Worse survival for TP53 (p53)-mutated breast cancer patients receiving adjuvant CMF


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Background: TP53 has been described as a prognostic factor in many malignancies, including breast cancer. Whether it also might be a predictive factor with reference to chemo- and endocrine therapy is more controversial.

Patients and methods: We investigated relapse-free (RFS), breast cancer-corrected (BCCS) and overall survival (OS) related to TP53 status in node-positive breast cancer patients that had received polychemotherapy [cyclophosphamide, methotrexate, 5-fluorouracil (CMF)] and/or endocrine therapy (tamoxifen). Sequence analyses of the whole TP53 coding region was performed in 376 patients operated on for primary breast cancer with axillary lymph node metastases between 1984 and 1989 (median follow-up time 84 months).

Results: TP53 mutations were found in 105 patients (28%). We found 90 (82%) of the 110 mutations in the more frequently analysed exons 5–8, while the other 20 (18%) were located in exons 3–4 and 9–10, respectively. Univariate analyses showed TP53 to be a significant prognostic factor with regard to RFS, BCCS and OS in patients who received adjuvant CMF.

Conclusions: TP53 mutations might induce resistance to certain modalities of breast cancer therapy. Sequence-determined TP53 mutation was of negative prognostic value in the total patient population and in the CMF treated patients.

Key words: adjuvant therapy, CMF, p53, sequence-based analysis, tamoxifen, TP53

Introduction

CMF (cyclophosphamide, methotrexate, 5-fluorouracil)-based regimens have been used as standard postoperative therapy for primary breast cancers, resulting in a relative mortality reduction of 27% [1]. Adjuvant tamoxifen administered to pre- and postmenopausal patients with hormone receptor-positi
tive breast cancers presents similar survival improvements [2]. The tumour suppressor gene TP53 (also known as p53) is critical for cell cycle control and apoptosis. TP53 status has been described as a prognostic factor associated with worse outcome in a variety of malignancies, including breast cancer, in which TP53 mutations or increased levels of protein have been found in ~20% to 30% of primary tumours [3]. The TP53 mutation frequency is higher in node-positive breast cancer [4]. In addition, some studies have claimed a prognostic and or predictive value for TP53 in relation to different cytotoxic agents and tamoxifen [5–8], while other studies have failed to demonstrate such correlations [9–12]. Hopefully, new TP53-reactivating strategies, by themselves and together with already known drugs like CMF, will help in the improvement of therapy response in patients with TP53-mutated tumours [13]. In this paper we describe the prognosis and prognostic value of TP53 mutations following adjuvant CMF and tamoxifen therapy in a population-derived breast cancer cohort of 376 women. Sequencing methodology of all protein coding exons of the TP53 gene was used, since previous studies have demonstrated superior results compared with immunohistochemistry [7, 14].

Patients and methods

Study population

The study was approved by the local ethics committee at the University of Gothenburg. Women with a node-positive primary breast cancer diagnosis from 1 January 1984 to 31 December 1989 were enrolled in the study. Patients receiving preoperative therapy or having a history of another
invasive malignancy were excluded from the study population. Our cohort of patients consisted of 376 women with a histopathologically verified invasive and axillary lymph node-positive breast cancer who had received adjuvant therapy. Median age at diagnosis was 60 years. The median tumour size was 25 mm. According to the medical records, 80 patients (21%) were premenopausal, 268 patients (71%) postmenopausal and 19 patients (5%) perimenopausal. We lacked information on menopausal status in nine women. Ductal carcinoma was the most common histopathological type (89%) and most tumours were poorly differentiated (60%). Patient and tumour characteristics are listed in Table 1. The patients' records were reviewed without any knowledge of $TP53$ status.

Locoregional therapy

The surgical treatment was modified radical mastectomy (339 patients) or breast conserving procedure (36 patients), both performed together with axillary dissection. Postoperative adjuvant radiotherapy was used in only 42 patients, of whom 29 had undergone breast conserving surgery with axillary dissection.

Primary systemic therapy

In this cohort of 376 women, 193 patients received systemic adjuvant chemotherapy. The majority (174 patients) received polychemotherapy with intravenous methotrexate and 5-fluorouracil together with oral cyclophosphamide (CMF) over 3–6 months. Adjuvant endocrine therapy was given to 251 women; 137 (of whom 127 were postmenopausal) received tamoxifen alone at doses of 20 mg/day. Another 46 women (of whom 44 were postmenopausal) received tamoxifen therapy alone, but at doses of 10, 30 or 40 mg/day. Sixty-eight patients (of whom 61 were postmenopausal) were treated with both adjuvant CMF and tamoxifen.

Clinical follow-up

The patients were mostly seen at regular outpatient visits at the different Departments of Surgery in the Gothenburg region and/or at the Department of Oncology at the Sahlgrenska University hospital during the first 5 years after breast cancer diagnosis. Women participating in prospective and randomised clinical studies (141 patients in the International Breast Cancer Study Group V–VII) were controlled according to the routines described for each study. Patients treated outside the clinical studies had laboratory and X-ray investigations taken on the basis of clinical signs and symptoms. Deaths were classified as death caused by breast cancer (145 patients), death due to other cause with signs of active breast cancer (seven patients) and death from another cause without signs of breast cancer (44 patients), based on medical records and death certificates.

Tumour material

All operated primary breast cancers were routinely brought, as soon as possible, on ice, to the Department of Pathology, Eastern University Hospital, Gothenburg, and were handled by one pathologist, Dr Johan Savel-Soderberg (deceased before the initiation of this study). Tumour material was removed for routine histopathology and locoregional staging. Additional tumour pieces were removed for receptor analyses and for future use (this material was stored at $-70^\circ$C). These were the standard procedures for all breast cancers in the Gothenburg area from 1983 and throughout the whole study period.

Sequence-based analysis of $TP53$ status

The details of this procedure have been published in detail elsewhere [14]. Since that publication, minor modifications and improvements have been introduced, described in more detail as follows. Total RNA was extracted

Table 1. Patient and tumour characteristics

<table>
<thead>
<tr>
<th>Covariate</th>
<th>All patients (n = 376) [n (%)]</th>
<th>CMF (n = 125) [n (%)]</th>
<th>TAM (n = 183) [n (%)]</th>
<th>CMF–TAM (n = 68) [n (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis, years (range)</td>
<td>60 (27–94)</td>
<td>47 (27–70)</td>
<td>69 (35–94)</td>
<td>60 (46–71)</td>
</tr>
<tr>
<td>Tumour size, mm (range)</td>
<td>25 (10–100)</td>
<td>26 (6–70)</td>
<td>25 (8–100)</td>
<td>20 (10–80)</td>
</tr>
<tr>
<td>Lymph node metastases</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–3</td>
<td>201 (53)</td>
<td>59 (47)</td>
<td>107 (58)</td>
<td>35 (51)</td>
</tr>
<tr>
<td>4–9</td>
<td>98 (26)</td>
<td>36 (29)</td>
<td>46 (25)</td>
<td>16 (24)</td>
</tr>
<tr>
<td>&gt;9</td>
<td>77 (20)</td>
<td>30 (24)</td>
<td>30 (16)</td>
<td>17 (25)</td>
</tr>
<tr>
<td>ER positive</td>
<td>286 (76)</td>
<td>78 (62)</td>
<td>159 (87)</td>
<td>49 (72)</td>
</tr>
<tr>
<td>PR positive</td>
<td>202 (54)</td>
<td>55 (44)</td>
<td>116 (63)</td>
<td>31 (46)</td>
</tr>
<tr>
<td>Level of differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>3 (1)</td>
<td>26 (21)</td>
<td>2 (1)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Medium</td>
<td>108 (29)</td>
<td>84 (67)</td>
<td>57 (31)</td>
<td>25 (37)</td>
</tr>
<tr>
<td>Low</td>
<td>224 (60)</td>
<td>15 (12)</td>
<td>105 (57)</td>
<td>35 (51)</td>
</tr>
<tr>
<td>Unknown</td>
<td>41 (11)</td>
<td>(10)</td>
<td>(10)</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ductal</td>
<td>336 (89)</td>
<td>114 (91)</td>
<td>165 (90)</td>
<td>57 (84)</td>
</tr>
<tr>
<td>Lobular</td>
<td>27 (7)</td>
<td>6 (5)</td>
<td>12 (7)</td>
<td>9 (13)</td>
</tr>
<tr>
<td>Other</td>
<td>10 (3)</td>
<td>(4)</td>
<td>4 (2)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Unknown</td>
<td>3 (1)</td>
<td>(1)</td>
<td>(1)</td>
<td></td>
</tr>
<tr>
<td>TP53 mutation</td>
<td>105 (28)</td>
<td>46 (37)</td>
<td>40 (22)</td>
<td>19 (28)</td>
</tr>
</tbody>
</table>

CMF, cyclophosphamide; methotrexate, 5-fluorouracil; TAM, tamoxifen; ER, estrogen receptor; PR, progesterone receptor.
from frozen tumour samples under stringent conditions (QuickPrep™ Total RNA Extraction Kit; Amersham-Pharmacia Biotech, Uppsala, Sweden). This procedure allowed the exclusion of phenol and chloroform. This procedure was followed by an enzymatic synthesis of cDNA by PCR, using four overlapping primer pairs covering the entire coding region of the TP53 gene. Two of 12 primers were altered compared with the original publication; both of them covered fragment 1 [14]. The used PCR primer for fragment 1 had the following sequence: B-5′-TCC ACG ACG GTG ACA CGC TTC-3′. The sequence primer consisted of the following basepairs: C-5′-GGA GTA CGT GCA AGT CAC A-3′. One of the primers in each primer pair was modified with biotin. Thus, biotin-labelled PCR products were generated, facilitating solid-phase sequencing [15]. The fluorescence-labelled primers were this time labelled with Cy5, owing to the fact that the ALF express sequencer was used instead of the original ALF version. A manifold-based version of solid phase sequencing was utilised, essentially as described by Lagerkvist et al. [16]. The sequencing products generated were analysed using an automated laser fluorescence sequencer (ALFexpress™; Amersham-Pharmacia Biotech). The generated sequence was then compared with the sequence of the following basepairs: C-5′-GGA GTA CGT GCA AGT CAC A-3′. When the mutation was located in an overlapping segment.

Statistical methods

Survival was estimated using the Kaplan–Meier method and log-rank tests were performed for comparison of survival between groups. To estimate simultaneous effects by different factors on survival, Cox proportional hazard regression models were used. We included the factors TP53 status, age at diagnosis, tumour size, number of positive lymph nodes (0–3 versus ≥4), estrogen (ER) and progesterone (PR) receptor status, S-phase and level of differentiation (low versus medium and high). All factors were dichotomised in the analyses except for tumour size and age at diagnosis, which were analysed as continuous variables. All hazard ratios (HRs) reported in this paper are derived from these estimated models. Survival was measured from date of diagnosis. In breast cancer-corrected survival (BCCS), dates of death from other causes were handled as censored observations in the analyses. In relapse-free survival (RFS), all end points except for recurrences were handled as censored observations.

Results

Median follow-up in the study was 7 years (maximum 12 years); at the latest follow-up, 180 patients were still alive and 145 patients had died of breast cancer. Seven out of the 51 women who had died due to other causes had signs of active breast cancer. cDNA sequencing of the whole coding region of the TP53 gene was successful in 370 of the 376 patients and mutations were found in the primary breast cancers from 105 patients (28%). We detected 66 missense mutations, six stop codons, four and 16 deletions in- and out-of-frame, respectively, and two and six insertions in- and out-of-frame, respectively. In five tumours there were two different types of mutations (missense mutation and a stop codon; missense mutation and deletion in-frame; deletion in-frame and insertion in-frame; two different deletions out-of-frame; deletion out-of-frame and insertion in-frame). Analyses of the mutation sites showed 44 of the mutations to be located outside the evolutionarily conserved regions, and 10, 12, 18 and 16 mutations within the evolutionarily conserved regions 2, 3, 4 and 5, respectively. We found 20 (18%) of the 110 mutations in exons 3–4 and 9–10, respectively. The other 90 mutations (82%) were located in the more frequently analysed exons 5–8.

TP53 status and survival analyses

We analysed the total patient population using RFS, BCCS and overall survival (OS). The same analyses were performed in three subgroups based on given adjuvant therapy: tamoxifen therapy only (TAM), CMF only (CMF) and CMF together with tamoxifen (CMF–TAM), respectively. In all patients and in the CMF treated patients TP53 status was a statistically significant prognostic factor with regard to RFS (P = 0.004 and P = 0.01 respectively; not shown). TP53 status was also of statistically significant importance when censoring for BCCS in all patients (P < 0.0001; not shown) and in the CMF group (P = 0.001; not shown). Patients with TP53 mutations had statistically significantly worse OS compared with wild-type TP53 patients (P = 0.0005; Figure 1). Worse OS for women

![Graphs showing survival outcomes](chart.png)
with TP53-mutated tumours was also seen in patients treated with CMF (P = 0.001; Figure 1).

In ER-positive TAM-treated patients (n = 159), TP53 revealed a borderline significant value with reference to BCCS (P = 0.05; not shown), while non-significant results were obtained for RFS and OS. TP53 status was of no significant importance with reference to RFS, BCCS and OS in the ER-negative tamoxifen-treated patients (n = 22).

In ER-positive patients (n = 286), regardless of given adjuvant therapy, univariate analyses showed TP53 to be a significant prognostic factor with regard to BCCS (P = 0.003; not shown) and OS (P = 0.009; not shown), but not RFS. In patients with ER-negative tumours (n = 82), regardless of given adjuvant therapy, TP53 was of no significant importance for RFS, BCCS or OS.

**Discussion**

At present, the role of TP53 as a predictive factor for treatment in breast cancer is unclear [8, 17]. Our study demonstrates that TP53 status is a prognostic factor in node positive patients receiving adjuvant CMF with reference to RFS, BCCS and OS (not confirmed in multivariate analyses), and we were able to confirm the previously observed negative

<table>
<thead>
<tr>
<th>Variable</th>
<th>All patients (n = 376)</th>
<th>CMF (n = 125)</th>
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<tbody>
<tr>
<td></td>
<td>HR 95% CI</td>
<td>P</td>
</tr>
<tr>
<td>TP53 mutation</td>
<td>1.33 0.92–1.93</td>
<td>NS</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>1.01 1.00–1.02</td>
<td>NS</td>
</tr>
<tr>
<td>Tumour size</td>
<td>1.02 1.01–1.03</td>
<td>0.001</td>
</tr>
<tr>
<td>≥4 positive lymph nodes</td>
<td>1.49 1.06–2.09</td>
<td>0.02</td>
</tr>
<tr>
<td>ER (positive)</td>
<td>0.62 0.40–0.98</td>
<td>0.04</td>
</tr>
<tr>
<td>PR (positive)</td>
<td>0.92 0.62–1.37</td>
<td>NS</td>
</tr>
<tr>
<td>S-phase (low)</td>
<td>0.50 0.20–1.24</td>
<td>NS</td>
</tr>
<tr>
<td>Level of differentiation (low)</td>
<td>0.69 0.47–1.01</td>
<td>NS</td>
</tr>
</tbody>
</table>

RFS, relapse-free survival; CMF, cyclophosphamide, methotrexate, 5-fluorouracil; HR, hazard ratio; CI, confidence interval; NS, not significant; ER, estrogen receptor; PR, progesterone receptor.
prognostic value of mutated TP53 [4, 18, 19]. Specific TP53 mutations may be associated with a poor response to CMF and tamoxifen therapy, while others are not. However, the present study lacks the statistical power for mutation-specific therapy outcome analyses. Survival analyses are indirect assessments of the response to therapy, which is why a retrospective study such as this one cannot address the true predictive value of TP53. There are limitations in the interpretation of our data, due in part to the relatively small subsets of patients, despite the fact that the present study is one of the largest studies using sequence-based determination of the whole coding region of the TP53 gene.

The mutation frequency was higher in the CMF group compared with the other subgroups (TAM and CMF–TAM). CMF patients were younger at the time of diagnosis and presented with more ER-negative tumours, as well as more poorly differentiated tumours. In summary, they had a more aggressive biology. Perhaps a functional TP53 is of greater importance in patients with more aggressive tumours compared with patients with less aggressive tumours.

At the time that our patient cohort received their adjuvant treatment, the recommendation for tamoxifen therapy was based on menopausal status rather than on hormone receptor expression. For this reason, we also analysed the prognostic value of TP53 mutations in tamoxifen-treated ER-positive and -negative tumours, respectively, as the subgroups of CMF, TAM and CMF–TAM might be considered to be arbitrary. By means of Cox proportional hazard statistics, TP53 was of no importance with reference to survival analyses in tamoxifen-treated patients, regardless of whether they were ER-positive or -negative. In contrast to our present results, we previously reported a worse survival in patients with TP53 mutations receiving adjuvant tamoxifen and radiotherapy in a subgroup of a small number of node-positive patients (48 patients) [5]. Our present study has the advantage that only a few patients (42/376, 11%; Table 1) received adjuvant radiotherapy, which thus largely avoids an interaction between radiotherapy and TP53 status [20].

In some reports, TP53 status has been shown to predict poor response to tamoxifen in metastatic disease, as well as being a prognostic factor associated with worse survival after tamoxifen therapy in the adjuvant setting [7, 21]. In contrast, relatively recent reports have claimed that TP53 status is of no prognostic importance with regard to survival after tamoxifen therapy [22, 23]. However, in the latter studies the duration of tamoxifen therapy was short (1 year) or unknown, and in both reports immunohistochemistry was the method used for TP53 evaluation. Several explanations are available for the conflicting results in literature with regard to the predictive value of TP53 status, such as heterogeneity of the patient cohorts and tumours, as well as TP53 methodology and therapeutic regimens. In addition, other studies have been under-powered to track potential differences between patients with tumours containing mutant TP53 and those with wild-type TP53. A review article published in 2000 stated that there is inadequate evidence to support TP53 as a guide when selecting endocrine, chemo- and radiotherapy, which is also our opinion [17].

This study included 376 population-derived patients operated on for primary breast cancer with positive axillary nodes during 1984–1989 (median follow-up time 84 months). TP53 mutations were present in 28% of the patients, which is in good concordance with previous reports in patients with lymph node metastases demonstrating 20% to 30% TP53 mutations [3–5, 14]. Slight differences in mutation frequency could be due to different proportions of node-positive patients and detection methodology. Different techniques are available to determine the status of the TP53 gene: immunohistochemistry, cDNA-based sequencing and denaturing gradient gel electrophoresis, among others [14, 24–26]. At present, relatively few studies have used more optimal methods, such as cDNA-based sequencing, rather than IHC for TP53 determinations [7, 14, 19, 27, 28].

Three years ago, Vogelstein and Kinzler [13] reasoned that TP53 is an Achilles’ heel of cancer. Its tumour specificity and wild-type function indicate a possibility of exclusively targeting cancer cells with compounds and strategies that enhance the effect of available drugs. Studies like ours may help in determining the use and feasibility of such TP53 reactivating strategies. Our data imply that women with TP53-mutated breast cancer tumours should be offered treatments other than CMF. For example, Kandolier-Eckersberger et al. [29] indicate that patients with mutated TP53 might benefit from paclitaxel, while the response to FEC is dependent on wild type TP53, while other data show the reverse [8]. The predictive therapeutic value of TP53 needs to be further investigated and is presently being studied in the ongoing randomised EORTC p53 study [30].

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