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Cytokeratin-positive cells in bone marrow of breast cancer patients and noncancer donors

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Abstract

Detection of disseminated tumor cells in the bone marrow may provide important prognostic information in breast cancer patients. With few exceptions the number of stained cells scored as cancer is very low; there may be only one cell per slide. This makes definitive interpretation of cancer in marrow challenging. False-positive staining of marrow cells with cytokeratin (CK) antibody is relatively common and makes interpretation more difficult. In this report we focus on false-positive staining of marrow specimens from breast cancer patients and noncancer controls and demonstrate that the frequency of false positive events is common.

Bone marrow was collected from 23 cancer-free donors and 60 breast cancer patients. Samples were processed by Ficoll density gradient centrifugation and slides were prepared for immunocytochemical staining with CK and irrelevant (IR) antibody. Slides were evaluated manually and positive cells were categorized as tumor cells (TCs), hematopoietic cells (HCs), or questionable cells (QCs).

False-positive staining events were commonly observed in noncancer cases stained with CK or IR antibodies and in breast cancer cases stained with IR antibody. There was little difference in the number of breast cancer marrow specimens scored as tumor cells regardless of whether the antibody used was CK or IR.

It is important to devise improved criteria and methods for accurate detection and interpretation of disseminated tumor cells in the marrow of breast cancer patients.

Keywords

disseminated tumor cells; cytokeratin; breast cancer; bone marrow

Introduction

Detection of disseminated tumor cells (DTCs) in the bone marrow may provide important prognostic information in breast cancer patients. In pooled multicenter data, the rate of DTC detection in bone marrow aspirates obtained pre-operatively using CK antibody (A45-B/B3) was 30% in patients with surgically resectable breast cancer.¹ Discovery of DTCs in the marrow of these breast cancer patients was the most important independent prognostic factor in these patients, surpassing tumor size or lymph node occult disease status. Other investigators reported pre-operative rates of DTC detection in the marrow in 13% to 42% of patients and in each study this finding was an independent predictor of disease recurrence.²⁻⁵ Patients that had pathologically negative regional lymph nodes and bone marrow aspirates free of detectable tumor cells had a disease recurrence rate of 5% or less.

While these results suggest prognostic importance, these techniques have not yet been tested in a large prospective multi-center trial with a standardized approach to tissue procurement, handling, staining, and interpretation, and the size of individual studies has been too small to quantify prognostic value across the spectrum of currently defined patient staging categories. Perhaps most importantly, the high rate of false-positive staining events needs to be understood and resolved before widespread clinical adoption is possible.

CK is the antigen most commonly used to detect breast cancer cells in the marrow⁶⁻⁸ but false-positive CK staining may occur. CK antibodies may bind to hematopoietic cells (HC) through the Fc receptor or bind CK present on a variety of primitive, nonmalignant, epithelial precursor cells.⁶⁻⁸ Cell morphology has been used to differentiate CK-stained cells as either true-positive and false-positive events⁹ and guidelines have also been proposed for defining when a sample meets the requirements of being called positive for cancer.¹⁰

We previously presented data on immunofluorescent staining of bone marrow aspirates from breast cancer patients using CK antibodies as the detection antibody, a set of HC antibodies as a counterstaining control, and morphological criteria.¹¹ We observed that false-positive CK staining was a relatively common event. We now focus on the frequency of false-positive events in marrow samples from both breast cancer patients and noncancer donors, using conventional brightfield staining procedures and morphological interpretation protocols.

Material and Methods

This study was undertaken after approval was obtained from local institutional review boards and was done in accord with an assurance filed with and approved by the US Department of Health and Human Services. Informed written consent was obtained from each participant in this study.

Bilateral bone marrow aspirates from the anterior iliac crest were obtained from 60 women undergoing surgery for breast cancer and processed as previously reported.¹¹ Bone marrow samples were also collected from 23 patients without a history of cancer who underwent surgery in which bone marrow was available as part of the surgical procedure. Samples were processed using density gradient centrifugation. Bone marrow samples were diluted with an equal amount of phosphate buffered saline and layered on Ficoll-Plaque Plus (GE Healthcare, Sweden). After centrifugation the mononuclear cell (MNC) layer was collected and washed with phosphate

buffered saline. Cells were counted on a Neubauer hemacytometer and 1.5 million MNCs were deposited per slide. Slides were frozen at -80°C until staining.

Slides were fixed with 10% buffered formalin or acetone and then with methanol. Blocking was done with Dual Endogenous Enzyme Block (Dako, California) and Image It (Invitrogen, Carlsbad, California). Four slides were incubated with CK antibody (CK8) CAM5.2 IgG2a isotype (BD Biosciences, California) and 4 additional slides were incubated with irrelevant (IR) antibody mouse IgG2a (Dako, California). A secondary anti-mouse IgG2a conjugated to alkaline phosphatase was detected using Fast Red chromagen. All antibody incubations were done for 30 minutes. Nuclear staining was done with DAPI (4'-6-Diamidino-2-phenylindole) and evaluated by fluorescence microscopy.

Slides were screened and evaluated by brightfield microscopy for the presence of red-stained cells. Stained cells were classified as tumor cells (TC), questionable cells/uninterpretable cells (QC), or hematopoietic cells (HC) based on criteria previously reported.^{10, 12, 13} A cell was considered a TC if it was enlarged, had an increased nuclear/cytoplasmic ratio, and was stained red in such a way as to overlap the nucleus. A HC was not enlarged, had a low nuclear/cytoplasmic ratio, and the staining was less intense. QCs were cells that fell between the morphologic criteria for the two groups.

A separate set of marrow specimens were used to evaluate lowering of background staining of HCs using a variety of blocking strategies and cytokeratin antibodies and (Table 3). Blocking reagents included CytoQ Background Buster and Fc Receptor Blocker (Innovex Biosciences, Richmond, California), different animal sera, BSA, Image It and isotype-specific irrelevant antibodies (Invitrogen, Carlsbad, California). Primary and secondary antibodies used in this set of experiments listed in Table 3.

Results

Typical staining patterns are shown in Figure 1. The upper panels are marrow cells from noncancer cases and the lower panels are marrow specimens from breast cancer patients. The left-side images are stained with CK and the right-side images are stained with IR antibody.

The frequency of staining events and the scoring of the stained cells for 23 noncancer control cases and 60 breast cancer patients is shown in Table 1. All cases were stained with both CK and IR antibodies and results are presented according to the antibody used. For example, 30% of noncancer control cases were scored as having TC with either CK or IR antibody. In the 60 breast cancer cases, 27% were scored as having TCs with either CK or IR antibody.

The staining and scoring results, tabulated according to the specific number of cells stained and scored as TCs per 10⁶ MNCs, are presented in Table 2. The overall frequency of cells stained with CK and IR per 10⁶ MNCs was very similar for noncancer control samples (0.32 and 0.28 respectively) and breast cancer case samples (0.33 and 0.25 respectively). These rates are similar to recent data from Borgen.¹³ The number of cells scored as TCs, per 10⁶ MNCs, was also similar between the noncancer control cases (CK 0.12 and IR 0.10) and the breast cancer cases (CK 0.07 and IR 0.06).

From these data, the following points were observed: (1) there were several positive staining events in the noncancer specimens; (2) several of the staining events in the noncancer cases were consistent with criteria for TCs, based on morphology; (3) there were frequent staining events in the breast cancer patient marrow with the IR negative control antibody; (4) the IR staining events in the breast cancer cases were frequently scored as TCs; (5) the frequency of breast cancer patient cases scored as having TCs was the same regardless of whether the antibody was CK or was IR; and (6) the frequency of cases scored as TCs was remarkably

similar across all categories. These data confirmed our previous observations that there are serious problems associated with the accuracy of this method for detecting DTCs in marrow specimens.

Nonspecific staining of HCs is common with many antibodies. The background cells in the marrow specimens are predominantly hematopoietic and there are about 1.5 million HCs per slide. We evaluated antibodies used in the reagent sets, including secondary antibodies and irrelevant antibodies, and found isolated staining of HCs to be a common event (Table 3). Different blocking strategies including CytoQ Background Buster, Fc Receptor Blocker, different animal serums, BSA, Image It and isotype-specific irrelevant antibodies did not eliminate isolated staining of HCs.

Isolated staining of HCs by non-HC antibodies has been previously reported, but the importance of the phenomenon may have been overlooked because the incidence is as low as 1 cell per slide (1 per million HCs).^{14, 16} However, in the clinical situation of detecting rare DTCs, this frequency is an important issue because the number of candidate TCs is so low. The frequency of HC staining by non-HC antibodies is similar to the frequency of TCs reported to be present when staining with epithelial antibodies.

Discussion

Staining events with CK antibody in a noncancer control patient are by definition false positive staining events. We observed that CK antibody staining of marrow cells is common in samples from noncancer control patients. False-positive staining events do not necessarily mean a false-positive interpretation, since morphological criteria allow additional discrimination of the stained cells. Application of morphological criteria did reduce the frequency of false-positive staining events. However, 30% of the noncancer control cases were still scored as positive for having tumor cells despite application of morphological criteria.

CK antibody staining events of noncancer marrow samples has been previously reported. For example, based on CK staining and morphology, Naume reported 8% incidence of false-positive staining in noncancer marrow specimens.¹⁴ Lower rates were reported by Braun: 2/191 (1%) of noncancer controls had false-positive staining events. It appears that Braun used staining only, without morphological criteria to make the interpretation.¹⁷

Variation of results may be related to the size of the evaluated sample. As gauged by the number of MNCs, sample volume varies between centers. Fixed-rate rare events may be observed more frequently when a larger sample is examined.¹⁵ For example, in the noncancer control cases our observed rate of events per 10^6 MNCs is similar to that reported by Borgen et al.¹³ Borgen reported a range between 1.02 to 0.21 stained cells per 10^6 MNCs which varied according to the type of CK antibody. We observed a rate of 0.32 stained cells per 10^6 MNCs, which falls within the range observed by Borgen.

We observed that cell staining events with IR antibody were common and almost half the noncancer cases had positive staining events. Staining events due to IR antibody are all false-positive, regardless of whether the marrow sample is from a cancer patient or a noncancer control case. Even after applying morphological criteria, nearly a third of the noncancer control cases were scored as having stained cells consistent with interpretation as cancer. This rate was similar in the 60 breast cancer cases.

Other reports also describe IR antibody-stained cells in noncancer control cases. For example, Naume observed that 5/50 (10%) noncancer cases had IR-stained cells that were interpreted as TCs.¹⁴ Using CK, the same noncancer control cases had a similar rate of cases rated as TC. Although this rate is somewhat lower than our observed rate, it still represents a clinically

relevant false-positive rate. Naume reported that the TC scoring rate was similar whether the antibody was CK or IR.

One report compared the results from 3 different laboratories evaluating the same patient marrow samples. In 48 noncancer control cases, the number of IR-stained cells was similar to the number of CK-stained cells. Although morphological interpretation of the IR-stained noncancer control cases was not reported, these results emphasize that false-positive staining events are common.¹³

The reported incidence of IR-stained cells falsely scored as cancer in breast cancer cases varies from 0% to 9.3%. Naume observed 64/685 (9.3%) of breast cancer cases had IR-stained cells classified as TCs.¹⁴ Wiedswang observed that in 22/421 (5.2%) breast cancer cases, IR-stained cells were scored as TCs.¹⁶ Borgen found that in 14/257 (5.4%) cases, cells were present that were IR-stained and lacked HC characteristics.¹² Bidard found no IR-stained cells with TC morphology in 621 patients.¹⁷ These results indicate that there is considerable variation in the final interpretation of IR-stained cells in breast cancer marrow specimens. Ideally all the results should be zero.

Our observed rate of breast cancer cases with IR-stained marrow cells scored as TCs was 27% and is higher than other reports. This may be due to variation in interpretation and may also be related to larger marrow samples as reported by Naume.¹⁴ We analyzed 6×10^6 MNCs, which is 3 times the number of MNCs analyzed in other reports. It is likely that as larger marrow samples are analyzed, more IR-positive cells will be scored in all categories. However, exact comparisons are difficult because it is uncommon for reports to present the number of IR-stained cells in breast cancer patient marrow specimens.

It appears that many non-HC antibodies will stain occasional HCs in marrow samples from breast cancer patients or from noncancer control samples. We evaluated several non-HC antibodies for staining of HCs (Table 3). Staining of HCs was observed with 5 different commercially available CK antibodies. Staining of occasional HCs was also observed with 5 different goat or rabbit secondary antibodies, 1 mouse anti-FITC, and 2 different mouse anti-aspergillus antibodies. These findings are consistent with previous reports describing HC staining with non-HC antibodies.^{12, 18} Non-HC antibodies may stain marrow cells when HCs bind the Fc receptor of mouse antibodies.^{9, 10} Alternatively, CK antibodies may bind to epithelial stem cells in the bone marrow.^{21, 22} The combined use of primary, secondary, and irrelevant antibodies in a reagent set for staining marrow specimens, all of which could independently stain noncancer marrow cells, increases the chance of false-positive staining of HCs.

In our 60 breast cancer cases, CK-stained cells were observed in 66% of cases. Application of morphological criteria to these cases resulted in scoring 27% of cases consistent with cancer. This scoring rate is similar to a large analysis of 9 pooled studies in which 30.6% of cases were scored as cancer.¹ The similar rates between our data and this large data set support the comparability of the staining methods and interpretation criteria employed in our study.

Importantly, we observed a similar rate of staining events and scoring of TCs in the breast cancer patient cases regardless of whether the antibody used was CK antibody or IR antibody. Even the overall frequency of cells stained per 10^6 MNCs and the number of cells scored as TCs was similar in the breast cancer group regardless of the type of antibody used. When considering both the breast cancer group and the noncancer controls, the frequency of cases scored as TCs was remarkably similar across both staining categories. Essentially, the staining rates and cancer cell scoring rates were similar regardless of whether the cases were cancer, noncancer controls, or whether the antibody was CK or IR. These observations present a serious

challenge to the confidence level of rendering an accurate clinical interpretation of DTCs using these methods.

There is a variety of possible solutions to the problems of false-positive staining events of breast cancer patient marrow specimens using CK antibodies and brightfield microscopy. Defining an improved set of morphological criteria for interpretation of stained cells may be possible. This will be a challenge due to the wide variety of noncancer cells present in the marrow, particularly cells that are progenitor cells that have fewer features of differentiation.

The rarity of cancer cells is also a problem since there may be only one stained cell with features consistent with cancer. There is almost no other area of clinical medicine that would consider a diagnosis as confirmed with such minimal patient material. For example, fine-needle aspiration of a primary breast cancer will typically yield thousands of candidate cells. Obtaining sufficient clinical material for confident interpretation of needle aspiration cytology is a necessary step in the evaluation of primary tumors of the breast.¹⁹ A minimum threshold number of cells may need to be established as criteria for successful interpretation of bone marrow aspirations.

Another option is to stain marrow specimens in parallel using CK on one set of slides and IR antibody on another set.²⁰ If the IR antibody slides have cells scored as cancer then that case would be considered not interpretable. This approach assumes that in the absence of cancer-scored cells on IR slides, the CK slide results are valid. Application of this strategy to our results and eliminating those cases where TCs were found on corresponding IR antibody control slides, the cancer score rate dropped to 7/60 (13%). This rate is similar to previously reported data in a larger group of patients in which the final scoring as cancer was 108/817 (13%).⁹ However, this approach decreases the number of cases that are considered evaluable and does not provide a diagnosis for the non-interpretable subgroup.

Another potential solution is to perform simultaneous dual staining with one reagent set that stains cancer cells and a second reagent set that stains HCs. Using fluorescent reagents, we previously reported data in 31 breast cancer patients using this strategy. The majority of CK-positive cases were demonstrated to be HCs by counterstaining with HC antibodies. Other reports using dual staining have observed HCs stained with CK antibodies.¹²

The data presented here demonstrate that staining of occasional marrow cells with antibodies, regardless of the antibody target, is a common event. The staining events occurred in both noncancer control cases and breast cancer cases, and the events were nearly equal in both groups. Interpretation of stained cells using morphological criteria diminished the number of false-positive scores. Even after scoring of cells there were still considerable false-positive events and these were evenly distributed throughout both groups with both CK and IR antibody. With as few as one or two cells present for interpretation, the relatively high rate of potential false-positive staining events makes it important to devise improved criteria and methods for accurate detection and interpretation of DTCs in the marrow of breast cancer patients.

Acknowledgments

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Figure 1. Typical Examples of Cells Classified as Tumor Cells

A, B: Noncancer control marrow specimens displaying intense red staining covering the nucleus and high nuclear/cytoplasmic ratio. A is stained with CK antibody and B is stained with IR antibody. **C:** Breast cancer patient marrow specimen stained with CK antibody displaying enlarged cell size, high nuclear/cytoplasmic ratio, and staining covering the nucleus. **D:** Breast cancer patient marrow specimen stained with IR antibody showing an enlarged cell with bright red stain covering the nucleus.

Table 1

Scoring of Marrow Specimens from 23 Noncancer Marrow Donors and 60 Breast Cancer Patients Stained with CK and IR Antibodies

			Percent of cases scored as:		
	Antibody	Total Cases	TC	QC	HC
Noncancer control cases	CK	23	30%	13%	17%
	IR	23	30%	4%	13%
Breast cancer patient cases	CK	60	27%	7%	32%
	IR	60	27%	5%	35%

Abbreviations: TC, tumor cell; QC, questionable tumor cell; HC, hematopoietic cell; CK, cytokeratin; IR, irrelevant antibody.

Table 2

Overall Frequency of Cells Stained with CK or IR Antibodies and Scored as TCs in 23 Noncancer and 60 Breast Cancer Patient Marrow Specimens

	Antibody	Total number of cells stained per group	Overall frequency of cells stained per 10 ⁶ MNC	Total number of cells scored as TCs	Overall frequency of cells scored as TCs per 10 ⁶ MNC
23 Noncancer BM specimens	CK	37	0.32	14	0.12
	IR	32	0.28	11	0.10
60 Breast cancer BM specimens	CK	138	0.33	22	0.07
	IR	103	0.25	18	0.06

Abbreviation: MNC, mononuclear cells

Table 3

Healthy control marrow stained with cytokeratin and irrelevant antibodies (both with alkaline phosphatase secondary antibody) and secondary antibody alone.

Cytokeratin antibodies	Secondary antibodies	Irrelevant antibodies
Mouse CAM5.2 IgG2a	Goat anti-mouse IgG1	Mouse anti-aspergillus IgG1
Mouse AE1/AE3 IgG1	Goat anti-mouse IgG2a	Mouse anti-aspergillus IgG2a
Mouse CK 8, 18 IgG1	Goat anti-rabbit	Mouse anti-FITC
Rabbit pan cytokeratin	Goat anti-chicken	
EpCam (BerEp4) IgG1	Rabbit anti-chicken fab/Goat anti-rabbit fab	