Clinical Significance of Occult Metastatic Cells in Bone Marrow of Breast Cancer Patients

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ABSTRACT

The early and clinically occult spread of viable tumor cells to the organism is increasingly considered a hallmark in cancer progression, as emerging data suggest that these cells are precursors of subsequent distant relapse. Using monoclonal antibodies to epithelial cytokeratins or tumor-associated cell membrane glycoproteins, individual carcinoma cells can be detected on cytologic bone marrow preparations at frequencies of 10^-5 to 10^-6. Prospective clinical studies have shown that the presence of these immunostained cells in bone marrow, as a frequent site of overt metastases, is prognostically relevant with regard to relapse-free and overall survival. This screening approach may be, therefore, used to improve tumor staging and guide the stratification of patients for adjuvant therapy in clinical trials. Another promising application is monitoring the response of micrometastatic cells to adjuvant therapies, which, at present, can only be assessed retrospectively after an extended period of clinical follow-up. The present review summarizes the current data on the clinical significance of occult metastatic breast cancer cells in bone marrow.

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INTRODUCTION

The occult hematogenous spread of tumor cells in patients with operable breast cancer may be considered a determinant of subsequent metastases formation, yet is usually missed by conventional tumor staging. Several groups (including ours) have therefore designed sensitive immunocytochemical and molecular assays to identify individual tumor cells that have successfully invaded the bone marrow [1-15] (Table 1).

However, their clinical significance has been questioned until methodological analyses signaled that the diversity of antibodies used to identify epithelial cells was the major confounding variable rendering the results of most of the cited studies almost incomparable. This substantial methodological variation, which resulted in detection rates of 4%-48% though comparing similar study populations [16], predominately accounted for the discrepant results among some clinical follow-up studies. Current data suggest that antibodies against polymorphic epithelial mucins, such as epithelial membrane antigen or mucin, label 2%-10% of the mesenchymal mononucleated cells of healthy volunteers [17]. In contrast, when using validated antibodies directed against cytokeratins as major constituents of the epithelial cell, no such cross-reactivity was observed.

Bone marrow has played a prominent role as an indicator organ of occult tumor cell dissemination because it is easily accessible by aspiration, and it represents a relevant site of distant metastases in breast cancer. The development of monoclonal antibodies to epithelial cytokeratins and tumor-associated cell membrane glycoproteins has opened a diagnostic window to detect individual disseminated tumor cells against the background of 10^-5 to 10^-6 normal bone marrow cells. The present review focuses on the clinical relevance of new diagnostic approaches for the identification and characterization of occult metastatic breast cancer cells in bone marrow.

PROGNOSTIC RELEVANCE OF BONE MARROW MICROMETASTASES

Metastatic disease occurs in about half of the cases with apparently localized breast cancer (stage M0) within 5 years after surgery. Even among patients with node-negative...
disease, approximately one-third will recur with distant disease. At first relapse, bone marrow metastases are detectable in 23% of patients by conventional diagnostic techniques, and this rate increases up to 80% in necropsy studies of patients with metastatic relapse [18].

Using conventional histopathologic techniques, the likelihood for the identification of isolated breast cancer cells in bone marrow is as low as 4% [19, 20]. Among the first investigators, Redding et al. used an antiserum against epithelial membrane antigen (EMA) and detected breast cancer cells in bone marrow at the time of primary surgery in 28% of females without overt metastases [21]. Although this marker is known to be rather nonspecific [22-26], several groups including ours were able to confirm these first results with an incidence of positive findings ranging from 20%-45%.

An important question was whether the presence of epithelial antigen-positive cells was correlated to established risk factors, such as tumor size or lymph node involvement. Diel et al. [6] found a significant correlation between bone marrow positivity and tumor size ($p < 0.001$), nodal status ($p = 0.001$), histopathologic tumor grading ($p = 0.002$), and postmenopausal status of the study patients ($p = 0.01$). The London Ludwig Cancer Institute Group described that the presence of EMA-positive cells in bone marrow was significantly related to lymph node involvement, peritumoral vascular invasion, and size of the primary tumor [27]. Studies using different anticytokeratin monoclonal antibodies demonstrated merely a tendency towards correlation between detection of cytokeratin-positive cells in bone marrow and locoregional lymph node involvement antibodies [3, 20, 28]. Applying the broad-spectrum anticytokeratin monoclonal antibody A45-B/B3 for tumor cell detection [1], we recently reported a significant association of bone marrow micrometastases with the diagnosis of inflammatory breast cancer, tumor size, extensive lymph node metastases of $\geq 10$ nodes (each $p < 0.0001$), and tumor grade ($p = 0.0044$).

In order to assess the significance of isolated tumor cells in bone marrow, clinical follow-up studies were initiated. While several studies confirmed the prognostic influence of occult metastatic cells on relapse-free and overall survival [1, 2, 4, 6, 7], other studies failed to do so [3, 10-13, 15, 29] (Table 1). Using a polyclonal EMA antibody, Mansi et al. found a significantly shorter relapse-free interval in patients with bone marrow positivity after an intermediate follow-up of 28 months [30]. Analysis of the sites of relapse showed that their immunocytochemical assay predicted predominantly for the occurrence of bone metastases. In the 6-year follow-up analysis (median: 76 months; range: 34-108), univariate statistical analysis revealed that this immunocytochemical finding predicts for an increased rate of relapse in bone ($p < 0.01$) and other distant sites ($p < 0.001$), as well as a decreased overall survival ($p < 0.005$) [31]. Multivariate analysis both after 6 [31] and more than 12 years of clinical follow-up [2] indicated that this prognostic influence of EMA-positive cells was not independent of established risk factors, such as tumor size, grade, and lymph node status.

In contrast, a recent follow-up examination of 727 primary breast cancer patients after a median follow-up time of 36 months (3-108 months) claimed that the presence of TAG-12-positive cells identified patients with poor prognosis.

### Table 1. Immunocytochemical detection of occult metastatic cancer cells in bone marrow of breast cancer patients

<table>
<thead>
<tr>
<th>Marker</th>
<th>Preparation</th>
<th>$n$ of patients</th>
<th>Detection rate</th>
<th>Prognostic value</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucin</td>
<td>Biopsy</td>
<td>159</td>
<td>16%</td>
<td>None</td>
<td>Porro et al. [15]</td>
</tr>
<tr>
<td>Mucin</td>
<td>Biopsy</td>
<td>121</td>
<td>17%</td>
<td>None</td>
<td>Salvadori et al. [12]</td>
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<tr>
<td>Mucin/CK</td>
<td>Biopsy</td>
<td>93</td>
<td>1%</td>
<td>None</td>
<td>Mathieu et al. [13]</td>
</tr>
<tr>
<td>Mucin</td>
<td>Cell smears</td>
<td>25</td>
<td>48%</td>
<td>None</td>
<td>Kirk et al. [14]</td>
</tr>
<tr>
<td>Mucin</td>
<td>Biopsy</td>
<td>50</td>
<td>8%</td>
<td>None</td>
<td>Courtemanche et al. [10]</td>
</tr>
<tr>
<td>Mucin/CK</td>
<td>Cell smears</td>
<td>71</td>
<td>38%</td>
<td>None</td>
<td>Singleton et al. [11]</td>
</tr>
<tr>
<td>Mucin/CK</td>
<td>Cell smears</td>
<td>49</td>
<td>37%</td>
<td>DFS, OS</td>
<td>Cote et al. [9]</td>
</tr>
<tr>
<td>Mucin/CK</td>
<td>Cell smears</td>
<td>100</td>
<td>38%</td>
<td>DFS, OS*</td>
<td>Harbeck et al. [7]</td>
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<tr>
<td>Mucin</td>
<td>Cell smears</td>
<td>727</td>
<td>43%</td>
<td>DFS, OS*</td>
<td>Diel et al. [6]</td>
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<tr>
<td>CK18</td>
<td>Cytospins</td>
<td>234</td>
<td>38%</td>
<td>n.d.</td>
<td>Funke et al. [5]</td>
</tr>
<tr>
<td>CK</td>
<td>Biopsy</td>
<td>128</td>
<td>19%</td>
<td>DFS, OS*</td>
<td>Landys et al. [4]</td>
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<tr>
<td>CK18</td>
<td>Cytospins</td>
<td>581</td>
<td>28%</td>
<td>None</td>
<td>Untch et al. [3]</td>
</tr>
<tr>
<td>Mucin</td>
<td>Cell smears</td>
<td>350</td>
<td>25%</td>
<td>DFS, OS</td>
<td>Mansi et al. [2]</td>
</tr>
<tr>
<td>CK</td>
<td>Cytospins</td>
<td>552</td>
<td>36%</td>
<td>DFS, OS*</td>
<td>Braun et al. [1]</td>
</tr>
</tbody>
</table>

Abbreviations: DFS = disease-free survival; DDFS = distant disease-free survival; OS = overall survival; CK = cytokeratin

*Prognostic value supported by multivariate analysis.
The detection of TAG-12-positive cells was described as an independent prognostic indicator for both metastasis-free and overall survival being superior to axillary lymph node status, tumor stage, and tumor grade. The discrepancy between both cited studies raised the question of the clinical value of bone marrow screening. A meta-analysis of 2,494 patients from 20 studies failed to substantiate an independent prognostic impact of positive bone marrow findings on the relapse-free period or overall survival [32]. This meta-analysis, however, jumped to conclusions based on results of various, largely incomparable detection methods. Thus, the finding of absence and presence of prognostic influence, respectively, might be related to varying technical factors, such as the choice of antibodies, labeling systems, or varying numbers of cells analyzed as well as the number of patients studied. The latter studies (Table 1) used either antibodies directed against membrane-bound mucins, which have been shown to be expressed by hematopoietic cells [17], or a monospecific antibody directed against a single cytokeratin peptide, which is less sensitive than a broad-spectrum antibody [23, 33].

Using different cocktails of monoclonal antibodies to cell-surface antigens and cytokeratins, Cote et al. [9] and Harbeck et al. [7] found prognostic information derived from the presence of occult metastatic cells in bone marrow. Harbeck et al. detected isolated tumor cells in 38% of the patients (n = 100) using a cocktail of monoclonal antibodies to EMA, TAG-12, and cytokeratin. After a median follow-up of 34 months (7-64 months) multivariate analysis using the Cox proportional-hazard model revealed that bone marrow positivity was a strong, significant prognostic indicator for relapse-free and overall survival. Using a cocktail of monoclonal antibodies to cell-surface antigens (monoclonal antibody C26 and T16) and cytokeratins (monoclonal antibody AE-1), Cote et al. reported that the tumor burden in bone marrow was an important risk factor [9]. In their analysis of bone marrow cell smears, the number of isolated tumor cells per sample (0 or <10 cells versus ≥10 cells) was the only independent predictor of early recurrence (p < 0.003). However, their study population (n = 49) was considerably small.

Thus, a large-scale study using an immunoassay with proven sensitivity and specificity was launched to determine whether a positive immunocytochemical finding reflects the presence of tumor cells and to evaluate the prognostic role of bone marrow micrometastases in breast cancer patients. To dispel the prevailing doubts as to the accuracy of methodology and size of study populations, we performed a prospectively planned study on 552 newly diagnosed patients with stage I-III breast cancer, using an anticytokeratin immunoassay that has been validated [23, 33] and could be reproduced at both centers of the study [1]. In this study, we found that the presence of occult metastatic cells in bone marrow, as exemplified in Figure 1, was associated with the occurrence of clinically overt distant metastases and death from cancer-related causes (Fig. 2). In addition, this study demonstrated that in clinically relevant subgroups (e.g., categorized by stage, grading, and tumor size), the presence of occult metastatic cells distinguished between marrow-negative patients with fairly good prognosis and marrow-positive patients with worse outcome in respect to disease-free and overall survival. Particularly, as verified by multivariate regression analyses, the presence of occult metastatic cells in bone marrow predicts a poor prognosis independently from lymph node metastases [1] (Fig. 3).

In this context it is interesting to mention that, as shown in animal models as early as the 1970s [34], the presence of lymph node metastases does not necessarily correlate with the presence of occult metastatic cells in bone marrow. Numerous studies so far have demonstrated that the presence of immunocytochemically identifiable lymph node...
micrometastases in presumed node-negative patients indicates a worse outcome [35-45]. However, these studies generally reported on a conversion of the nodal status in a percentage below the ~30% of patients that will recur within 5 years after diagnosis. In a first study to compare directly the presence of lymph node micrometastases with that of bone marrow micrometastases in presumed node-negative patients, we found a prevalence of 9% and 29%, respectively [46]. Interestingly, a coincidence of isolated tumor cells in bone marrow and lymph nodes was assessed in only two patients. Reduced distant disease-free interval and overall survival were only associated with a positive bone marrow finding ($p = 0.039$ and $p = 0.014$, respectively) but not with lymph node micrometastases.

**POTENTIAL SURROGATE MARKER OF THERAPEUTIC EFFICACY**

Beyond merely adding another prognostic factor to the plethora of such markers in breast cancer, the potential of occult metastatic cells in bone marrow as a predictor for therapeutic efficacy should be emphasized. The efficacy of adjuvant therapy can thus far only be assessed retrospectively in large-scale clinical trials following an observation period of at least 5 years. Consequently, progress in this form of therapy is extremely slow and cumbersome and, in addition, it is difficult to tailor therapy to the special needs of an individual patient. The importance of a surrogate marker assay that would permit the immediate assessment of therapy-induced cytotoxic effects on residual cancer cells is therefore obvious.

A recent study showed that cytokeratin-positive metastatic breast cancer cells rarely proliferate (i.e., they are negative for the proliferation marker Ki-67) at the time of primary diagnosis [47]. Current cytotoxic chemotherapy regimens might therefore fail to eliminate dormant, nonproliferating tumor cells, which may explain metastatic relapse even after high-dose chemotherapy. Patients with high-risk breast cancer (e.g., metastasis of >3 lymph nodes or extensive invasion of cutaneous lymph vessels) who received standard taxane or anthracycline-containing chemotherapy were monitored before and after treatment. Beyond the fact that the overall prevalence of positive bone marrow findings before and after chemotherapy remained essentially unchanged, the presence of tumor cells after therapy was associated with an extremely poor prognosis and pointed to a heterogeneous response to treatment (Fig. 4). In the high-dose chemotherapy setting, two pilot studies on breast cancer patients undergoing either ifosfamide-carboplatin-epirubicin ($n = 18$) or vinblastin-ifosfamide-carboplatin ($n = 10$) chemotherapy with autologous stem cell transplantation described the presence of cytokeratin-positive cells in 15 (83%) and three (30%) bone marrow specimens obtained after completion of treatment with the majority of patients being in complete remission [48, 49]. These findings again point to the discrepancy between clinical diagnosis and the yet imminent risk of relapse represented...
by the presence of occult metastatic breast cancer cells that may serve as an explanation for treatment failure of high-dose chemotherapy.

Thus, we feel that there is an urgent need of complementary strategies, such as antibody-based immunotherapy, with proven efficacy and improved specificity for tumor cells. In a recent pilot study by Schlimok et al. [50], 40 patients with breast and colorectal cancer were treated in a randomized fashion with either 6 × 100 mg ABL 364 over 2 weeks or human serum albumin as placebo. Monoclonal antibody ABL 364 (murine IgG3) is directed to the Lewis Y (LeY) blood group precursor carbohydrate antigen which is widely expressed on most epithelial tumors [51], and on disseminated tumor cells in bone marrow (Figs. 5A and B). Cytokeratin-positive cells in marrow were monitored on days 15 and 60 after initiation of treatment. Even in patients with an extremely low number of cytokeratin-positive cells (1-11/4 × 10^5 mononuclear cells [MNC]), a tendency for reduction of cytokeratin-positive cells was seen after antibody therapy. Significant data, however, were only obtained from the 10 breast cancer patients who displayed an initial cell count of more than 20 cytokeratin-positive cells per 4 × 10^5 MNC. Of the seven patients treated with antibody, five showed a distinct reduction or eradication of cytokeratin-positive/LeY-positive cells (96%-100%), while in two patients with cytokeratin-positive/LeY-negative cells no response was registered. Similarly, in the three patients receiving human serum albumin, no significant tumor cell reduction was observed.

We conducted a similar pilot study [52] in 10 patients with advanced breast cancer disease who received a single dose of 500 mg edrecolomab which is directed against the epithelial cell adhesion molecule EpCAM widely expressed on breast cancer cells [53]. In all patients a marked reduction of the pretherapeutic tumor load could be monitored by a
second follow-up bone marrow aspiration within 5-7 days of antibody treatment (Fig. 5). In 4 of the 10 patients, no metastatic cells could be identified after treatment with edrecolomab. Because of the marked antibody-dependent cellular cytotoxicity, and complement-dependent cytotoxicity that both antibodies (ABL 364 and edrecolomab) exhibit in ex vivo experiments with serum of treated patients [51], we postulate that the observed disappearance of tumor cells from bone marrow can be reduced to the action of the administered antibodies. Due to the ample metastatic tumor masses in these study patients, no immediate alteration of the clinical course of disease was observed.

Despite their preliminary character, these studies signal a new approach towards a more rational selection of antibodies for adjuvant studies in minimal residual disease. The proposed use of cytokeratin-positive cells as surrogate markers for the prediction of therapeutic response may benefit from the recent improvements of the bone marrow assay (e.g., immunomagnetic enrichment of tumor cells [54]), which allows a more precise quantification of the individual tumor load. Prospective clinical studies are now required to evaluate whether the eradication of cytokeratin-positive cells translates into a longer disease-free period and overall survival. Availability of such a surrogate marker would considerably enhance our abilities to rationally design new therapies for the elimination of minimal residual disease.

**CONCLUDING REMARKS**

Various immunocytochemical and molecular methods have been applied to detect disseminated carcinoma cells in bone marrow. The current strategies provide intriguing opportunities for improved tumor staging, therapeutic targeting, and for the first time, a possibility to monitor the efficacy of adjuvant therapy. At present, we feel that international concerted activities rather than meta-analysis of extremely heterogeneous sets of data [32] are now required to establish standardized procedures that may then also serve as a gold standard. Although the development of new polymerase chain reaction (PCR)-based methods [55] allows for increased assay sensitivity, the clinical significance of this finding needs to be further evaluated in clinical follow-up studies.

Thus far, the biology of bone marrow micrometastases is poorly understood, particularly in patients who remain free of overt metastatic disease despite the presence of tumor cells at the time of diagnosis. Our present results indicate that cytokeratin-positive micrometastatic tumor cells represent a selected population of cancer cells; these cells, however, still express a considerable degree of heterogeneity [47, 56-58]. With the development of new techniques like single-cell PCR [59, 60] and the in vitro expansion of micrometastatic cells [61, 62], it may be possible to determine the characteristic genotypic features of those cells.

The outlined current strategies for detection and characterization of cancer micrometastasis might help to design and control new therapeutic strategies for secondary prevention of metastatic relapse in patients with operable primary carcinomas. Minimal residual disease offers the advantage of a small burden of dispersed tumor cells which are more accessible to intravenously applied drugs than gross metastases. In view of the dormant nature of micrometastatic cells in bone marrow [47, 58], therapies that are also directed against quiescent cells, such as antibody-based immunotherapy, might be complementary to chemotherapy.

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