Most Early Disseminated Cancer Cells Detected in Bone Marrow of Breast Cancer Patients Have a Putative Breast Cancer Stem Cell Phenotype

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Abstract

Purpose: The presence of disseminated tumor cells (DTC) in the bone marrow of breast cancer patients is an acknowledged independent prognostic factor. The biological metastatic potential of these cells has not yet been shown. The presence of putative breast cancer stem cells is shown both in primary tumors and distant metastases. These cells with a $CD44^+CD24^{-/low}$ phenotype represent a minor population in primary breast cancer and are associated with self-renewal and tumorigenic potential. Recognizing the potential effect of prevalence of putative stem cells among DTC, we evaluated the bone marrow DTC.

Experimental Design: We employed the double/triple-staining immunohistochemistry protocol and modified the established bone marrow cytokeratin (CK) staining protocol by adding steps for additional antigens, CD44 and/or CD24. We evaluated 50 bone marrow specimens, previously categorized as CK^+ from early breast cancer patients. CK^+ cells were examined for CD44 and CD24 expression by light microscopy, fluorescence microscopy, and spectral imaging.

Results: We detected the putative stem cell–like phenotype in all CK^+ specimens. The mean prevalence of putative stem/progenitor cells was 72% and median prevalence was 65% (range, 33-100%) among the overall DTC per patient, compared with primary tumors where this phenotype is reported in <10% of cells.

Conclusions: This is the first evidence of the existence of the putative stem-like phenotype within the DTC in bone marrow in early breast cancer patients. All patients had a putative stem cell phenotype among the DTC and most individual DTC showed such phenotype. Future molecular characterization of these cells is warranted.

The most important factor influencing the outcome of patients with an invasive cancer is whether the tumor has spread regionally or systemically (1). Metastases start out as single cells that detach from the primary tumor and travel to distal locations; these cells in transit, however, are not detectable by routine histologic or radiologic methods. Tumor cell dissemination to the bone marrow is an early event in progression of breast cancer (1) and is associated with poor prognosis (2–4). Disseminated tumor cells (DTC) are commonly detected by immunohistochemistry for epithelial antigens, such as cytokeratins (CK). The biology of these cells is not well understood and is critical for therapeutic improvements. Recent evidence suggests that only a certain type of tumor cells, cancer stem cells or cancer initiating cells, harbor tumorigenic potential (5). The hypothesis that the initiation of malignancy has to take place in cancer stem cells derives from the observation that it often takes many months or years for the promotion stage of carcinogenesis to occur, suggesting that the cancer stem cells must stay viable over a long period (6). Such cells have been identified for hematologic malignancies (7), brain tumors (8), and breast cancers. For breast cancers, such cells have been identified to have a $CD44^+CD24^{-/low}$ phenotype (9) and represent a minor population (10-20%) within primary tumors (10). Cells with this phenotype have been shown to be tumorigenic and multipotent, capable of generating cells with all the different lineages. One recently published study further characterized these cells with additional neoangiogenic and cytoprotective markers (11). These cells may be responsible for therapeutic failure (12).

CD44 is a cell adhesion molecule known to be expressed in most cell types (13, 14) and has been associated with stem cells

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in normal breast tissue (15). CD24 is expressed in the early stages of B-cell development and is highly expressed on neutrophils, but is absent in normal T cells or monocytes (16). Whereas CD24 is not present in adult human tissues, it has been shown to be expressed in human carcinomas (17, 18). Although considered in one study as a prognostic marker in breast cancer, where higher CD24 expression as seen by immunohistochemistry was considered to favor the worse prognosis (19), recent data suggest a different role for CD24 in the carcinogenesis of breast cancer, such that decreased expression or loss of CD24 seems to be the characteristic of the stemness of a tumor cell (9, 11). CD24 reduces stromal cell–derived factor-1–mediated migration and signaling via CXCR4 in breast cancer cell lines with enhanced CD24 expression, suppressing their metastatic potential. On the contrary, the metastatic potential of $CD24^-$ cells is increased as evidenced by small interfering RNA inhibition (20). This role of CD24 is also supported by *in vivo* analysis of nonobese diabetic severe combined immunodeficient mouse models.

Taking into account the significance of DTC in breast cancer (2–4), a reasonable next step is that we analyze the compartments known to be customarily associated with such cells—the bone marrow and the lymph nodes—to identify the potential cancer stem cells and characterize them. The underlying hypothesis, therefore, is that cancer stem cells comprise a subpopulation of DTC, and that immunohistochemical identification of this subpopulation may provide a tool to assess the malignant potential of specific cells and thus may help to present the clinical course of the malignancy. In the present study, we show for the first time an application in bone marrow of a newly developed immunohistochemical protocol to stratify DTC into subpopulations, and further show that, surprisingly, even in the early-stage breast cancer bone marrow samples studied, a vast majority of the DTC match the phenotype criterion of putative cancer stem/progenitor cells.

Materials and Methods

Patients and specimen collection

Bone marrow specimens were obtained from early breast cancer patients, participating in the American College of Surgeons Oncology Group Z-0010 clinical trial for detection and molecular characterization of DTC. Following patient informed consent, unilateral bone marrow aspirates from upper iliac crest were processed as described below. For the present analysis, frozen cytospin slides from early breast cancer patients previously categorized as CK^+ were used. Because the analysis of patient samples is still ongoing, the study remains blinded for clinical details and follow-up data are not yet available. The study was approved by local Institutional Review Boards.

Preparation of cultured cell slides

Breast cancer cell lines MCF-7, MDA-MB-231, SK-BR-3, HCC-38, HCC-1395, and HCC-70 were purchased from American Type Culture Collection (Manassas, VA). Cells were grown in appropriate growth medium as suggested by American Type Culture Collection and harvested by trypsinization before reaching confluence. The cells were subjected to cytocentrifugation (at a density of $\sim 1{,}000/mm^2$) onto positively charged slides by centrifugation at 1,000 rpm for 10 minutes. Slides were air-dried overnight, followed by acetone fixation for 10 minutes, and stored at −20°C until use for immunohistochemical analysis.

Bone marrow preparation

The procedure for processing the bone marrow aspirates has previously been published (21). Briefly, the mononuclear cell fraction containing any DTC was enriched by Ficoll-Hypaque density gradient centrifugation, using Beckman GS-6 centrifuge, at $400 \times g$ for 35 minutes and washed twice with PBS before transferring the cells onto microscope slides by cytocentrifugation at a density of $5 \times 10^5/mm^2$. Subsequently, the cells were fixed in acetone for 10 minutes and stored at −20°C.

Immunohistochemistry

Antibodies. A cocktail of two different mouse monoclonal antibodies against CK was used as primary immunohistochemistry detection reagent for circulating tumor cells: AE-1 (Signet, Dedham, MA) against low and intermediate type I acidic keratins and CAM5.2 (Becton Dickinson, San Jose, CA) against CK8 and CK18. Further, mouse monoclonal primary antibodies anti-CD44 (clone 156-3C11, LabVision, Fremont, CA), binding to standard isoform of CD44, and anti-CD24 (clone SN3b, LabVision) were used to detect the putative breast cancer stem/progenitor cell markers (10, 19).

Single-marker immunohistochemistry

Antibodies were titered until optimal concentration was determined by two independent trained personnel (M.B. and D.H.), viewing under 40× and 100× objective lenses. The following procedure was used for evaluating the expressions of the three markers (CK, CD44, and CD24) on different cell lines and bone marrow specimens. Slides were brought to room temperature before blocking with normal horse serum for 30 minutes and incubated with primary antibody diluted in blocking buffer for 1 hour. Subsequently, the slides were washed thrice with PBS and incubated with biotinylated secondary antibody (antimouse). After washing in PBS thrice to remove the unbound secondary antibody, the immune complexes were made visible by 30-minute incubation with avidin-biotin-horseradish peroxidase complexes (Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine (DAB; Biogenex, San Ramon, CA) as substrate. Cultured MCF7 and MDA-MB-231 were used as positive controls for each run in single-, double-, and triple-marker immunohistochemistry.

Double-marker immunohistochemistry

We optimized the double marker immunohistochemistry procedure using slides with MDA-MB-231 cell line following the completion of the single marker immunohistochemistry. The optimized protocol is briefly described below. Slides were brought to room temperature and incubated with normal horse serum blocking solution for 30 minutes, with Universal block solution for 10 minutes, and with Tris-HCl for 5 minutes. Subsequently, the slides were incubated with a cocktail of first primary antibodies for 2 hours (AE1 at 1:200 dilution and CAM5.2 at 1:100 dilution from stock solution) following similar detection system with DAB as described above in single-marker immunohistochemistry. The second primary antibody was incubated either overnight (CD24 antibody at 1:100 dilution from stock solution) or with similar procedure with a different detection system, alkaline phosphatase with Vector Red as substrate (Vector Laboratories) and the addition of a 30-minute blocking step of endogenous alkaline phosphatase activity with 0.1 mol/L levamisole along with PBS with 5 mmol/L levamisole for all the washes before the application of the avidin-biotin alkaline phosphatase complexes. When the slides were assessed for CK and CD44 staining, the procedure was slightly modified. The slides were first incubated with anti-CD44 antibody (at 1:300 dilution from stock solution) for 1 hour, followed by detection using horseradish peroxidase/DAB system, and then with the cocktail for anti-CK antibodies as described above, followed by detection with alkaline phosphatase-Vector Red system.

Triple-marker immunohistochemistry with immunofluorescence

Additional CD44 assessment was done on the slides where the immunodetection was already completed for CK and CD24. The slides were first soaked in warm water for a few minutes to remove the coverslips. After washing with PBS thrice, additional blocking was done with normal horse serum for 30 minutes and incubated with primary antibody for 2 hours (CD44 at dilution 1:200 from stock solution). After washing in PBS thrice, Alexa Fluor 488 goat anti-mouse immunoglobulin G (Molecular Probes, Invitrogen, Carlsbad, CA) was added at 1:100 dilution from the stock solution and incubated for 1 hour. Following nuclear staining with 4′,6-diamidino-2-phenylindole (Vecatshield mounting medium containing 4′,6-diamidino-2-phenylindole), the slides were coverslipped and sealed with nail polish. The protocol optimized for triple staining in cultured cell line slides was used to analyze the clinical bone marrow samples.

DTC identification

All preparations were analyzed by two independent trained personnel (M.B. and D.H.) using 40× objective lens and 100× objective lens with oil immersion.

When slides were analyzed for CK and CD44 staining, CK was represented by red cytoplasmic staining and CD44 by brown membranous staining. When slides were assessed for CK and CD24, cells stained for CK, which was brown cytoplasmic staining, and CD24, which was membranous red staining, were classified as CK^+CD24^+ cells (see Fig. 1A), and cells dominant with only brown cytoplasmic staining were classified as CK^+CD24^- (putative breast cancer stem/progenitor cells; Fig. 1B). By changing the focal plane of the objective, some cells previously considered negative for membrane CD24 staining did show some membrane staining, and were characterized as CK^+CD24^+.

Slides assessed for all the three antigens, CK, CD24, and CD44, were first analyzed with light microscopy. CK^+ cells were marked and subsequently examined for CD44 expression using a Leica DM LB2 microscope equipped with Diagnostic Instruments 7.3 3 shot color camera viewed with a Chroma filter set consisting of an excitation filter of 480/40 nm, dichroic filter of 505-nm long pass, and an emission filter of 5,350/50 nm (Diagnostic Instruments, Sterling Heights, MI).

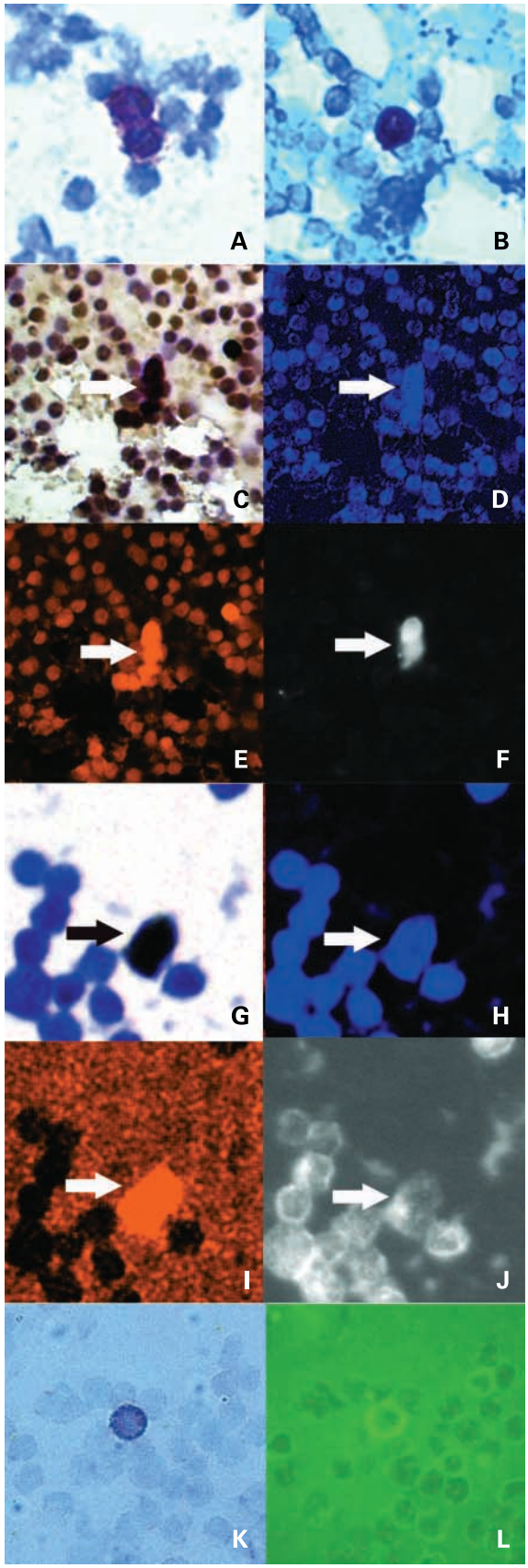

Fig. 1. *A* and *B,* double staining immunohistochemistry results for breast cancer bone marrow samples. The bone marrow samples show presence of distinct stem-like and non-stem tumor cells (magnification, ×40). *A,* two tumor cells with brown cytoplasmic staining for CK (DAB) and red membranous staining for CD24 (Vector Red). *B*, an example of a CK^+CD24^- cell (putative breast cancer stem cell). *C* to *F*, the applicability of spectral imaging analysis for immunohistochemistry double-stained samples, where the chromogens are not easily distinguishable with the naked eye. Here, the cytospun bone marrow aspirates were assessed for CK (*red*; stained with Fast Red) and CD44 (*brown*; stained with DAB). The cells were counterstained with hematoxylin. *C*, transmitted light image, showing overall cell population subjected to double-staining for CK and CD44. Arrow, two CK positive cells that are difficult to assess for their CD44 positivity. *D* and *E,* spectrally unmixed images with pseudocolors assigned to hematoxylin (*D*) and DAB (CD44; *E*) using SpectraView software, showing that all the cells in the field show nuclear hematoxylin as well as CD44 staining, including the two cells denoted by arrow. *F,* fluorescence image obtained for CK with Cy3 filter for Fast Red. *G* to *J,* applicability of spectral imaging analysis for immunohistochemistry double-stained samples for CK and CD24. In this case, the expression of CK was assessed using DAB (*brown*) and that of CD24 with Fast red (*red*). The cells were counterstained with hematoxylin. *G,* transmitted light image, showing overall cell population subjected to double-staining for CK and CD24. Arrow, a CK-positive cell that appears as $CD24^{-/low}$. To confirm that this cell is indeed $CD24^{-/low}$, we did further spectral imaging analysis. Spectrally unmixed images with pseudocolors assigned to hematoxylin (*H*) and DAB (CK; *I*). These images show that, whereas all cells in the field show nuclear hematoxylin, only the cell denoted is positive for CK (DAB). *J,* fluorescence image obtained for CD24 staining with Cy3 filter for Fast Red. Spectral imaging analysis of the cell denoted in (*G*) shows that the cell at this location expresses no/low CD24 (*red*). *K,* another CK-positive cell; *L,* the same cell with specific membranous fluorescence demarking the CD44 positivity; note the surrounding $CD44^+$ lymphocytes.

Spectral imaging

When it was not possible to distinguish with the naked eye if the cell is single or double stained, spectral imaging was done. Images were acquired at the CHLARI Congressman Julian Dixon Cellular Image Core with a Leica DM RXA microscope using a HC Plan 20×/0.70 numerical aperture Ph2 circular differential interference contrast microscopy objective lens, 1 optovar, and Koehler illumination (Leica Microsystems, Inc., Bannockburn, IL). A SKY/SD-300/VDS-1300 spectral imager with Spectral Imaging 2.5 and SpectraView 1.1 software (Applied Spectral Imaging, Inc., Carlsbad, CA) was used. Transmitted light spectral images were acquired at maximum tungsten-halogen lamp power (12 V) through a CB11 color balancing filter and ND4 neutral density filter. Spectral image exposure time was 20 ms per frame to avoid saturating the charge-coupled device detector. Spectral image wavelength range was 400 to 750 nm. Vector Red fluorescence was viewed through a Chroma Technology HQ Cy3 filter set (Rockingham, VT) with a Sutter Instruments LS300/LLG380 Xenon arc lamp/1-m liquid light guide (Novato, CA). DAB is not fluorescent and did not attenuate the red fluorescence from these dyes. Fluorescence images were acquired in SD-300/VDS-1300 direct view mode as 12-bit monochrome images and saved as 16-bit TIFF images. Spectral color images were saved from SpectraView and merged with monochrome images using Adobe Photoshop 6.0 (San Jose, CA). Figure 1C to F shows the applicability of spectral imaging analysis for immunohistochemistry double-stained samples for analysis of CK^+ cell for CD44 expression, and Fig. 1*G* to *J* for analysis of CK^+ cell for CD24 expression.

Results

We evaluated bone marrow samples for the presence and prevalence of putative cancer stem/progenitor cells. We accomplished this by using pan-CK expression to first identify tumor cells, followed by characterization of these DTC into $CD44^+CD24^-$ (cancer stem/progenitor cell subpopulation) and $CD44^+CD24^+$ (non-stem population). We first assessed six different breast cancer cell lines for CD44, which were all found to be immunohistochemistry positive. Furthermore, as proof of principle, we stained 10 slides, selected from the 50 breast cancer cases, for CK and CD44. In these 10 cases, we detected 22 CK^+ cells, and all of them were $CD44^+$. In three cases where $CD44^+$ reactivity was difficult to distinguish with the naked eye, we did spectral imaging analysis to evaluate the cells for the expression of CD44 (as shown in an example in Fig. 1C-F). These observations, coupled with the evidence that most cells (breast as well as hematopoietic; refs. 13, 14) and breast cancer cell lines are $CD44^+$, led us to reason that most of the breast cancer DTC are both CK and CD44 positive. We employed the double immunohistochemistry staining protocol optimized for cell lines on cytospin preparations from bone marrow, which were previously characterized as CK^+.

Of 100 slides (from 50 clinical samples, 2 slides each), 2 were excluded from the analysis due to poor sample quality. The remaining 98 slides were evaluated for the presence and prevalence of CK^+CD24^+ or CK^+ $CD24^-$ cells.

Only 3 of 50 (6%) clinical bone marrow specimens have not been confirmed for being positive on repeat immunohistochemistry because no CK^+ cells could be detected in either slide. This finding is attributable to sampling variability during cytospin preparations, an acknowledged clinical reality. In all 47 (100%) clinical bone marrow specimens with CK^+ cells, CK^+CD24^- phenotype was identified (Fig. 1B, G-J). The mean percentage of cells with CK^+CD24^- phenotype detected in DTC per clinical sample was 74.87% (range, 33-100%). The median percentage of such cells per clinical sample was 71%. Table 1 provides the numbers of detected cells with either of the two phenotypes and percentages of CK^+CD24^- cells in each bone marrow specimen. On analyzing a total of 332 CK^+ cells, 232 (70.59%) cells showed CK^+CD24^- phenotype.

To further ascertain the prevalence of CD44 expression across CK^+ cells, we have additionally done reevaluation of the bone marrow specimens where possible. Of the 100 slides that were assessed for CK and CD24, 71 slides underwent the additional analysis. We used the cultured cell line MDA-MB-231 as positive control, and the lymphocytes as internal positive control, where the majority are positive for CD44. When specific membranous fluorescence was undetectable in a slide, it was regarded as insufficient immunohistochemical reaction. Six of 71 slides showed insufficient staining for CD44. Two hundred-eighteen cells could be identified in the remaining 65 slides as CK^+. Of these, 19 (9%) were categorized as $CD44^-$; the rest of the cells (91%) showed specific positive membranous reaction (see Fig. 1*L*). The $CD44^-$ cells were evenly distributed between the CK^+CD24^- (4%) and CK^+CD24^+ (5%) cell populations. In none of the specimens could we identify only CK^+CD44^- cells. From 199 $CD44^+$ cells, 141 (71%) were $CD24^-$ and 58 (29%) were $CD24^+$. $CK^+CD44^+CD24^-$ cells represented 65% of the total pool of 218 cells, whereas $CK^+CD44^+CD24^+$ cells represented 26%.

Discussion

Whereas identifying the rare tumor cells in a background of numerous hematopoietic cells from bone marrow is itself a labor-intensive task requiring much expertise, distinguishing the tumor cells further between different subpopulations (stem/non-stem) poses a new challenge. Using an optimized immunohistochemistry procedure, we show here that the majority of CK^+ cells (DTC) have the putative stem cell phenotype ($CD44^+CD24^+$; refs. 9, 11). Further, we show here for the first time the usefulness of spectral imaging for analysis of single DTC and expression of additional markers. Potential enhancement of the above assay can come through application of newer techniques such as quantum dot labeling, which would allow the labeling of cells with up to six different markers and simultaneous analysis of their expression.

To our surprise, all the patients with CK^+ DTC had cells with putative breast cancer stem cell phenotype, and in fact, even when the assessment for CD44 was completed on the substantial portion of the slides, the majority (71%) of DTC were found to have a putative breast cancer stem cell–like phenotype. The majority of DTC isolated from bone marrow have previously been shown to be viable tumor cells with proliferative potential under specific culturing conditions (22); our results may be attributable to this finding because the vast majority of the detected cells have a stem cell–like phenotype. Somewhat unexpected, the high percentage of CK^+ cells having CD44 expression in bone marrow may be attributable to the previous findings in cell lines that, as an adhesion factor, CD44 also represents a homing factor of breast cancer cells to settle down in bone marrow (23).

Although DTC are regarded as the prerequisite for relapse and metastasis (24), no studies have yet examined these cells

Table 1. Presence and distribution of putative stem cells across all the bone marrow specimens analyzed

Patient no.	No. CK^+CD24^- cells	No. CK^+CD24^+ cells	Total no. CK^+ cells	% CK^+CD24^- phenotype
1	10	6	16	62.5
2	9	5	14	64.3
3	2	2	4	50
4	0	0	0	
5	4	2	6	66.7
6	5	3	8	62.5
7	6	2	8	75
8	7	2	9	77.8
9	6	2	8	75
10	3	0	3	100
11	11	1	12	91.6
12	5	6	11	45.5
13	5	1	6	83.3
14	7	4	11	63.6
15	9	3	12	75
16	9	5	14	64.2
17	3	2	5	60
18	6	3	9	66.7
19	11	5	16	68.8
20	6	2	8	75
21	2	0	2	100
22	1	2	3	33
23	7	2	9	77.8
24	1	0	1	100
25	7	5	11	58.3
26	14	6	20	70
27	1	0	1	100
28	2	0	2	100
29	2	0	2	100
30	7	2	9	77.8
31	2	0	2	100
32	4	3	7	57.1
33	5	3	8	62.5
34	0	0	0	
35	5	3	8	62.5
36	6	0	6	100
37	8	3	11	72.7
38	7	3	10	70
39	1	0	1	100
40	4	0	4	100
41	3	0	3	100
42	4	2	6	66.7
43	5	2	7	71.4
44	3	0	3	100
45	4	1	5	80
46	3	2	5	60
47	5	2	7	71.4
48	0	0	0	
49	1	1	3	50
50	2	2	4	50
Average of CK^+CD24^- phenotype/total DTC per patient				74.87
% CK^+CD24^- phenotype in overall total DTC				70.59 (240 of 340)

NOTE: The table provides the total number of CK^+CD24^-, CK^+CD24^+, and CK^+ cells, analyzed from two cytospun slides from bone marrow of each patient. For each patient the percentage of CK^+ cells with CK^+CD24^- phenotype is presented along with the mean percentage per patient and the overall percentage of all CK^+ cells with CK^+CD24^- phenotype.

for the existence of putative stem cell phenotype. Abraham et al. (10) have recently analyzed primary breast tumors for prevalence of $CD44^+CD24^{-/low}$ cells and their effect on clinical outcome. They found that 70% of patients had <10% of cells with $CD44^+CD24^{-/low}$ phenotype and that higher prevalence of these cells correlated with distant metastases, in particular, bone metastases. However, in their study, the finding had no effect on overall survival of breast cancer patients. Kristiansen et al. (19) considered higher CD24 expression as a prognostic marker in breast cancer to favor the worse prognosis. Their immunohistochemistry data, however, show differential expression of CD24 in tumor cells, with a high number of $CD24^+$ cells, but cell nests can be recognized with low or no expression of $CD24^-$. These data can be explained given the recent evidence that $CD24^-$ stem cells give rise to both $CD24^+$ and $CD24^-$ cells (9) and that the

larger proportion of cells in tumors is represented by $CD24^+$ cells. In addition, it is still not clear if the number of cancer stem cells—or simply their presence—in the primary tumor is critical for further disease development.

We are well aware of the fact that our present study as well as previous studies has analyzed these cells for a predefined phenotype, and that this is intended only as a preliminary morphologic characterization of these cells. Whereas the finding of DTC with putative stem/progenitor cell–like phenotype in majority of breast cancer patient bone marrow is somewhat unexpected, it must be noted that this phenotypic characterization needs to be supplemented with functional analysis of these cells, which would inform on their capability of self-renewal. Therefore, further molecular characterization and biological analysis of the putative stem cells in prospectively collected patient samples must be done. Nonetheless, considering the present evidence, which suggests the effect of such cells on the progression of breast cancer (9, 11, 25) and other solid tumors (8, 24), we strongly believe that our assay permits an initial identification of putative stem-like cell subpopulation among the DTC, which can then permit further molecular and functional characterization.

Using the assay we present here as a basic morphologic assay, it should also be possible to better characterize DTC for additional stem cell–like markers. Some of the molecular features of putative stem cells are emerging in the recent literature (11, 26). Their data suggest that only a small proportion of the $CD44^+CD24^{-/low}$ cells may have the self-renewal capability. However, even if only a proportion of the putative stem-like cells detected in our study have an ability to perpetuate indefinitely, this finding indicates that at least a proportion of the cancer cells detected in the bone marrow of patients with early-stage breast cancer are capable of forming metastases. Further, the majority of the DTC detected in the bone marrow have the putative stem cell phenotype, as compared with what has been reported in the primary and metastatic tumor formations (where only a minority of cells have this phenotype; refs. 9, 10), clearly suggesting that there is a selection for these stem-like cells and that DTC detected in the bone marrow of patients with early-stage breast cancer are not merely shed from the primary tumor.

Breast cancer patient population has a lifetime enhanced risk of death from cancer when compared with similar normal population group (27). This may be attributable to the potential effect of the existence of cancer stem cells among the DTC in patients. Recent pooled analysis by Braun et al. (3) in a large number of patients analyzed shows that although the presence of DTC is associated with a poor prognosis, a significant proportion of patients still do well even after 10 years. The reason for this variability could be that a proportion of these cells may be capable of remaining "dormant," settled in bone marrow and/or distant organs, which are driven towards the recurrent tumorigenesis only after unknown initiation events occur in these cells. In addition, besides their presence, the environment of DTC may be as crucial in determining the destiny of the cells and final patient outcome (24).

This is the first evidence of the existence of the putative stem/progenitor cell ($CD44^+CD24^-$) subpopulation within the DTC component in bone marrow. Because we could identify the putative breast cancer stem cell phenotype in all patients where we detected CK^+ cells, our data suggest that the majority of the patients with DTC may have a lifetime risk for relapse, an idea that has been suggested previously (28). Existence of putative stem cells will be a clinically relevant issue that we must address. Recognizing that it is desirable to correlate these findings of existence of putative stem cells among the DTC population with the actual clinical progression of breast cancer patients, we will in the future examine the clinical correlates for these patients once the clinical outcome data become available from American College of Surgeons Oncology Group clinical trial.

Whereas the possible presence of early tumor dissemination is the rationale behind the use of systemic adjuvant chemotherapy in patients who have undergone definitive local treatment of the primary tumor (29, 30), the presence of cancer stem cells may explain the failure of adjuvant chemotherapy in a proportion of early-stage breast cancer patients. Further research aimed at enrichment and expression profiling of $CD44^+CD24^-$ population of DTC in a larger set of prospectively collected bone marrow specimens can help identify additional, novel potential therapeutic targets and further define the biological potential of these cells.

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