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<tr>
<td>95</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>9</td>
<td>1</td>
<td>2</td>
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<td>No</td>
<td>No</td>
<td>0</td>
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<td>No</td>
<td>Yes</td>
<td>1</td>
<td>0</td>
<td>9</td>
<td>F22</td>
</tr>
</tbody>
</table>

* p53 immunohistochemistry given as staining index.
* LOH: loss of heterozygosity.
* bcl-2: bcl-2 expression.

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### Table 4 Correlations between different parameters evaluated as Ps

<table>
<thead>
<tr>
<th>TP53 mutations</th>
<th>LOH</th>
<th>Intensity</th>
<th>Index</th>
<th>Intensity</th>
<th>Index</th>
<th>&lt;0.001</th>
<th>0.003</th>
<th>0.003</th>
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<tbody>
<tr>
<td>0.021</td>
<td>0.003</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.003</td>
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<tr>
<td>0.056</td>
<td>0.002</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.005</td>
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<tr>
<td>0.031</td>
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<td>0.001</td>
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<td>0.117</td>
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<td>0.548</td>
<td>0.181</td>
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* p53 immunohistochemistry given as staining intensity and staining index.
* LOH: loss of heterozygosity.

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### Table 5 Mutations grouped as wild-type versus all mutations.

<table>
<thead>
<tr>
<th>TP53 mutations</th>
<th>p53 IHC</th>
<th>bcl-2 IHC</th>
<th>c-erbB-2 IHC</th>
<th>Tumor grade</th>
<th>Tumor stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.021</td>
<td>0.003</td>
<td>0.003</td>
<td>&lt;0.001</td>
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<td>0.219</td>
</tr>
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<td>0.056</td>
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<td>0.005</td>
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<td>0.002</td>
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<td>0.117</td>
<td>0.087</td>
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<tr>
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<td>0.548</td>
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<td>0.181</td>
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</table>

* p53 immunohistochemistry given as staining intensity and staining index.
* LOH: loss of heterozygosity.
* TP53 mutations grouped as wild-type versus all mutations.
* TP53 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).
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 Kandoler-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 alterations to chemoresistance in breast cancer. 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-

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**Table 5 LOH in relation to response to therapy**

<table>
<thead>
<tr>
<th>Clinical response</th>
<th>PR (n = 21)</th>
<th>StD (n = 29)</th>
<th>PD (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53 gene mutations aff. L2/L3 and LOH&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4/5</td>
<td>5/6</td>
<td>3/3</td>
</tr>
<tr>
<td>TP53 gene mutations not aff. L2/L3 and LOH&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2/3</td>
<td>3/4</td>
<td>0/0</td>
</tr>
<tr>
<td>LOH without TP53 gene mutation&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6/13</td>
<td>10/19</td>
<td>2/4</td>
</tr>
</tbody>
</table>

<sup>a</sup> TP53 gene mutations affecting L2 or L3 domains with concomitant LOH.
<sup>b</sup> TP53 mutations not affecting L2 or L3 domains with concomitant LOH.
<sup>c</sup> LOH without TP53 mutation.

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 NH2-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-

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**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.
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 TP53 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 affects 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. Eke, B. Nordanger, H. Berntsen, B. Leirvaag, L.-M. Jorgensen, and P. Vu. We also thank R. Skjarven for the statistical support needed to perform the survival analysis.

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

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