Clinical relevance of cancer stem cells in bone marrow of early breast cancer patients

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Background: Cancer stem cells (CSCs) are epithelial tumor cells that express CD44\(^+\)CD24\(^−\)\(^{high}\). CSCs can be further divided into those that have aldehyde dehydrogenase (ALDH) activity (Aldefluor\(^+\)) and those that do not. We hypothesized that if CSCs are responsible for tumor dissemination, their presence in bone marrow (BM) would be prognostic in early stages of breast cancer (EBC) patients.

Patients and methods: BM aspirates were collected at the time of surgery from 108 patients with EBC. BM was analyzed for CSCs and ALDH activity by flow cytometry. Overall survival and disease-free survival (DFS) were calculated from the date of diagnosis and analyzed with Kaplan-Meier survival plots. Cox multivariate proportional hazards model was also carried out.

Results: Patients with CSCs in BM had a hazard ratio (HR) of 8.8 for DFS (\(P = 0.002\)); patients with Aldefluor\(^+\) CSCs had a HR of 5.9 (\(P = 0.052\)) for DFS. All deceased patients (\(n = 7\)) had CSCs in BM. In multivariate analysis, the presence of CSCs in BM was a prognostic factor of DFS (HR = 15.8, \(P = 0.017\)).

Conclusions: The presence of BM metastasis is correlated with CSCs and these CSCs irrespective of ALDH activity are an independent adverse prognostic factor in EBC patients.

Key words: bone marrow, breast cancer, cancer stem cells, CD24, CD44, circulating tumor cells

Introduction

Breast cancer is confined to the primary site and regional lymph nodes in more than 90% of cases. Up to 16% of women with early breast cancer (EBC) die within 5 years from diagnosis due to the development of distant metastasis [1]. Adjuvant and primary treatments in EBC patients are supposed to eliminate the micrometastatic cells responsible for vital organ cancer involvement that lead to an incurable disease. Identifying patients who would benefit from adjuvant therapy is still a significant challenge for medical oncologists.

In 2003, Al-Hajj and coworkers [2] showed that heterogeneous populations of cells in breast cancers consisted of a phenotypically distinct tumorigenic population, as well as a much larger population that lacks this tumorigenic potential. They demonstrated that CD44\(^+\)CD24\(^−\)\(^{low}\) tumors...
that micrometastases that ultimately form gross metastases represent the small population of cancer stem cells (CSCs) capable of recapitulating the heterogeneity of the tumor, Balic et al. [13] and Reuben et al. [14] showed that the majority of DTC had a putative breast CSCs-like phenotype based on immunofluorescent microscopy and multiparameter flow cytometry, respectively. A consensus of the biological significance of DTCs in BM, as well as for circulating tumor cells (CTCs) in peripheral blood (PB), is yet to be established due to the low concordance of the presence of these cells [15]. Therefore, using multiparameter flow cytometry to incorporate the nuances of expression of the best available combination of putative stem cell markers, we assessed the presence of CD44+/CD24low expression and ALDH activity in BM of EBC patients and correlated these findings with CTCs, DTCs and clinical outcome.

**patients and methods**

**study population**

This is part of a prospective laboratory study (Protocol 04-0657) conducted in the Departments of Hematopathology and Surgical Oncology, at The University of Texas MD Anderson Cancer Center in Houston, TX, and was approved by the institutional review board. Enrollment eligibility criteria included women with a diagnosis of EBC who elected to undergo definitive surgery for primary tumor and lymph node dissection [14]. All patients provided informed consent according to institutional guidelines. The baseline frequency of CD44+/CD24low cells and ALDH activity in patients without epithelial malignancy was assessed in 21 BM aspirate samples procured as part of clinical staging of patients with lymphoma. Nineteen samples were confirmed to be negative for malignancy by morphology and immunophenotyping studies that were reviewed by a Board-certified hematopathologist (JK).

**clinical-pathological analysis**

All tumor specimens were assigned a study identification number that was distinct from the patient’s medical record number. The histological type and grade of invasive disease were coded according to the World Health Organization classification system [16] and modified Black nuclear grading system, respectively [17]. Tumor specimens were analyzed in a Clinical Laboratory Improvement Act (CLIA)-certified clinical pathology laboratory at this institution for estrogen and progesterone receptor status by immunohistochemical (IHC) staining. Patients with at least one positive hormonal receptor (≥1% positive nuclei) were considered hormone receptor positive. HER-2/neu status was determined using IHC analysis or fluorescent in situ hybridization (FISH) [18]. Specimens with no evidence of staining for HER2 protein on IHC analysis or no HER2 gene amplification by FISH were considered HER-2/neu-normal (HER2−). Specimens that stained 3+ for HER2 protein on IHC analysis or demonstrated HER2 gene amplification on FISH were considered HER2+.

**detection of CTCs by cellSearch**

The CellSearch system (Veridex, Raritan, NJ) was used to detect CTCs in each of three tubes of 7.5 ml of PB per patient, as previously described [19]. In brief, PB samples were subjected to EpCAM+ cell enrichment with anti EpCAM-coated ferrous particles. Thereafter, EpCAM-enriched cells were stained with 4',6-diamidino-2-phenylindole (DAPI) and reacted with anti CD45 antibody to identify leukocytes, and with a cocktail of antibodies against cytokeratin (CK)-8, -18 or -19 [20]. CTCs were defined as EpCAM+ nucleated cells that lacked surface expression of CD45 leukocyte antigen, but expressed cytoplasmic CK and had a DAPI-stained nucleus [20]. Samples were considered positive if they had ≥1 CTC in any of the triplicate samples or per 22.5 ml of PB.

**detection of CSCs and DTCs in BM**

Techniques to isolate and identify CSCs in BM of EBC patients were already described in a previous paper [14]. Briefly, from 5 to 8 ml of BM were aspirated from each iliac crest of patients and processed within 4 h. Mononuclear cells (MNC) were isolated by Ficoll-Hypaque density gradient centrifugation at 400 g for 30 min. MNC samples (n = 66) were interrogated for ALDH activity using the Aldefluor assay, according to the manufacturer’s protocol (StemCell Technologies). Purified anti-CD44 monoclonal antibody (BD Pharmingen, San Diego, CA) was conjugated with Alexa700 using the Zenon antibody labeling kit (Invitrogen, Carlsbad, CA) before reaction with the Aldefluor-labeled cells. Additionally, preconjugated antibodies to CD24 (PE) and CD45 (PE-Cy7) both from BD Pharmingen (San Diego, CA), and CD326 (APC, Miltenyi Biotec, Auburn, CA) were used to label cells at room temperature protected from light for 30 min. An additional tube of Aldefluor-labeled cells was stained with the appropriate isotype-matched controls. The stained cells were washed twice with phosphate-buffered saline (PBS), and the cell pellet suspended in 200 µl of PBS before analysis on a LSRII flow cytometer capable of discriminating 6-color fluorescence (BD Biosciences, San Jose, CA). Cellular debris was excluded from the analysis based on low forward light scatter. For analysis and throughout the manuscript, epithelial cells in BM were defined as cells exhibiting the phenotype CD326+ and CD45−. Within the epithelial cell population (data available for 103 patients), a subset of CSC was defined as epithelial cells with a CD44+CD24low phenotype. Furthermore, based on the Aldefluor reaction, CSCs were further divided into those with Aldefluor (Aldefluor+ CSCs, CD326+CD44+CD24low) and those without Aldefluor activity (data available for 66 patients; Figure 1). Within the CSCs

**Figure 1.** Detection of cancer stem cells (CSCs) in patient bone marrow (BM). Up to 10 ml of BM were aspirated from bilateral iliac crests of patients, mononuclear cells (MNC) were isolated by Ficoll-Hypaque density gradient centrifugation; 103 patients underwent analysis by flow cytometry to detect CD326, CD45, CD24 receptors; 66 patients were assessed for ALDH activity by the Aldefluor assay. CSCs were defined as CD45+CD326+ cells with a CD44+CD24low phenotype. Furthermore, according to the Aldefluor reaction, CSCs were divided into those with ALDH activity (Aldefluor+ CSCs) and those without.
population, about half of the cells (median 53%, range 7%–88%, data available for 66 patients) were Aldefluor®.

Enrichment of tumor cells from BM was achieved by preparing MNC using Ficoll-Hypaque density gradient centrifugation. Ten cytospin slides of the BM MNC were prepared from each BM aspirate and reacted with a cocktail of antibodies, including AE1/AE3, CAM5.2, MNF116, CK8 and CK18, to identify epithelial cells (DTC) by IHC, as previously described [21–23]. A Board-certified pathologist (SK) reviewed all BM samples from 86 patients for the presence of DTCs.

statistical analysis

One hundred and eight (108) patients with EBC were included in this study. Patient characteristics were summarized using the frequency distributions. Fisher’s exact test was used to assess the association between presence of CSCs and basal clinical pathological patient characteristics. We used recursive partitioning on the null martingale residuals from a Cox proportional hazards regression model to establish cut-points for DTC, CSC and Aldefluor® CSC presence in BM. We determined overall survival (OS) and disease-free survival (DFS) hazard ratios by Kaplan–Meier curves and log-rank test according to the presence of either CSCs or ALDH+ CSCs. OS duration was defined as the time of cancer diagnosis to the date of death. All living patients were censored at the last follow-up date. The DFS duration was defined as the time from cancer diagnosis to the date of first disease recurrence or death, whichever came first. All data, such as survival and treatments, were collected from patients’ medical records. A multivariate Cox regression test was used to model and assess the relationship between OS, DFS and CSCs presence in BM. A P value of ≤0.05 was deemed statistically significant. All statistical analyses were conducted using SPSS 19 software (IBM, Somers, NY).

results

patient characteristics

From October 2006 to June 2009, 108 EBC patients were enrolled and provided a BM specimen either at the time of placement of a central venous catheter for delivering neoadjuvant chemotherapy (n = 46) or at the time of primary surgery (n = 62). Of the 108 patients enrolled in this protocol, 103 were assessable for the presence of CSCs in BM. Table 1 describes patient characteristics. Primary tumor ≥2 cm was the only characteristic associated with the presence of CSCs (P = 0.016). Nine of 23 (39%) patients treated with only endocrine therapy after surgery presented CSCs in the BM. As we have shown in a previous report [14], patients who have received neoadjuvant chemotherapy before the BM aspiration (n = 26) tend to show a higher CSC rate in comparison to patients who did not receive neoadjuvant chemotherapy (69% versus 49%, P = 0.11). Two-thirds of patients receiving neoadjuvant chemotherapy presented with CSCs in the BM at the time of surgery (Table 1). We identified a significantly higher frequency of CSC and Aldefluor® CSC in the EBC patient group compared with disease-free staging BM samples from lymphoma patients (P = 0.0006 and 0.012, respectively) (supplementary Figure S1, available at Annals of Oncology online). Among the 19 patients with disease-free staging BM and lymphoma, only one patient had CSC and Aldefluor® CSC ≥ 0.5%.

CTCs and DTCs evaluation

We evaluated the presence of CTCs in the PB of 74 patients and DTC in BM of 86 patients. Seventeen (23%) of patients had at least 1 CTC per 22.5 ml of blood. Presence of CTC ≥ 1 was not associated with presence of CSC in BM (P = 0.78) and the presence of DTCs was inversely correlated with presence of CSCs (P = 0.012) (Table 2). Moreover, we found DTCs in 34 (39.5%) of patients evaluated and the presence of DTCs was not associated with the presence of CTCs (the incidence of CTCs
was 28% in 46 patients without DTCs and 9% in 11 patients with DTCs, \( P = 0.26 \), data not shown).

We also evaluated the presence of DTCs by multiparameter flow cytometry (CD326−CD45−) in 104 patients. Sixty-one (75%) patients had positive BM samples for DTC (CD326−CD45− BM cells ≥0.53%). No association was found between DTC in BM as determined by multi-parameter flow cytometry and CTCs in PB determined by CellSearch (Table 2). Surprisingly, the detection of DTCs by multiparameter flow cytometry was inversely correlated with the presence of DTCs in BM, as evaluated by IHC (\( P = 0.021 \)) (Table 2).

### DFS and OS according to CSCs

After a median follow-up duration of 43 months, 7 of 103 patients had died and 11 had experienced disease recurrence. Three-year OS and DFS were 95% and 89%, respectively. Among patients receiving only endocrine therapy \((n = 23)\), no disease recurrence was observed. On the other hand, among the 45 patients who received neoadjuvant chemotherapy, eight (18%) had experienced recurrence of disease and six (13%) had died. Finally, among 35 patients who received adjuvant chemotherapy, three (9%) had experienced recurrence of disease and one (3%) had died. Only 1 (2%) patient without CSCs in BM had experienced recurrence of disease compared with 10 (18%) patients with CSCs in BM. All patients who died \((n = 7)\) had CSCs in BM. Patients with CSCs in BM had a hazard ratio (HR) of 8.8 for DFS \([95\% \text{ confidence interval} (CI) 1.1–69; P = 0.006]\) compared with patients without CSCs in BM. Patients with Aldefluor− CSCs had a HR of 5.9 (95% CI 1–35; \( P = 0.052 \)) for DFS compared with patients without Aldefluor− CSCs \((P = 0.013)\) (Figure 2). Because there were no deaths in the lower risk groups, the HR for OS could not be estimated and reached statistical significance \((\text{OS HR for CSC} \geq 0.5\% \text{ not estimateable}, P = 0.005; \text{OS HR for Aldefluor− CSC} \geq 0.5\% \text{ not estimateable}, P = 0.013)\) (Figure 2). DFS and OS according to DTC determined by flow cytometry and CTC are summarized in Figure 3.

### Cox proportional hazard models

We calculated the univariate Cox proportional hazard regression models for the presence of CTCs in PB, and the presence of DTCs, CSCs and Aldefluor− CSCs in BM. We found that CTCs, CSCs and Aldefluor− CSCs were strong prognostic factors for DFS: HR for CTCs \( \geq 1 = 5.9, (95\% \text{ CI } 1.7–20.8; P = 0.006) \); HR for CSCs \( \geq 0.5\% = 8.8, (95\% \text{ CI } 1.1–69; P = 0.038) \); HR for Aldefluor− CSCs \( \geq 0.5\% = 5.9, (95\% \text{ CI } 1–35; P = 0.052) \). The HR for presence of DTCs was not associated with DFS: \( \text{HR} = 1.03 (95\% \text{ CI 0.3–3.7 } P = 0.96) \) (Table 3).

Finally, a multivariate Cox proportional hazards regression analysis was carried out to determine the association between factors of interest and DFS. After adjusting for age (<45 years old), clinical T stage, N stage, ER, PR, HER2 status and nuclear grading, the presence of CSCs was found to be an independent predictor of DFS \((\text{HR} = 15.8, P = 0.017)\) as well as the presence of CTCs \((\text{HR} = 13.9, P = 0.007)\). The presence of Aldefluor− CSCs and DTCs was not found to be predictive of DFS in the multivariate model including the same factors as listed above \((\text{Aldefluor− CSCs} \geq 0.5\% \text{ HR} = 2.2, P = 0.47; \text{DTC} \geq 1 \text{ HR} = 0.8, P = 0.77)\).

**Discussion**

In this study, for the first time, we demonstrated that epithelial cells with stem cell features (CSC) identified in the BM of EBC patients predict for lower overall survival. Moreover, we demonstrated the independent prognostic value of CSCs providing much needed clinical proof of principle that CSC biology is relevant in tumor recurrence.

In an interesting study conducted on 431 primary breast cancer patients, Fehm et al. [15] have shown that there is a weak correlation between the presence of DTCs in the BM and CTCs in PB and that CTCs and not DTCs are more closely related to the biology of the primary tumor. As with other groups, we confirmed that the frequency of DTCs (40%) is significantly higher than that of CTCs (23%) and that CTCs are not correlated with DTCs in EBC \([11, 24–26]\). Other researchers have also reported that most DTCs expressed the stem cell phenotype CD44+ and CD24− when analyzed by immunohistochemistry \([13]\) and by multiparameter flow cytometry \([15]\). However, in a recent study, markers associated with epithelial mesenchymal transition (EMT) \((\text{such as Akt2, PI3K and TWIST1})\) and stem cell characteristics (ALDH1) were found in CTCs of EBC patients \([27]\). Nevertheless, DTCs and CTCs seem to represent two different categories of cells with different characteristics and morphology \([15]\). We suggest that DTCs may represent a reservoir of quiescent form of cancer cells with stem cell-like features homing to the BM, while CTCs are seeding cancer cells with intravasion and extravasion properties and EMT plasticity, circulating in the peripheral blood. Currently, it is not clear whether there is a relationship between the role of CSCs, bone metastasis and BM involvement. We are unable to state whether the cancer stem-like cell population \((\sim 10^3–10^5 \text{ cells counted at flow cytometry per sample})\) represents the self-renewal subset of cancer cells in the BM. Interestingly, with the intent to simulate healthy donor BM samples, we found that in disease-free staging BM of patients with lymphoma, the CSC and Aldefluor− CSC frequencies were significantly lower compared with those of early breast cancer patients. However, the frequency of epithelial stem cells in the BM of patients with a cancer of nonepithelial origin was still considerably high \((\text{CSC average count 0.19%, median 0.11%})\), and additional morphological study should be undertaken to clarify the function of the different cell populations. Unfortunately, a BM biopsy is a very invasive procedure and presents a considerable challenge to enroll healthy donors to provide negative control samples.

The outcome analysis of DTCs, determined by IHC in a small subset of patients \([21–23]\), are in contrast with previous reports \([9, 24–26]\) and we are cognizant that our data represent only a part of the complete database included in previous papers \([14, 21–23]\). In our study, the identification of DTC by flow cytometry was based on a single parameter (EpCAM staining) in contrast with the detection of DTCs by IHC analysis \((\text{pan-cytokeratin antibody cocktail})\) followed by meticulous pathological review. Moreover, Effenberger et al. \([28]\) have showed that the clinical value of DTCs is dependent on the type of cytokeratin expression and the cocktail of antibodies used in our protocol may represent a further confounding factor. The absence of a cytokeratin marker analysis during flow cytometer
may explain the lack of correlation between the two DTC detection methods used in our study and the considerably high count we observed using flow cytometry.

Previous studies provided only a preliminary morphologic characterization of DTCs [13]. In our trial, we were able to identify the epithelial clusters of cancer stem-like cells (CD44+/CD24−/lo) in BM with and without ALDH activity. In particular, we observed that CSCs are a promising predictive tool of adjuvant and neoadjuvant chemotherapy failure. However, we did not observe any recurrence of disease in patients receiving only endocrine treatment. With longer follow-up, it would be possible to determine whether the presence of CSCs in BM can be validated as a predictor of DFS in EBC patients receiving only endocrine treatment. Two-thirds of patients receiving primary chemotherapy presented with a high rate of CSCs in BM along with a high-disease recurrence rate. Additional adjuvant treatments might be considered for patients showing persistence of CSCs even after neoadjuvant chemotherapy. CSCs, as well as CTCs, may be a prognostic marker in primary chemotherapy in addition and independently of a pathologic complete response [29, 30].

Moreover, we found that EBC patients with ≥1 CTC in 22.5 ml of blood had a risk of relapse of 5.9 compared with patients with no CTCs. Others have also shown the feasibility of

Figure 2. (A) Disease-free survival (DFS) and (C) overall survival (OS) Kaplan–Meier curves for all 103 patients according to the presence of cancer stem cells (CSCs) in bone marrow (BM); dashed lines represent patients with CSCs < 0.5% (n = 47); continuous lines represent patients with CSCs ≥ 0.5% (n = 56). (B) DFS and (D) OS Kaplan–Meier curves for 66 patients according to ALDH+ CSCs in BM; dashed lines represent patients with ALDH+ CSCs < 0.5% (n = 52); continuous lines represent patients with Aldefluor+ CSCs ≥ 0.5% (n = 14). Time was measured from the diagnosis to the death for OS and to the first disease recurrence (DFS).
detecting CTCs [29–31] and the prognostic value of CTCs in nonmetastatic patients [29, 30, 32]. The SUCCESS-trial, a randomized study evaluating the role of CTCs at primary diagnosis and during adjuvant chemotherapy, endocrine and bisphosphonate treatment, will better clarify the prognostic relevance of CTC in EBC patients.

Considering the cumulative data of this study, we speculate that CTCs and DTCs represent a heterogeneous group of cells dislodged from primary tumor with the ability to spread and establish micrometastases in distant organs: dormant and chemo-resistant DTCs homing to the BM with more stem cell-like features (CSCs) thus functioning as a reservoir of CTCs in EMT that are potentially responsible for metastatic spread. We strongly believe that further molecular and functional characterization of CSCs might be carried out to completely understand the dynamics of tumor cell metastasis. These cells might be responsible for drug resistance and serve as a reservoir of tumor seeding cells even many years after chemotherapy [33].

In conclusion we demonstrated: (i) identification of a population of epithelial cells with stem cell-like features in the BM of EBC patients is feasible; (ii) CSCs can be detected after neoadjuvant chemotherapy in BM of some patients; (iii) the presence of CD44+CD24−/low CSCs, irrespective of Aldefluor activity, is an independent prognostic factor in EBC patients.

### Table 3. Univariate analysis for DFS and OS

<table>
<thead>
<tr>
<th>Marker</th>
<th>Patients (N)</th>
<th>Outcome</th>
<th>HR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTC (≥1 cell per 22.5 ml of blood)</td>
<td>74</td>
<td>DFS</td>
<td>5.9 (1.7–20.8)</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OS</td>
<td>4.3 (0.97–19.3)</td>
<td>0.06</td>
</tr>
<tr>
<td>Presence of DTC (by IHC) (≥1 cell per 8 ml of BM)</td>
<td>86</td>
<td>DFS</td>
<td>1.03 (0.3–3.7)</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OS</td>
<td>0.7 (0.14–3.8)</td>
<td>0.7</td>
</tr>
<tr>
<td>Presence of DTC (by flow) (≥0.5% cells)</td>
<td>104</td>
<td>DFS</td>
<td>7.3 (0.93–57)</td>
<td>0.058</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OS</td>
<td>0.7 (0.14–3.8)</td>
<td>0.7</td>
</tr>
<tr>
<td>CSC in BM (≥0.5% cells)</td>
<td>103</td>
<td>DFS</td>
<td>8.8 (1.1–69)</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OS</td>
<td>NA</td>
<td>0.236</td>
</tr>
<tr>
<td>Aldefluor+ CSC in BM (≥0.5% cells)</td>
<td>66</td>
<td>DFS</td>
<td>5.9 (1–35)</td>
<td>0.052</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OS</td>
<td>NA</td>
<td>0.013</td>
</tr>
</tbody>
</table>

HR, hazard ratio; DFS, disease-free survival; OS, overall survival; CTC, circulating tumor cell; DTC, disseminated tumor cell; IHC, immunohistochemistry; CSC, cancer stem cell; BM, bone marrow.

Figure 3. (A and B) Disease-free survival (DFS) and (C and D) overall survival (OS) Kaplan–Meier curves according to different prognostic markers: (A) DFS and (C) OS Kaplan–Meier curves for 104 patients according to DTC determined by flow cytometry (CD326); (B) DFS and (D) OS Kaplan–Meier curves for 74 patients according to CTC determined by CellSearch (22.5 ml of peripheral blood). Time was measured from the diagnosis to the death for OS and to the first disease recurrence (DFS).

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disclosure

The authors have declared no conflicts of interest.

references