Correlation of p53 status with outcome of neoadjuvant chemotherapy using paclitaxel and doxorubicin in stage IIIB breast cancer

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Background: The role of p53 in modulating apoptosis has suggested that it may affect efficacy of anticancer agents. We prospectively evaluated p53 alterations in 73 patients with locally advanced breast cancer (IIIB) submitted to neoadjuvant chemotherapy.

Patients and methods: Patients received three cycles of paclitaxel (175 mg/m2) and doxorubicin (60 mg/m2) every 21 days. Tumor sections were analyzed before treatment for altered patterns of p53 expression using immunohistochemistry and DNA sequencing.

Results: An overall response rate of 83.5% was obtained, including 15.1% complete pathological responses. The regimen was well tolerated with 17.7% grade 2/3 nausea and 12.8% grade 3/4 leukopenia. There was a statistically significant correlation between response and expression of p53. Of the 25 patients who obtained a complete clinical response, two were classified as positive ($P = 0.004$, $\chi^2$-square). Of 11 patients who obtained a complete pathological remission, one was positive ($P = 0.099$, $\chi^2$-square).

Discussion: The combination is highly effective in locally advanced breast cancer. A negative expression of p53 indicates a higher chance of responding to this regimen. The p53 status may be used as a biological marker to identify those patients who would benefit from more aggressive treatments.

Key words: breast cancer, doxorubicin, locally advanced, p53, paclitaxel

Introduction

A combination of doxorubicin and paclitaxel has been tested in a variety of schedules and sequences to exploit the high therapeutic potential of the two drugs in metastatic breast cancer [1]. Initial studies indicated that the tolerability of the combination was sequence-dependent if paclitaxel was infused over at least 24 h, independently of the schedule for doxorubicin administration [2]. In order to use the combination as an outpatient treatment, a dose- and sequence-finding trial was performed at the National Cancer Institute of Milan. Taking advantage of the safety and feasibility of a short infusion of paclitaxel, the study showed that tolerability did not depend on sequence and that the combination was highly effective in metastatic breast cancer [3].

Although the interactions of paclitaxel with the cytoskeleton are well characterized, the molecular mechanisms by which such an interaction leads to cell cycle arrest and cytotoxicity are not well understood. Recent evidence suggests that paclitaxel alters certain intracellular signal-transduction events, such as activation of mitogen-activated protein kinase and transcriptional activation of genes encoding a number of cytokines [4, 5]. In breast cancer, mutations in the p53 gene have been demonstrated by our group and others as the most frequently observed single gene alteration [6]. DNA damage caused by various chemotherapeutic agents leads to an increase in the level of tumor-suppressor gene p53, followed by a G1 cell cycle arrest, and subsequently apoptosis. The p53 protein is a multifunctional transcriptional regulator involved in the cellular response to DNA damage, and has been implicated as a putative determinant of tumor cell sensitivity to cytotoxic agents [7, 8].

We conducted a prospective, non-randomized trial to investigate the efficacy and feasibility of a combination of paclitaxel and doxorubicin as preoperative treatment for locally advanced breast cancer and the impact of the expression of the tumor-suppressor gene p53 on response rate and overall survival.

Patients and methods

Between October 1995 and September 1999, 73 women who had primary locally advanced breast cancer (IIIB, T4b, N1–2, M0, according to the tumor, node, metastasis staging system) were enrolled in the trial. The median tumor size of this population was 8.4 cm by physical examination and mammography. All patients received an incisional biopsy to provide a histological diagnosis. The protocol was approved by the institutional internal review board and all patients gave written informed consent before entering the trial.

Patients were required to have histological proof of invasive ductal carcinoma, to be at least 18 years of age, have a performance status of 90% by the
Karnofsky scale, have a serum bilirubin level <0.5 mg/dl, serum creatinine level <1.5 mg/dl, absolute granulocyte count ≥1500/mm², platelet count ≥100'000/mm² and normal cardiac function. All patients performed an ejection fraction test (multigated acquisition scan) before the first cycle and 3 weeks after the third cycle of chemotherapy. Mammographies, computed tomography scans of the chest and abdomen and bone scans were performed before entering the trial to ensure the absence of metastatic disease.

Treatment schedule
Following standard premedication with glucocorticoids and histamine type H1/H2 receptor blockers, doxorubicin (60 mg/m²) was administered as a bolus infusion followed by paclitaxel (175 mg/m²) infused over 3 h. Chemotherapy was given every 3 weeks for three cycles. Upon the completion of chemotherapy, all patients were submitted to a modified radical mastectomy with axillary dissection. After recovery from surgery, all patients received systemic chemotherapy and adjuvant tamoxifen for 5 years when the estrogen receptor was positive. All patients underwent external beam irradiation using a 6 MeV linear accelerator.

Evaluation of tumor response
The size of primary breast tumors was determined immediately before administration of each cycle of chemotherapy and before surgery. Before the first cycle and in the week before surgery, a mammography was performed. At each assessment, the product of the two greatest perpendicular diameters was used to quantify the tumor. In the absence of clinical evidence of tumors in the breast, response to therapy was categorized as a complete clinical response (cCR). When the diameters decreased by 50% or more, the response was judged to be a partial response (cPR). Patients with reductions between 25% and less than 50% were categorized as having stable disease (cSD). Progression of disease was categorized as an increase of at least 25%. Surgical specimens were evaluated for their pathological tumor status and were further classified as complete pathological responders with no histological evidence of invasive tumor cells or with histological evidence of invasive cells.

Determination of p53 status
Immunostaining was performed on histological sections prepared from the biopsy sample taken before treatment. Buffered-formalin-fixed, paraffin-embedded tissue sections were analyzed immunohistochemically for altered patterns of p53 expression, using a standard streptavidin–biotin technique. Sections (4 µm thick) were deparaffinized in xylene, rehydrated in a graded ethanol series, and incubated in 3% hydrogen peroxide for 20 min. Specimens were then placed in a plastic Coplin jar containing citrate buffer and heated in a microwave processor at 95°C. After the microwave processing, sections were left at room temperature for 30 min. Specimens were covered with normal goat serum for 15 min to reduce non-specific staining and incubated with a 1:100 dilution of primary antibody D0-7 (Dako Co., Carpinteria, CA, USA) at room temperature overnight. Sections were washed with Tris-buffered saline, and then covered with a 1:100 dilution of streptavidin–biotin–peroxidase complex at room temperature for 30 min. The antibody was localized with 3′,3′-diaminobenzidine tetrahydrochloride. Tissue sections were counterstained with Harris’ hematoxylin, dehydrated with ethanol, and mounted under a coverslip. Immunohistochemical staining of tumors with this antibody shows primarily a nuclear localization of p53 staining.

The staining results were interpreted independently by one pathologist who was unaware of the clinical outcome. In each case, the entire section was systematically examined under high-power fields (×40) for p53 immunoreactivity. Among all immunoreactive nuclei, only those clearly and strongly immunostained were recorded as being p53-positive. The level of immunoreactivity was expressed as the percentage of p53-positive cancer cell nuclei. Staining was considered positive if at least 10% of tumor cell nuclei stained positively compared with corresponding controls.

Samples classified as positive were also analyzed at the molecular level by DNA sequencing. Genomic DNA was extracted from paraffin blocks and sequences corresponding to the functional domains L2, L3 and the loop-sheet-helix of the p53 protein were amplified by polymerase chain reaction. DNA sequencing was performed using the ABI Prism™ 377 DNA Sequencer (Perkin-Elmer, Wellesley, MA) and the DNA Sequencing Kit-Big Dye Terminator Cycle Sequencing (Perkin-Elmer) as described elsewhere [8]. Patients were cross-classified by p53 expression and by clinical responses to chemotherapy.

Statistical analysis
For statistical analysis, differences in proportions were evaluated by the χ²-square test or Fisher’s exact test. Survival was estimated by use of Kaplan–Meier method, and differences between groups were tested by the log-rank test. For all statistical tests, differences were considered as significant at P <0.05.

Results
Response to chemotherapy
All patients completed the planned three cycles of therapy and therefore were assessable for overall clinical loco-regional tumor response. A cCR as previously defined was documented in 25 patients (34.2 %). Thirty-six patients (49.3 %) achieved a CPR and 12 patients (16.4 %) were classified as cSD, resulting in an overall response rate to the regimen of 83.5 %. We did not observe any cases of progressive disease during treatment.

Pathological examination of breast tissue from all 73 patients showed no evidence of residual cancer in 11 specimens (15.1 %) and only non-invasive tumor (ductal carcinoma in situ) in three (4.11 %).

Toxicity
Chemotherapy was generally well tolerated. In 219 delivered cycles, grade 2/3 nausea and vomiting was present in 16.8 %, grade 3/4 leukopenia in 13.2 %. Eight patients required hospitalization and intravenous antibiotics due to febrile neutropenia. No grade 3/4 mucositis was observed. Alopecia was universal in all patients. There were no toxicity-related deaths.

The median ejection fraction before chemotherapy was 66% (range 54–84%). After the three cycles of chemotherapy the median ejection fraction was 62% (range 44–76%). Seven patients (9.6 %) had a cardiac ejection fraction test below 50% after the three cycles of treatment. One patient developed cardiac failure, requiring clinical intervention 10 months after completion of treatment.

This relatively good tolerance of the drug regimen was reflected in the high relative dose intensity that could be achieved during the three cycles of primary chemotherapy, with 90.4% of the cycles being delivered on the scheduled date.
Correlation of p53 protein expression with response and survival

A high level of p53 immunoreactivity was seen in 22 of 73 patients (30.1%). Direct sequencing with these tumors identified two mutations on codon 259, causing an amino acid change from asparagine (GAC) to tyrosine (TAC).

The response to chemotherapy was correlated with p53 expression as shown in Table 1, where 23 patients with p53-negative tumors obtained a complete clinical remission compared with two patients with p53-positive tumors ($P = 0.004$, $\chi^2$-square). There was also a trend towards statistical significance when p53 expression was correlated with the achievement of a complete pathological response. Eleven patients achieved a complete pathological response, of whom 10 were classified as p53-negative ($P = 0.099$, $\chi^2$-square).

Overall survival was also influenced by p53 expression, showing a statistical advantage for those patients with p53-negative tumors ($P = 0.0023$, log-rank) as shown in Figure 1. The overall survival is also changed among those patients who achieved a complete pathological response as shown in Figure 2, with $P = 0.017$ (log-rank).

Discussion

Paclitaxel is one of the most promising anticancer agents for the therapy of breast cancer, where it has shown activity also in tumors resistant to doxorubicin [9]. The combination of both drugs resulted in high response rates in metastatic disease, with no impact, however, on overall survival and on disease-free survival [10–12]. This combination has been previously used in the neoadjuvant setting by Moliterni et al. [13] who reported an overall response rate of 88%. Notable in this trial is that only 41% of the women were clinically staged as having locally advanced disease, favoring the high response rate in more initial stages. In our trial we achieved an overall response rate of 83.5%, including 15.1% complete pathological response, confirming the high efficacy of this regimen. It is, therefore, important to understand if there are cellular factors that can play a role in determining the response of breast tumors to the combination of paclitaxel and doxorubicin. The p53 protein plays a central role in the response to anticancer treatment. It has, in fact, been shown that in different cell types, the presence of a wild-type p53 induces sensitization to DNA-damaging agents, although more recent evidence of a wild-type p53-induced chemoresistance has been described.

The role of p53 in the intrinsic sensitivity of human cancer cells to paclitaxel remains controversial. While paclitaxel resistance is primarily conferred by the tubulin mutations, the loss of functional p53 observed in some cell lines suggests that this loss may facilitate the development of resistance potentially by providing a clonal advantage which promotes the isolation of paclitaxel-resistant cells [14]. In a study published by the European Organization for Research and Treatment of Cancer, 114 women with metastatic breast cancer were treated with paclitaxel or doxorubicin and all specimens were assessed by immunohistochemistry using monoclonal antibodies against human epidermal growth factor receptor-2, p53 and Bcl-2. The results were correlated with clinical response to therapy and the authors found no correlation between the expression of any of the markers and the clinical response to either agent [15]. Paclitaxel, which does not interact directly with DNA, was found to be able to activate p53 in some cell types, and this increase has been associated mainly with its ability to activate the Raf-1 cascade [16]. In other cell types, including one human ovarian cancer cell line, p53 expression was not increased after paclitaxel treatment, and the presence of a wild-type p53 observed in some cell types, and this increase has been associated mainly with its ability to activate the Raf-1 cascade [16]. In other cell types, including one human ovarian cancer cell line, p53 expression was not increased after paclitaxel treatment, and the presence of a wild-type p53 did not result in a change in sensitivity to paclitaxel with respect to cells expressing mutated p53 [17]. Recently, the presence of wild-type p53 has been reported to decrease the cytotoxicity of paclitaxel compared with the same cell lines not expressing wild-type p53. This was explained on the basis of a p53-dependent block in $G_1$ after treatment that would prevent the

Table 1. p53 expression and response to preoperative chemotherapy

<table>
<thead>
<tr>
<th>Clinical response</th>
<th>Stable disease</th>
<th>Partial response</th>
<th>Complete response</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53-positive</td>
<td>3</td>
<td>17</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>p53-negative</td>
<td>9</td>
<td>19</td>
<td>23*</td>
<td>51</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>36</td>
<td>25</td>
<td>73</td>
</tr>
</tbody>
</table>

*P = 0.004, $\chi^2$-square test.
cell from progressing to G2–M, where paclitaxel is known to exert its activity [17, 18]. Another report, however, showed that disruption of wild-type p53 did reduce the cytotoxicity induced by paclitaxel [19].

Several studies have shown overexpression of p53 to be a strong prognostic indicator in infiltrating ductal carcinoma, although a recent consensus statement by the College of American Pathologists categorizes p53 protein overexpression a category two parameter for this reason. This category means that the clinical relevance should be tested in well-designed studies with validation of the methodology in individual laboratories [20]. p53 immunohistochemistry assays detect overexpression of the gene, which is often related to conformational alterations and a prolonged half-life of the encoded protein. p53 mutations which generate truncated proteins, like nonsense and splicing mutations, do not correlate with p53 overexpression [21], but this kind of mutation is observed only in a minority of cases [22]. Routine sequencing of the p53 gene in all breast cancers would be highly costly and time consuming in daily practice, and it is not so easy. So, the validation of immunohistochemistry is the most critical step in using it as a prognostic factor [23]. In our laboratory, the p53 immunohistochemical procedure is very well standardized, and only cases with distinct and strong nuclear staining of more than 10% of nuclei were considered positive.

In our study, the response to chemotherapy was correlated with p53 expression, with a significant statistical advantage among those patients with p53-negative tumors. The same advantage was observed when p53 expression was correlated with the odds of a complete pathological response. Since overall survival is changed among those patients who achieved a complete pathological response, determination of the p53 status may be used as a biological marker to identify those patient who would benefit from more aggressive treatments.

Acknowledgements

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References