The Prognostic Value of Isolated Tumor Cells in Bone Marrow in Breast Cancer Patients: Evaluation of Morphological Categories and the Number of Clinically Significant Cells

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ABSTRACT
Purpose/Experimental Design: Immunocytochemical detection of isolated tumor cells (ITCs) in the bone marrow (BM) is a prognostic factor in breast cancer. However, hematopoietic cells (HCS) can occasionally be stained by the techniques used. Morphological evaluation improves the specificity of ITC detection, but optimal separation of ITCs from false-positive HCS needs to be determined. Here, predetermined morphological categories of immunocytochemically (ICC)-positive cells in the BM and the number of detected ITCs were analyzed for association with clinical outcome in 817 early-stage breast cancer patients (median 49 months of follow-up). All ICC+ cells detected were categorized into one of the following groups: (a) tumor cell (TC); (b) uninterpretable cell (UIC); (c) probable HC; or (d) HC.

Results: Among the TC+ patients, 30.6% and 25.9% experienced systemic relapse (SR) and breast cancer death (BCD), respectively, as compared with 13.3% and 8.5% of patients without TCs in the BM (survival analyses: P < 0.001, log-rank). The SR and BCD rate was 19.7% and 15.8% for TC+/UIC+ patients versus 12.5% and 7.4% for TC+/UIC- patients. Survival analyses confirmed that the UIC+ group contained clinically significant cells (P = 0.018, log-rank). No difference in clinical outcome was observed, regardless of whether probable HCs or HCs were present. Analyzing the number of ITC+ cells, SR and BCD occurred in 12.4% and 7.4% of patients with 0 ITCs present, 21.3% and 18.5% of patients with 1 ITC present, 19.4% and 16.7% of patients with 2 ITCs present, and 42.5% and 32.5% of patients with ≥3 ITCs present.

Conclusions: Morphological categorization of ICC+ cells improves the clinical value of ITC detection in the BM. The presence of only one ITC reduces survival, and a greater number of ITCs further aggravates the prognosis.

INTRODUCTION
During the last two decades, methods have been developed to discover very early bone marrow (BM) dissemination in carcinoma patients (1–5). The challenge of these techniques has been to detect single epithelial cells in the mononuclear cell (MNC) fraction of the BM. The most established method is immunocytochemical analysis of MNCs through visualization of the binding of monoclonal antibody to epithelial cells by alkaline phosphatase-based color reactions (6).

The immunocytochemical detection of ≥1 isolated tumor cell (ITC) in the BM in breast cancer patients predicts a shorter disease-free interval and reduced overall survival (1, 2, 7–10). Most larger studies show an independent prognostic value of detection of ITCs in the BM (2, 7–10). Previous studies have shown that immunocytochemistry (ICC) can result in the staining of single hematopoietic cells (HCS) as well (11). With one ITC in the analyzed sample as the threshold for positivity, the controls are important and should be included when performing ICC of ITCs in patient samples. In addition, morphological evaluation of the immunopositive cells can help to differentiate between ITCs and false-positive cells.

We have reported the clinical significance of detection of ITCs in 817 early-stage breast cancer patients (10). The immunocytochemical detection of ITCs was based on staining with pan-cytokeratin antibodies, followed by screening and morphological evaluation of the immunopositive cells. The criteria for an ITC+ result have been defined in published guidelines from the European Working Group for Standardization of Tumor Cell Detection (11). The reported results showed that the detection of immunopositive cells in the BM with a morphology compatible with tumor cells (TCs) is an independent prognostic factor for both distant disease-free survival (DDFS) and breast cancer-specific survival (BCSS; Ref. 10). Before any clinical correlation, all immunostained cells were categorized according to morphological criteria into one of four groups: (a) TC; (b) uninterpretable cell (UIC); (c) probable HC (PHC); or (d) HC.

Here, we explore the clinical significance of detection of immunopositive cells within these predefined morphological categories in 817 breast cancer patients. In addition, the aim of this study was to disclose the correlation between the number of clinically significant cells and clinical outcome.
MATERIALS AND METHODS

Patients
From May 1995 to December 1998, 920 patients were included from five hospitals in the Oslo region (Ullevaal University Hospital, Norwegian Radium Hospital, Baerum Hospital, Aker University Hospital, and Buskerud Hospital). After informed written consent was obtained from patients, BM aspiration was performed under general anesthesia, just before primary surgery for suspected breast cancer. The diagnostic workup included mammography of the contralateral breast, chest X-ray, blood sampling, and clinical examination. Ultrasound and bone scans were not routinely performed. The treatment was conservative breast surgery in combination with postoperative radiotherapy or breast ablation. All patients with invasive carcinomas had axillary clearance. Adjuvant chemotherapy, hormone therapy, and/or radiotherapy were given according to national protocols. After completing primary therapy, patients had a clinical examination at 6–12-month intervals at the hospitals’ outpatient departments or by their primary doctors, with annual mammography. Further diagnostic work up was performed only if the patients had symptoms or signs of progression.

Preparation of the BM
A total of 40 ml of BM was aspirated from anterior and posterior iliac crests bilaterally (10 ml/site) and processed as described previously (12). Briefly, after separation by density centrifugation, MNCs were collected, and cytospins were prepared (5 × 10^5 MNCs/slide).

Immunocytochemical Staining
The immunocytochemical staining has been described previously (12). Briefly, four slides (2 × 10^6 BM MNCs) were incubated with the anti-cytokeratin monoclonal antibodies AE1 and AE3 (Sanbio, Uden, the Netherlands), and the same number of slides was incubated with a negative control antibody of the same immunoglobulin isotype (IgG1). The visualization step included the alkaline phosphatase/anti-alkaline phosphatase technique, and the slides were counterstained with hematoxylin to visualize nuclear morphology. BM samples from 98 healthy donors were also analyzed following the same procedures. Four slides (2 × 10^6 MNCs; 50 samples) and two slides (1 × 10^6 MNCs; 48 samples) were immunostained with AE1/AE3, and the corresponding number of slides was immunostained with a negative control antibody. In 4 of the 98 BMs, ≥1 stained cell was detected, showing a morphology compatible with TCs, without similar cells in the negative control.

Detection of ITCs
The cytospins were manually screened by light microscopy using the ×10 lens. All immunostained cells were closely evaluated by one of the pathologists (E. B.) and morphologically categorized into the following groups.

TC. In this group, cells with a clearly enlarged nucleus as compared with the neighboring HCs and cells appearing in clusters as a sign of their epithelial nature, were registered. Furthermore, cells lacking these pathognomonic features could be classified as TCs if they lacked recognizable hematopoietic features, most often showing strong cytoplasmic staining, often partly covering the nucleus, irregular/atypical appearance, and/or fine-stippled nuclear chromatin (11).

UICs. As UICs were classified stained objects with a badly conserved morphology, and looking destroyed or degenerated, making the distinction between TC and HC impossible or making them difficult to differentiate from artifacts.

PHCs. These cells were considered as questionable HCs during the screening process, mostly because they exhibited some HC features as described below, in addition to an anonymous appearance or sharing some characteristics with TCs.

HCs. Easy recognizable HCs have a low nuclear/cytoplasmic ratio, with nuclear size and chromatin texture resembling those of the neighboring BM cells, weak or moderate staining intensity, and cytoplasmic microvacuolization. Some HCs have pinpoint vacuoles; others have typical plasma cell appearance with characteristic chromatin condensations. This categorization is based on published guidelines (11). In doubtful cases, a second pathologist (J. M. N.) was consulted, and consensus was obtained. The presence of positive cells classified as TCs in both AE1/AE3-stained slides and the corresponding negative controls resulted in exclusion of the sample from diagnostic conclusion (46 samples).

Patients Included in the Analysis
Of the 920 patients initially enrolled in the study, histopathological tumor analysis revealed 7 cases with a benign lesion, 38 in situ carcinomas, and 2 nonepithelial cancers. Eight patients had distant metastasis at the time of the primary operation. For two of the patients, the cytospin quality was inappropriate for ITC conclusions, and 46 patients were noninterpretable because of a TC+ result in both the specific test and the negative controls. Excluding these patients from the survival analysis, 817 evaluable patients remained for further exploration. When analyzing the different categories of cells (TC, UIC, PHC, and HC), positive samples with similar cells in negative control analysis were excluded from further study. Samples with PHC+ cells were also excluded if the negative control contained cells compatible with UIC or TC. Furthermore, HC+ samples were also excluded if cells resembling PHC, UIC, or TC were present in the negative control.

Statistical Analysis
The end point for the survival analysis was BCSS and systemic disease-free survival, measured from the date of surgery to breast cancer-related death or SR and otherwise censored at the time of the last follow-up visit or at non-cancer-related death. Metastases to the skeletal system, liver, lungs or CNS were recorded as SR. Kaplan-Meier curves for time of survival free of distant recurrences and BCSS were constructed. *p* were computed by log-rank test. For statistical analysis, the SPSS (10.1) software was used.

RESULTS

Cell Categories and Clinical Outcome. Before any correlation to clinical outcome or any patient or histopathological characteristics, the immunopositive cells detected in the 817 breast cancer patients were morphologically categorized into
four groups. Examples of cells within the groups are presented in Fig. 1. Based on an initial evaluation of the probability for these categories to include true TCs, the data were analyzed in the following order: (a) samples positive for cells with morphology included in the TC group; (b) samples negative for TC but positive for cells included in the UIC group; (c) samples negative for TC and UIC but positive for cells included in the PHC group; and (d) samples negative for TC, UIC, and PHC but
positive for cells included in the HC group. The frequency of positive samples in each group is presented in Table 1. Overall, 13.2% of the evaluable patients had TC+ samples, 9.3% had TC−/UIC+ samples, 7.8% had TC−/UIC−/PHC+ samples, and 7.7% had TC−/UIC−/PHC−/HC+ samples. The frequencies of SRs and breast cancer deaths (BCDs) within the different categories are presented in Table 2 (median follow-up, 49 months). Samples with positive cells in both the specific test and the negative control were excluded from the analyses.

The patient categories described in Table 2 were then used for Kaplan-Meier survival analysis of DDFS and BCSS. As shown in Fig. 2, a marked reduction in DDFS and BCSS was observed in TC+ patients as compared with TC− patients (P < 0.001). The TC−/UIC+ patients experienced a significant reduction in BCSS (P = 0.018) versus the TC−/UIC− patients. A reduction in DDFS was also indicated (P = 0.081). No difference in DDFS and BCSS was detected between TC−/UIC− patients with and without PHC (P = 0.865 and P = 0.415) and HC (P = 0.412 and P = 0.626) present.

Combined Analysis of the TC and UIC Groups and Clinical Outcome. These results show that clinically significant immunopositive cells are present in the uninterpretable group (UIC). Therefore, for optimal categorization of clinically significant immunopositive cells, the TC and UIC groups were merged. Accordingly, the total number of positive samples increased to 22.5%. As shown in Fig. 3, the inclusion of both TC and UIC in the ITC+ group further improved the survival curve for ITC− patients as compared with the inclusion of only TC (Fig. 2). In our previous publication, the overall subgroup analysis by nodal status showed that the prognostic relevance of ITC detection was confined to the node-positive patients (10). The same result holds true when TC/UIC+ versus TC/UIC− samples were analyzed separately for the N0 and N1 group (Fig. 3).

The Number of Positive Cells and Clinical Outcome. Having identified the cell categories associated with reduced survival, the association between the number of positive cells in the TC/UIC category and clinical outcome was analyzed. Table 3 shows that the frequency of SR and breast cancer-specific death is already increased when only 1 ITC is detected (21.3% and 18.5%, respectively). The same frequency of clinical events was found for patients with 2 ITCs detected in the sample (19.4% and 16.7%, respectively). With no ITC+ cells present, 12.4% and 7.4% experienced SR and BCD. Because relatively few samples had ≥3 ITCs (22% of the positive cases), all patients with ≥3 positive cells were analyzed together. An increased frequency of SRs and BCDs was observed in this group, as compared with those with 1 or 2 ITCs present (42.5% and 32.5%, respectively).

Accordingly, Kaplan-Meier survival analyses for DDFS and BCSS were performed. Patients with 1 or 2 ITCs were categorized together because of nearly identical survival curves (data not shown). As shown in Fig. 4, the survival is reduced with increasing number of positive cells. However, both DDFS and BCSS are also significantly reduced when only 1–2 ITCs are detected in the sample.

### Table 1 Categorization of immunopositive patients by the ICC analysis of bone marrow (n = 817)

<table>
<thead>
<tr>
<th>Patient category</th>
<th>Frequency of positive cases (%)</th>
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<tbody>
<tr>
<td>TC+</td>
<td>108 (13.2)</td>
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</tr>
<tr>
<td>TC− UIC+</td>
<td>89 (10.9)</td>
<td>13 (1.6)</td>
<td>76 (9.3)</td>
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<td>TC− UIC− PHC+</td>
<td>106 (13.0)</td>
<td>42 (5.1)</td>
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* ICC, immunocytochemistry; TC, tumor cell; UIC, uninterpretable cell; PHC, probable hematopoietic cell; HC, hematopoietic cell.
* The samples have been immunostained by AE1/AE3 monoclonal antibodies and all detected immunopositive cells were categorized into the morphologic groups described in “Materials and Methods.” The patients were categorized according to the presence of cells in the cell groups, as presented.
* Negative control analysis was performed in parallel with the specific test (AE1/AE3), using an isotype-specific irrelevant monoclonal antibody.
* Only positive cases without ICC-positive cells in the negative control belonging to the corresponding morphological group were considered evaluable.

### Table 2 Clinical outcome in the patients categorized by the ICC analysis of bone marrow

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DISCUSSION

Although most clinical studies thus far have used ICC analysis for the detection of ITCs in the BM, the conclusion of a positive sample has been based on different criteria. In some studies, morphology has been used for separation between TCs and false-positive HCs (2, 9, 10, 13). In other studies, a positive result has been based on the presence of a stained cell, irrespective of the morphology (8, 14–16). Most of these studies have used negative control analysis for exclusion of patient samples with false-positive reactions. In addition, analyses of BM from non-carcinoma patients have been used for validation of the ICC staining technique (8, 10, 14–16). Only a few studies have combined the use of morphology and negative control analysis of the same number of cells as for the specific test (10). False-positive reactions may be caused by monoclonal antibody cross-reactivity or illegitimate expression of epithelial or tumor-associated antigens in normal HCs or by the visualization step of the ICC staining procedure (11, 17). The use of anti-cytokeratin antibodies ensures a high specificity of the immunostaining process. However, previous studies have shown that extremely rare HCs nevertheless may be stained during the ICC staining procedure (11, 17). Because the threshold for ITC positivity often is set to ≥1 positive cell/sample, false-positive HCs should be identified, if present. The Oslo micrometastasis study in early breast cancer has used morphological criteria (and negative control analysis) to determine ITC positivity or negativity. At the beginning of the study, it was decided that all stained cells were to be registered, irrespective of cell morphology, and categorized as described in the present paper. Before the correlation of BM results to clinical outcome was performed, it was decided that a positive BM required the presence of at least 1 ICC+ cell with morphology compatible with TC and lacking hematopoietic characteristics (12). This resulted in a frequency of 13% BM+. As shown in the present report, without use of morphological criteria, the fraction of positive samples would have reached the frequencies reported in several other studies [36–43% (7, 8, 18)]. The categorization of single immunopositive cells can be difficult because, to some extent, there is a morphological overlap between ITCs and false-positive HCs. Because the categorization of cells was predetermined as described, the morphological criteria used for ITC diagnosis can be validated and corrected, after correlation to clinical outcome. Interestingly, patients with ≥1 positive cell categorized in the UIC group experienced a significantly reduced BCSS (Table 1; Fig. 2). This indicates that a proportion of the cells within the UIC group represents destroyed or degenerated TCs. Therefore, for optimal detection of clinically significant breast cancer patients included in this study have an excellent prognosis, and therefore the ICC results can be hampered with false-positive cells (both immunocytochemically and morphologically) overwhelming a small number of true positives in this group (10). We have performed another ITC study in the BM in breast cancer. In that study, ICC+ cells categorized as UIC and/or TC were included as a ITC+ result, and an independent prognostic value was observed. The presence of

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Fig. 2 Distant disease-free survival (A, C, E, and G) and breast cancer-specific survival (B, D, F, and H) among patients with (+) or without (−) immunocytochemistry-positive cells in bone marrow within the indicated morphological cell categories. Ps were computed by log-rank test. HC?, PHC.
cells categorized as PHC and HC did not show association with SR or BCD. This is also supported by the fact that the frequency of these cells in the specific tests is comparable with the frequency in the negative controls (26% in both). If PHCs or HCs were present, the number of these cells together was 1 cell/10^6 MNCs in 96% of the cases.

Both the overlapping morphology between HCs and epithelial cells and the fact that not all ITCs may have malignant potential support the use of several markers to identify and characterize the TCs. It is possible to analyze ICC cells for hematopoietic markers as well as other epithelial markers (17, 19–23). Furthermore, it is possible to characterize the cells for factors of importance for the prediction of response to therapy or prognosis (21–23). It has also been shown that single-cell gene analyses can be performed for characterization and determination of the malignant nature of the cells (24). However, these methods are often time-consuming, complicated, and require expensive reagents. Therefore, a selection of candidate cells is needed before further characterization. Screening for ICC+ cells, followed by morphological evaluation for selection of cells belonging to the clinically significant cell group, is a feasible first step in the analysis of ITCs in BM. For some purposes, such as for the selection of good prognosis groups (BM- patients), this might be the only analysis needed. For tailored therapy or biological studies, further characterization may follow.

In a small breast cancer study, Cote et al. (4) categorized the BM samples as positive when ≥10 TCs were detected. With this detection limit, the presence of ITCs was shown to be an independent prognostic factor. The analysis of ITCs was performed differently from that in most of the larger recent studies (8–10). A direct comparison is therefore difficult. To our knowledge, no other studies have reported data on the number of ITCs present and the association with clinical outcome in breast cancer. Here, we clearly show that the detection of 1 ITC among 2 × 10^6 cells is enough to predict an increased frequency of SR and BCD (Table 2; Fig. 4). Therefore, the detection limit for prognostic relevance should be the presence of ≥1 ITC(s)/2 × 10^6 cells. Moreover, the prognosis is further aggravated when ≥3 ITCs are present in the sample, in accordance with a study in gastric cancer (15). Depending on the reason for performing the BM analysis, it can be argued that a higher number of cells (for example, ≥3 cells/sample) may be used as threshold for positivity. This could be the case if intensified treatment is planned for high-risk patients in a poor prognosis group.

In conclusion, the ICC detection of clinically significant ITCs in the BM can be improved by morphological evaluation of the stained cells. The scoring of cells with an uninterpretable morphology as ITC+, as defined in this study, increases the prognostic value of the analysis. An elevated number of ITCs in the sample predicts high frequency of early SR and death. However, the detection of a single ITC is enough to affect clinical outcome.

Table 3  Total number of positive cells within the TC\(^a\) and UIC\(^b\) categories and the correlation to clinical outcome (n = 804)\(^c\)

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<td>46 (7.4)</td>
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<tr>
<td>1</td>
<td>108 (13.4)</td>
<td>23 (21.3)</td>
<td>20 (18.5)</td>
</tr>
<tr>
<td>2</td>
<td>36 (4.5)</td>
<td>7 (19.4)</td>
<td>6 (16.7)</td>
</tr>
<tr>
<td>≥3</td>
<td>40 (5.0)</td>
<td>17 (42.5)</td>
<td>13 (32.5)</td>
</tr>
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</table>

\(^a\) TC, tumor cell; UIC, uninterpretable cell.

\(^b\) Of the 817 patients included in the study, 13 were excluded because of UIC-positive cells in both specific test and negative control.

Fig. 3  The association between the presence (BM+) or absence (BM−) of immunocytochemistry-positive cells in bone marrow categorized within the tumor cell group and uninterpretable cell group in combination and breast cancer-specific survival. Ps were computed by log-rank test.

Fig. 4  The association between the number of isolated tumor cells detected, as determined by the total number of tumor cell-positive and uninterpretable cell-positive cells present, and survival. Ps were computed by log-rank test.
ACKNOWLEDGMENTS
We acknowledge the skilled processing and sample analyses by members of the micrometastasis laboratory under the leadership of Anne Renolen.

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