Micrometastatic bone marrow cells at diagnosis have no impact on survival of primary breast cancer patients with extensive axillary lymph node involvement treated with stem cell-supported high-dose chemotherapy

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Background: To determine the impact of micrometastatic bone marrow cells (MMC) on survival in high-risk primary breast cancer (HRPBC) patients treated with high-dose chemotherapy (HDCT) and autologous stem cell transplantation (ASCT).

Patients and methods: Ninety-one HRPBC patients (73 patients with ≥10 involved axillary lymph nodes (ALN), 18 premenopausal women with ≥4 involved ALN) received one cycle (eight patients) or two cycles of HDCT and ASCT. Bone marrow aspiration was performed before systemic treatment to search for MMC using a cocktail of four monoclonal epithelial-specific antibodies (5D3, HEA125, BM7 and BM8). The influence of MMC and other prognostic factors on disease-free survival (DFS), distant DFS (DDFS), and overall survival (OS) was analysed.

Results: In 23 of 91 patients (25%) we detected a median of three MMC (range, 1–43) among 10⁶ mononuclear cells. With a median follow-up of 62 months (range, 10–117), the detection of MMC was not associated with DFS (P = 0.929), DDFS (P = 0.664) or OS (P = 0.642). In multivariate analysis the strongest predictor was nodal ratio for DFS (P = 0.012) and expression of p53 for OS (P < 0.001).

Conclusion: The detection of MMC at diagnosis has no impact on survival in HRPBC patients treated with HDCT and ASCT.

Key words: high-dose chemotherapy, micrometastatic bone marrow cells, primary breast cancer, prognostic impact, survival

Introduction

Despite adjuvant chemotherapy, patients with high-risk primary breast cancer (HRPBC) often suffer relapse and ultimately die of the disease. Preliminary results with myeloablative high-dose chemotherapy (HDCT) and autologous stem cell transplantation (ASCT) were encouraging [1, 2], and a comparison with historical control groups fuelled the enthusiasm for using HDCT as a treatment option. However, with a median follow-up period of 2.8–6.9 years, randomized trials have not shown any statistically significant overall survival (OS) benefit so far [3–8]. One trial, however, found a significantly longer disease-free survival (DFS) in the high-dose group [7]. Nevertheless, even if this benefit translates into an improved OS with longer follow-up, considering the toxicity and expense, HDCT with ASCT might not emerge as the optimal approach for all HRPBC patients. Therefore, it is worthwhile to characterize subgroups who may experience a major benefit from this treatment option.

Detection of micrometastatic bone marrow cells (MMC) at diagnosis has been identified as an independent prognostic factor of primary breast cancer in several studies [9–15]. Other investigators, however, did not find any impact of these cells on survival, either in univariate analysis or after adjustment for established prognostic factors [16–19]. Furthermore, no data are available on the subgroup of HRPBC patients with extensive axillary lymph node (ALN) involvement, in particular for those who received HDCT with ASCT.
In order to elucidate the prognostic value of MMC in this distinct subgroup of HRPBC patients, we prospectively evaluated the impact on DFS, distant DFS (DDFS), and OS together with all established and a panel of potential prognostic factors in 91 primary breast cancer patients with extensive ALN metastases who received stem cell-supported HDCT at the University of Heidelberg between 1992 and 1999.

Patients and methods

Patients

Between September 1992 and December 1999, 91 patients with HRPBC were enrolled in a single arm trial with HDCT and ASCT at the University of Heidelberg. The main inclusion criterion was stage II or III HRPBC, defined as primary breast cancer with at least 10 ALN involved (73 patients), or at least four involved ALN in case of premenopausal hormone levels (18 patients). Patients had to be between 18 and 65 years old with a Karnofsky performance score ≥90%, normal hematological, cardiac, renal and hepatic function, and no relevant concomitant disease. Initial work-up comprised chest X-ray, abdominal ultrasound, bone scan and computed tomography of the brain, thorax and abdomen. Patient characteristics in detail are given in Table 1. The number of tumor-involved ALN was divided by the number of sampled nodes to give the nodal ratio. The study was approved by the Joint Ethical Committee of the University of Heidelberg. All patients gave informed consent to participate in the study.

Immunohistochemistry and flow cytometry

Estrogen receptor (ER) and progesterone receptor (PR), Her2/neu, p53, Bcl-2 and Ki67 were measured by immunohistochemical staining on primary tumor sections. The following primary antibodies were used (clones in parentheses, all reagents by Dako): ER (1D5), PR (PR88), Her2/neu (A0485), p53 (DO7), Bcl-2 (124), Ki67 (MIB-1). ER and PR staining were evaluated for staining intensity and number of positive nuclei. Receptor positivity was assumed when the semiquantitative score was at least 3 points (out of a maximum of 12 points). Her2/neu and Bcl-2 immunoreaction as cell membrane staining was scored from 0 to 3. The antibody staining was recorded as the percentage of positive tumor nuclei for p53 and Ki67. In all patients bone marrow aspiration from the iliac crest was performed as a staging procedure before any systemic treatment was initiated. MMC were detected using an immunostaining method with a cocktail of four monoclonal epithelial-specific antibodies (antibody 5D3, BioGenex, San Ramos, CA; HEA 125, Progen Biotechnik GmbH, Heidelberg, Germany; and two murine monoclonal antibodies, BM7 and BM8, with a specificity for glycosylated side-chains of breast mucin) as previously described [20]. Per specimen, 4 × 10^6 cells were screened using ACIS image analysis (Chroma Vision, CA) for tumor cells. Bone marrow aspirate was scored as positive when at least one tumor cell per 10^6 bone marrow cells was detected. DNA index and S-phase fraction were measured by DNA flow cytometry.

Cytotoxic and endocrine therapy (Figure 1)

In 80 patients induction chemotherapy consisted of two cycles (78 patients) or three cycles (two patients) of ifosfamide 2500 mg/m^2 on days 1–3 and epirubicin 40 mg/m^2 on days 1–3, repeated on day 22. Ten patients received one cycle (seven patients) or two cycles (three patients) of paclitaxel (Taxol) 45 mg/m^2 on days 1–3, ifosfamide 2000 mg/m^2 on days 1–3 and epirubicin 30 mg/m^2 on days 1–3, repeated on day 22. In one patient, the University of Heidelberg between 1992 and 1999.

### Table 1. Characteristics of HRPBC patients treated with HDCT and ASCT

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All</th>
<th>MMC−</th>
<th>MMC+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients (%)</td>
<td>91 (100)</td>
<td>68 (75)</td>
<td>23 (25)</td>
</tr>
<tr>
<td>Age at diagnosis, years</td>
<td>43 (22–59)</td>
<td>45 (22–59)</td>
<td>38 (26–55)</td>
</tr>
<tr>
<td>Median (range)</td>
<td>18/37/36</td>
<td>9/28/31</td>
<td>9/9/5</td>
</tr>
<tr>
<td>Number of involved ALN</td>
<td>13 (4–40)</td>
<td>14 (4–40)</td>
<td>12 (4–23)</td>
</tr>
<tr>
<td>Median (range)</td>
<td>185/2/21</td>
<td>124/1/18</td>
<td>6/1/3</td>
</tr>
<tr>
<td>Number of involved ALN</td>
<td>0.7 (0.1–1)</td>
<td>0.6 (0.3–1)</td>
<td>0.7 (0.1–1)</td>
</tr>
<tr>
<td>Stage</td>
<td>44/34/13</td>
<td>33/31/4</td>
<td>11/3</td>
</tr>
<tr>
<td>Grade</td>
<td>1/39/51</td>
<td>1/27/40</td>
<td>0/12/11</td>
</tr>
<tr>
<td>Hormone receptor status</td>
<td>52/39</td>
<td>40/28</td>
<td>12/11</td>
</tr>
<tr>
<td>ER or PR positive/both negative</td>
<td>0–2+/3+/na</td>
<td>51/29/11</td>
<td>39/21/8</td>
</tr>
<tr>
<td>Her2/neu-overexpression, score</td>
<td>0–1+/2–3+/na</td>
<td>54/42/25</td>
<td>40/9/20</td>
</tr>
<tr>
<td>p53, % positive tumor nuclei</td>
<td>52/14/25</td>
<td>40/10/18</td>
<td>12/4/7</td>
</tr>
<tr>
<td>≤50/&gt;50/na</td>
<td>39/25/27</td>
<td>30/18/20</td>
<td>9/7/7</td>
</tr>
<tr>
<td>Ki67, % positive tumor nuclei</td>
<td>31/28/32</td>
<td>21/24/23</td>
<td>10/4/9</td>
</tr>
<tr>
<td>≤50/&gt;50/na</td>
<td>34/34/23</td>
<td>25/27/16</td>
<td>9/7/7</td>
</tr>
<tr>
<td>S-phase fraction, %</td>
<td>≤4.9/&gt;4.9/na</td>
<td>31/28/32</td>
<td>21/24/23</td>
</tr>
<tr>
<td>DNA-index, %</td>
<td>≤1.5/&gt;1.5/na</td>
<td>34/34/23</td>
<td>25/27/16</td>
</tr>
<tr>
<td>MMC in bone marrow aspirate</td>
<td>68/23</td>
<td>68/0</td>
<td>0/23</td>
</tr>
<tr>
<td>Surgical procedure</td>
<td>32/59</td>
<td>23/45</td>
<td>9/14</td>
</tr>
<tr>
<td>Adjuvant locoregional radiotherapy</td>
<td>41</td>
<td>34</td>
<td>7</td>
</tr>
<tr>
<td>chest wall after MRM</td>
<td>75</td>
<td>57</td>
<td>18</td>
</tr>
<tr>
<td>draining lymph nodes</td>
<td>41</td>
<td>34</td>
<td>7</td>
</tr>
</tbody>
</table>

ALN, axillary lymph nodes; ASCT, autologous stem cell transplantation; BCT, breast-conserving therapy (including adjuvant radiotherapy of the breast); ER, estrogen receptor; MMC, micrometastatic bone marrow cells; MRM, modified radical mastectomy; na, not available; PR, progesterone receptor.
induction chemotherapy consisted of three cycles of epirubicin 90 mg/m² on day 1 and cyclophosphamide 600 mg/m² on day 1, repeated on day 22. R-metHuG-CSF (filgrastim, 300 μg/day 1 subcutaneously; Neupogen®, Amgen Inc., Thousand Oaks, CA) was administered after induction chemotherapy in order to accelerate neutrophil recovery and to mobilize progenitor cells in the bloodstream. Peripheral blood stem cell collection began when CD34+ cells were measurable in the peripheral blood. Leukapheresis was performed using Fenwal CS3000 (Baxter Deutschland GmbH, Munich, Germany) or Spectra (Cobe Laboratories, Lakewood, CA) equipment.

The first stem cell-supported HDCT followed ~4 weeks after the last cycle of induction chemotherapy. As scheduled, the HDCT consisted of two cycles of peripheral blood stem cell-supported high-dose ifosfamide 12 000 mg/m² and epirubicin 180 mg/m² in three patients and high-dose ifosfamide 12 000 mg/m², epirubicin 180 mg/m² and carboplatin 900 mg/m² in 75 patients. Due to toxicity in five patients the regimen of the second course of HDCT was changed (one patient received cyclophosphamide 6000 mg/m² instead of ifosfamide and four patients received high-dose paclitaxel 180 mg/m², etoposide 1500 mg/m² and thiotepa 600 mg/m²). Eight patients were withdrawn from the study after the first cycle of HDCT. The reasons for withdrawal were refusal to proceed in four patients, severe hemorrhagic colitis in one patient, severe neurologic disturbances in one patient, alloreactive antibodies against platelets and no appropriate cross-matched donors available in one patient and retrospectively pre-existing chronic hepatitis B in one patient. The median time interval between the first and second cycle of HDCT was 7 weeks (range, 4–16). Peripheral blood stem cells were reinjected 48 h after the end of HDCT, and no cytokines were given following transplantation. The patients received prophylactic antimicrobial therapy with ciprofloxacin (1000 mg/day) and fluconazole (400 mg/day).

To treat hormone receptor-positive tumors, premenopausal patients additionally received goserelin 3.6 mg subcutaneously (s.c.) once a month starting from the first cycle of induction chemotherapy for 2 years or until the disease relapsed. Postmenopausal patients received tamoxifen 20–30 mg/day orally (p.o.) starting at least 6 weeks after the last peripheral blood stem cell-supported HDCT for 5 years or until the disease relapsed. After primary therapy, patients had regular check-ups at our hospital that included a careful history and thorough clinical examination every 3 months, as well as chest radiograph and liver ultrasound every 6 months and a bone scan every year.

Locoregional radiotherapy
All patients who underwent breast-conserving surgery received locoregional radiotherapy of the breast at a dose of 50 Gy with a boost of 10 Gy to the tumor bed. For patients undergoing modified radical mastectomy, there was no general recommendation for radiotherapy of the chest wall and draining lymph nodes at the beginning of the trial. From August 1996 on, locoregional irradiation was included in the protocol for all patients. As a result, 41 of 59 (69%) patients who received a mastectomy underwent locoregional radiotherapy of the chest wall. In 75 patients radiotherapy to the draining lymph nodes was administered.

Statistical analysis
DFS, DDFS and OS were taken as clinical outcome variables. DFS was measured from the start of induction chemotherapy until the time of relapse, death or last contact. DDFS was measured from the start of induction chemotherapy until the time of distant relapse, death or last contact. Distant relapse was defined as relapse other than in the ipsilateral breast, ipsilateral chest wall, ipsilateral ALN or above the ipsilateral clavicle. OS was calculated from the start of induction chemotherapy to death or to the date of the last patient contact. Survival curves were estimated using the Kaplan–Meier product limit method [21]. Univariate and multivariate analyses were performed to identify risk factors associated with DFS, DDFS and OS. Differences between the survival curves were compared using the log rank test [22]. The following risk factors were examined in a univariate analysis: age at diagnosis (<35 years versus 35–45 years versus >45 years), menopausal status (pre- versus postmenopausal), tumor size (T1 versus T2 versus T3 versus T4), number of involved ALN (<10 versus 10–19 versus >19), nodal ratio (<0.8 versus ≥0.8), stage (I versus II versus III), grade (G1 versus G2 versus G3), combined hormone receptor status (ER or PR positive versus ER and PR negative), Her2/neu expression (0–2+ versus 3+), proportion of p53-positive tumor nuclei on primary...
tumor section (≤50% versus >50%), Bcl-2 expression (0–1+ versus 2–3+), proportion of Ki67-positive tumor nuclei on primary tumor section (≤50% versus >50%), S-phase fraction (≤4.9% versus >4.9%), DNA index (≤1.5% versus >1.5%), presence of MMC (no versus yes), surgical procedure (breast-conserving surgery with adjuvant radiotherapy of the breast versus mastectomy), and adjuvant locoregional radiotherapy of the chest wall or draining lymph nodes (yes versus no). Variables that showed statistical significance in univariate analysis \( (P<0.05) \) were included in a multivariate analysis [23]. Model selection was performed using forward selection (entry into the model if \( P<0.1 \)) and backward selection (removal from the model if \( P>0.1 \)). All tests were performed using SAS software (version 8.2; Cary, NC).

**Results**

**Survival**

For all 91 HRPBC patients who received HDCT and ASCT with a median follow-up of 62 months (range, 10–117) DFS, DDFS and OS were 53%, 58% and 78%, respectively. So far, relapses after HDCT occurred in 43/91 (47%) patients and were observed locoregionally only as first relapse (ipsilateral breast, ipsilateral chest wall, above the ipsilateral clavicle or in the ipsilateral ALN) in 8/43 (19%) patients. Distant metastases as first relapse developed in 35 (81%) patients (bone, 20 patients; viscera, 13 patients; central nervous system, four patients; soft tissue, five patients). In seven patients first relapse occurred at more than one site. Median survival after first relapse amounted to 17 months (range, 0–75).

**Prognostic impact of MMC**

In 23 of 91 patients (25%), we detected a median of three MMC (range, 1–43) among 10^6 normal mononuclear bone marrow cells. Characteristics of patients with and without detection of MMC are given in Table 1. In univariate analysis the detection of these cells had no impact on DFS \( (P=0.929) \), DDFS \( (P=0.664) \) or OS \( (P=0.642) \). The corresponding DFS, DDFS and OS curves according to the presence or absence of MMC are shown in Figure 2A–C.

**Uni- and multivariate analysis**

According to univariate analysis, a longer DFS and DDFS were associated with nodal ratio <0.8 (for DFS \( P=0.011 \); for DDFS \( P=0.025 \)) and lower UICC stage (for DFS \( P=0.030 \); for DDFS \( P=0.013 \)), respectively. Prognostic factors for longer OS were age above 45 years \( (P=0.035) \), lower UICC stage \( (P=0.037) \), ER or PR positivity \( (P=0.005) \), lower expression of Her2/neu \( (P=0.033) \) and lower expression of p53 \( (P<0.001) \).

In multivariate analysis only nodal ratio <0.8 remained an independent predictor of longer DFS [relative risk (RR) = 1.8 (95% confidence interval (CI) 1.14–2.85); \( P=0.012 \)] and lower UICC stage for longer DDFS [RR = 1.9 (95% CI 1.13–3.14); \( P=0.015 \)]. The only independent prognostic factor for a longer OS was lower expression of p53 [RR = 5.0 (95% CI 2.0–12.3); \( P<0.001 \)].

**Discussion**

With a median follow-up of 62 months, DFS and OS of our cohort of 91 HRPBC patients with extensive ALN involvement who received HDCT with ASCT were 53% and 78%,
respectively. These survival rates are well within the range of survival rates reported from phase III studies available so far [3–8]. In our cohort, detection of MMC before systemic treatment had no impact on DFS, DDFS or OS. In contrast, in primary breast cancer patients who received conventional adjuvant treatment, detection of MMC has been identified as an independent prognostic factor in several studies [9–14]. These discordant results are best explained by varying follow-up periods of different patient cohorts with heterogeneity in size, in the extent of tumor disease, and in the antibodies and staining procedures used.

So far, five studies with median follow-up periods of between 34 and 54 months reported an independent impact of MMC on OS considering all established prognostic factors [9–12, 14]. In addition, a recently reported pooled analysis considering data of 4199 primary breast cancer patients from eight trials confirmed the independent prognostic impact of MMC on survival with a median follow-up of 58 months [15]. Most patients in these studies, however, had no or minimal ALN involvement, and neither in the single trials nor the pooled dataset was a separate analysis performed on the subgroup of patients with extensive ALN involvement, comparable to the patients in our cohort. The patients in our trial probably represent a certain subgroup with near-site metastatic disease, in which the detection of MMC no longer distinguishes between high and low risk for relapse and death.

Fields et al. [24] reported a correlation between micrometastatic bone disease detected by polymerase chain reaction for K19 and earlier relapse in 83 patients with stage II–IV breast cancer undergoing induction chemotherapy followed by stem cell-supported HDCT. Along the same line, Vredenburgh et al. [25] found an association between MMC detected with a panel of four anti-breast cancer monoclonal antibodies and DFS and OS in 83 HRPBC with at least 10 positive nodes who also received induction treatment followed by HDCT with ASCT. Both investigations, however, examined bone marrow aspirated after induction chemotherapy, i.e. they searched for chemotherapy-resistant MMC, while we analysed bone marrow aspirated before any systemic treatment was initiated. Chemotherapy-resistant MMC represent a selected subpopulation of occult bone metastases that might carry a worse prognosis [26].

Evaluating the established prognostic factors used to distinguish prognostic groups in the adjuvant setting in combination with nodal ratio, Her2/neu, p53, Bcl-2, K167, S-phase fraction, DNA index, surgical procedure, locoregional radiotherapy and presence of MMC, only nodal ratio ≥0.8 proved to be an independent predictor of shorter DFS in the current analysis [RR = 1.80 (95% CI 1.14–2.85); P = 0.004]. Considering 23 variables in 176 HRPBC patients with at least four involved ALN or inflammatory breast cancer treated with HDCT and ASCT, Nieto et al. [27] determined an axillary nodal ratio of ≥0.8, negative hormone receptors, and larger tumors (≤2 cm versus 2–5 cm versus >5 cm) to be independent predictors for earlier relapse. The subsequent analysis in which the prognostic value of Her2/neu was evaluated in a subgroup of 146 patients revealed a significantly poorer relapse-free survival (RFS) and OS in case of Her2/neu overexpression. After adjustment for nodal ratio, hormone receptor status and tumour size, Her2/neu overexpression remained an independent predictor for earlier relapse and death [28]. Probably due to the small number of patients with available Her2/neu status (n = 80) we only found a significant association with earlier death (P = 0.03), but no association with earlier relapse in our analysis.

The strongest predictor for shorter OS in this study was an overexpression of p53 in >50% of tumor cells [RR = 5.0 (95% CI 2.0–12.3); P < 0.001]. This confirms our results obtained with shorter follow-up [29] and data published by Somlo et al. [30], who also found p53 overexpression to be associated with inferior RFS and OS following HDCT for HRPBC. Nieto and co-workers, however, could not identify such a correlation [28]. We have no satisfactory explanation for this discrepancy but, in addition to differences in the conditioning regimen, above all methodological differences in defining p53 mutation, e.g. proportion of p53 overexpressing tumors cells or immunological versus molecular biological analyses, might be responsible.

In conclusion, data from randomized trials evaluating HDCT with ASCT in HRPBC patients are inconclusive so far. Even if a positive trend continues, HDCT might save only a certain subgroup of patients from relapse and ultimately death from the disease. The detection of MMC, however, does not help to define such a subpopulation among patients with extensive ALN involvement. According to this analysis it could be hypothesized that patients with a nodal ratio <0.8 and without p53 and Her2/neu overexpression might have a favorable outcome following HDCT and ASCT and, therefore, might be ideal candidates to further evaluate this approach.

References

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