Detection of Circulating Cytokeratin-positive Cells in the Blood of Breast Cancer Patients Using Immunomagnetic Enrichment and Digital Microscopy

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ABSTRACT

Purpose: **To examine the feasibility for identifying and enumerating cytokeratin positive (CK+) cells in the peripheral blood of breast cancer patients.**

Experimental Design: **Blood specimens from 34 normal donors (negative controls), 15 samples to which carcinoma cells were added (positive controls), and 84 breast cancer patients [27 node-negative (N−), 29 node-positive (N+), and 28 metastatic] were studied. RBCs were lysed with ammonium chloride and the resulting cell suspension incubated with anti-EpCAM-conjugated immunomagnetic beads for carcinoma cell enrichment. Immunomagnetically selected cells were placed on slides; stained for CKs 8, 18, and 19; and evaluated with an automated digital microscopy system that rapidly scanned the slide and collected images of cells meeting predefined staining and cytomorphological criteria. A montage of the CK+ cells was reviewed to confirm tumor cell morphology.**

Results: **Eighteen specimens (9 normal, 2 N−, 4 N+, and 3 metastatic) were excluded because of poor cytomorphology or staining artifact. All 15 of the positive controls [95% confidence interval (CI), 78–100%] and none of the 25 negative controls (95% CI, 0–14%) demonstrated CK+ cells. Twenty-one of the 75 (28%; 95% CI, 18–40%) samples from breast cancer patients demonstrated CK+ cells including 76% of patients with metastatic disease (95% CI, 55–91%), 8% with N+ disease (95% CI, 1–26%), and none of those with N− disease (95% CI, 0–14). The mean number of CK+ cells detected in the 21 CK+ patients was 18.4 (range, 1–120).**

Conclusions: **Breast carcinoma cells can be detected in the blood from a significant fraction of metastatic breast cancer patients using immunomagnetic cell enrichment and digital microscopy. The incidence of CK+ cells was low in those with resected N+ disease (at most 26%) and those with resected N− breast cancer (at most 14%). This technique could be used in large prospective studies of patients with breast cancer to learn whether the detection of rare carcinoma cells is a useful predictive or prognostic factor.**

INTRODUCTION

The finding of rare carcinoma cells in the blood of patients with malignancy is not unexpected. Indeed, Ashworth (1) demonstrated the presence of malignant cells in the blood of patients with various carcinomas including breast cancer more than 100 years ago. The search for metastases in the lymph nodes of patients with newly diagnosed breast cancer remains a part of standard care of breast cancer patients, and is a strong prognostic factor for recurrence and survival. The presence of metastatic cells in the bone marrow or peripheral blood is another potential prognostic factor. There have been several recent studies that have used light microscopy and manual enumeration of cancer cells in bone marrow aspirates of patients with breast cancer (2–8). In a recent study of 552 patients with breast cancer (2), CK^2+ cells were detected in the bone marrow aspirates of 36% (199 of 552) of patients. Patients with bone marrow micrometastases had a higher risk of tumor recurrence and death from cancer-related causes compared with those with carcinoma cell-negative bone marrow ($P < 0.001$).

Searching for rare tumor cells in blood or bone marrow with light microscopy is tedious and not amenable to the routine diagnostic laboratory. Technological advances such as immunomagnetic enrichment (9), digital microscopy (10), and molecular methods such as RT-PCR (11–14) have improved the assays and have made them more applicable to clinical research. Enrichment of the sample for carcinoma cells is especially useful when blood samples are being evaluated. By use of these immunomagnetic enrichment methodologies, rare (approximately 10^{-7}–10^{-8}) tumor cells may be detected in the peripheral circulation of patients with breast cancer (11). The detection of rare tumor cells in the blood has been additionally improved with the use of a novel automated digital microscope (ACIS; ChromaVision Medical

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[2] The abbreviations used are: CK, cytokeratin; RT-PCR, reverse transcription-PCR; N−, node-negative; N+, node-positive; ACIS, Automated Cellular Imaging System; CI, confidence interval.

Systems, Inc.). Key features of this technology are automated (unattended) scanning of up to 100 slide-based specimens followed by the active participation of the laboratory professional (pathologist) in reviewing interpreting collected images, thereby minimizing risk for false positive determinations. A recent report (10) suggests that this technology demonstrates superior sensitivity for rare tumor cell detection relative to manual microscopy with excellent reproducibility.

Flow cytometry (9, 15) and molecular techniques also have been shown to offer a sensitive means for rare circulating tumor cell identification. Mammaglobin is a glycoprotein, which is expressed preferentially in adult breast tissue, in 80–95% of malignant breast tumors, and lymph node metastases from breast cancer patients (13). RT-PCR using primers to mammaglobin or to CKs has been used to detect metastatic tumor cells (14, 16). Although RT-PCR and flow cytometry are sensitive techniques, they do not permit visual confirmation of malignant cells, and false positive results on normal controls have been reported (11, 12, 15). Nonetheless, these early studies suggest that CK+ cells can be detected in the blood and bone marrow with multiple methods and that this may be an important new addition to the staging of breast cancer and the predicting of prognosis.

Our investigation details the technical performance of combined immunomagnetic enrichment and digital microscopy for the detection and enumeration of CK+ cells in peripheral blood specimens. Initial results are based on the analysis of biological controls [blood from normal donors (negative controls) and blood samples spiked with BT474 breast cancer cells (positive controls)]. The false positive rate of the assay to detect CK+ cells is next evaluated using clinical specimens from normal donors and the prevalence of CK+ cells in women with breast cancer.

MATERIALS AND METHODS

Patient Eligibility. Patients with breast cancer provided verbal consent to donate 20 ml of blood for this research study. The patients were required to have either N−, N+, or metastatic adenocarcinoma of the breast. The protocol required that all of the patients with N− disease or N+ disease be newly diagnosed and that the blood be drawn before any adjuvant therapy. All of the samples from these patients were obtained after the patient had undergone primary surgery and were being evaluated in the postoperative setting. Patients with metastatic disease were outpatients returning for follow-up and were allowed to be on therapy at the time of phlebotomy. Blood samples from normal human volunteers were also tested. The patient record was reviewed only to confirm the disease status, and no information on treatments or clinical course in the case of metastatic disease was abstracted. No research test results were entered into the patient record or released to the patient or the patient physician. This study plan as described above was approved by the Mayo Foundation Institutional Review Board.

Epithelial Cell Enrichment Procedure. Peripheral blood specimens were processed for the detection of epithelial cells within 24 h of phlebotomy. In 12 cases, <20 ml was obtained by phlebotomy (all cases ≥14 ml). Peripheral blood was first enriched for nucleated cells using RBC lysis then enriched for epithelial cells by positive immunomagnetic selection. All of the enrichment steps and centrifugations were performed at room temperature. Erythrocytes were lysed by adding 4 volumes of $1\times$ RBC lysis buffer [155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA (pH 7.2); Sigma Chemical Co., St. Louis, MO], mixing by inverting, and incubating for 5 min. Lysed blood was then centrifuged at $300 \times g$ for 5 min. The lysis step was repeated and the cell pellet was then resuspended in 7 ml PEB buffer. PEB buffer is $1 \times$ PBS, 0.1 mM EDTA (Sigma Chemical Co.), and 0.5% BSA fraction V (Sigma Chemical Co.); $1 \times$ PBS is 0.144 g/liter KH_2PO_4, 0.795 g/liter Na_2HPO_4-$7H_2O$, and 9 g/liter NaCl (pH 7.4; Life Technologies, Inc., Gaithersburg, MD). Samples were centrifuged at $300 \times g$ for 5 min. Finally, the cell pellet was resuspended into 0.9 ml PEB.

Positive Selection Procedure. HEA125-microbeads (100 μl; Miltenyi Biotec Inc., Bergisch Gladbach, Germany) recognizing EpCAM were added to the 0.9 ml cell suspension. The cell-microbead mixture was incubated for 30 min on a shaker (Labquake; Barnstead/Thermolyne). An LS+ column was assembled on a MidiMACS magnet (Miltenyi Biotec) and washed with 3 ml PEB buffer. The cell-microbead suspension was then loaded on the LS+ column. The flow-through was collected and used for negative selection in 46% (54 of 118) of cases. The column was washed first with 2 ml of PEB and then twice with 2 ml of PBS. The column containing the positively selected cells was then disassembled from the magnet, and the cells were eluted from the column with 3 ml of PBS and collected into a Hettich cytospin chamber assembled with a silanized microscope slide (DAKO, Carpinteria, CA). The column was washed again with 3 ml of PBS, which was gently pushed through with the column plunger. Cytocentrifugation was performed at 500 rpm for 15 min with a Universal 16A cytocentrifuge (Hettich/RevPro, Monroe, CT). The supernatant was finally aspirated and the slide dried at room temperature. The slide was kept a maximum of 4 days in a dry box with desiccant before CK staining.

Negative Selection Procedure. In 54 cases (9 normal, 18 N−, 25 N+, and 2 metastatic) the flow-through was also processed by negative immunomagnetic selection. An equivalent maximum of 75×10^6 cells from the flow-through was depleted with 150 μl CD45 microbeads (Miltenyi Biotec Inc.) in a 1-ml cell suspension using a LS+ column assembled on a MidiMACS magnet. The flow-through was collected, and this negatively selected fraction was then cytocentrifuged onto a microscope slide with a maximum of 2×10^6 cells/slide. As necessary, samples with more cells were cytocentrifuged onto additional slides with the same maximum cell number/slide.

BT474-spiked Controls. To evaluate the performance (*i.e.,* tumor cell recovery) of immunomagnetic cell enrichment, control studies were performed using the BT474 breast carcinoma cell line. BT474 cells were maintained in culture medium (RPMI 1640, 10% fetal bovine serum, $1 \times$ penicillin/streptomycin/glutamine; Life Technologies, Inc.). After trypsinization, cells were counted using a hemacytometer to determine the cell titer. A calculated volume corresponding to ~20 cells (in 1 case 10 cells) based on the cell titer was spiked into 20 ml of normal blood and processed as for patient specimens by immunomag-

netic enrichment, cytocentrifugation and anti-CK immunocyto-chemistry, and ACIS digital microscopy analysis.

To more accurately estimate the number of carcinoma cells spiked in these experiments, three additional slides were prepared in parallel from each BT 474 cell suspension but without immunomagnetic enrichment. For each of the three slides, a volume of BT474 cells identical to the control sample submitted for immunomagnetic enrichment was added to 1 ml of blood from a normal donor and cytocentrifuged. These three slides were then submitted to anti-CK immunocytochemical staining and ACIS-assisted analysis. The average number of detected CK+ cells was used to calculate the cell recovery for the immunomagnetic enrichment experiments described in Table 3.

Immunocytochemistry. Specimens were first fixed in 0.5% neutral buffered formaldehyde (Sigma Chemical Co.) diluted in PBS for 10 min at room temperature, gently washed 2 × in PBS (DAKO), and then permeabilized according to the manufacturer's instructions available in the EPIMET cell detection kit (Micromet, Planegg, Germany). Immunocytochemical staining for CKs 8, 18, and 19 was performed with the alkaline phosphatase-conjugated A45-B/B3 Fab antibody fragment included in the EPIMET kit along with 0.5 μg/ml CAM5.2-AKP (BD PharMingen, San Diego, CA). The antibody mixture was incubated 150 min at room temperature in a moist chamber, and the slides were then washed three times with PBS. The alkaline phosphatase reaction was performed according to the EPIMET kit instructions, except that the reaction time was prolonged to 15 min at room temperature. Counterstaining was performed with a 4-s incubation in undiluted Mayer's hematoxylin (DAKO) followed by 10 dips in deionized water, a 30-s incubation in 0.08% ammonium hydroxide (Sigma Chemical Co.), and three 3-min washes in deionized water. The slides were dried and then coverslipped with a cellulose triacetate film using a Tissue-Tek SCA automated coverslipper (SAKURA Finetek USA Inc., Torrance, CA) with xylene. Each staining batch included a slide with ~1 × 10^6 WBC and a second slide containing 1 × 10^6 WBC spiked with cells from a carcinoma cell line (BT474 or HeLa S3) to serve as negative- and positive-staining controls, respectively.

CK+ Cell Detection by Digital Microscopy. The ACIS (ChromaVision Medical Systems, Inc.) is a computerized microscope, which includes an image processing system that has been optimized for the detection of rare carcinoma cells in hematological or solid tissue (*e.g.,* lymph node) specimens. A complete description of this system can be found in Ref. 10. Briefly, for rare cell detection, the ACIS makes use of proprietary software allowing for highly sensitive color detection along with the capability for analysis of a variety of morphometric features. The application software available on the instrument involves first scanning a microscope slide at low magnification (×10). The instrument next returns to objects that were originally identified for a second analysis at higher magnification (×40 or ×60). In this case, more sophisticated image analysis of color and morphometric characteristics (nuclear size and nuclear shape) is undertaken in an effort to exclude cellular debris, large clumps, and cells with morphological features typical of normal hematological mononuclear cells as opposed to CK+ carcinoma cells. Cellular objects that meet color- and morphometry-based criteria as likely tumor cells are collected

and presented as montage images for review and classification by a pathologist or other laboratory professional. Finally, the pathologist reviews all of the collected images to assure that they meet appropriate cytomorphometric criteria for classification as carcinoma cells (*e.g.,* excluding squamous cells, which arise as a result from venipuncture based on nucleus:cytoplasm ratio and chromatin texture). In the data file generated after the analysis of the peripheral blood or bone marrow specimen, the x-y coordinates of each cell are stored. On the basis of saved coordinates, a "revisit" capability allows the user to return to the proper location on the specimen slide for additional review of the cells of interest under manual control of the microscope.

RESULTS

Blinded Study with Peripheral Blood Samples from Normal Donors and Breast Cancer Patients. Peripheral blood samples from 34 normal volunteers and 84 breast cancer patients were obtained and processed for immunomagnetic enrichment at one site (Mayo Clinic Cell Kinetics Laboratory, Rochester MN). After cytocentrifugation, barcoded microscope slides were shipped overnight to the second site (ChromaVision Medical Systems). This site was "blinded" (*i.e.,* uninformed) as to whether individual specimens were obtained from normal volunteers or breast cancer patients. Slides were stained for CK, and ACIS digital microscopy analysis was performed. Finally, ACIS montage images (Fig. 1 and Fig. 2) of collected cells were reviewed and classified based on CK staining and cytomorphological characteristics in the absence of knowledge of the specimen source (*i.e.,* normal volunteer or carcinoma patient).

Results of ACIS-assisted analysis for the presence of CK+ cells is shown in Table 1. A total of 18 specimens were excluded from this study for various reasons. Specimens with poor cytomorphology (poor nuclear morphology and/or cell integrity; 5 of 34 normal and 3 of 84 cancer patient samples) were excluded before their clinical status was revealed. Slides from 6 additional cases (1 of 34 normal and 5 of 84 cancer patient specimens) demonstrated very sparse cellularity. For these cases, although no CK+ cells were identified, poor quality autofocusing also led to their exclusion before clinical status was revealed. Finally, specimens affected by a staining artifact, which appeared to arise from staining errors (3 of 34 normal and 1 of 84 cancer patient samples) were also excluded. In these cases, false positive immunocytochemical staining was observed in WBCs, which could be recognized by their distinctive nuclear morphology.

No CK+ cells were identified in any of the 25 normal donor samples (0%; 95% CI, 0–13.7%). In contrast, 28% (21 of 75; 95% CI, 18–40%) of the patients with breast cancer demonstrated one or more CK+ cells after ACIS-assisted analysis. One or more CK+ cells were detected in 0 of the 25 patients with N− breast cancer (0%; 95% CI, 0–13.7%), 2 of the 25 patients with N+ disease (8%; 95% CI, 1–27%), and in 19 of the 25 with distant metastatic disease (76%; 95% CI, 55–90%). For the 21 CK+ patients, the mean number of CK+ cells detected was 18.4 (range, 1–120). Among the 25 patients with metastatic disease, the mean number of CK+ cells per sample was 15 (median of 3). Six of 25 (24%) of CK+ specimens from
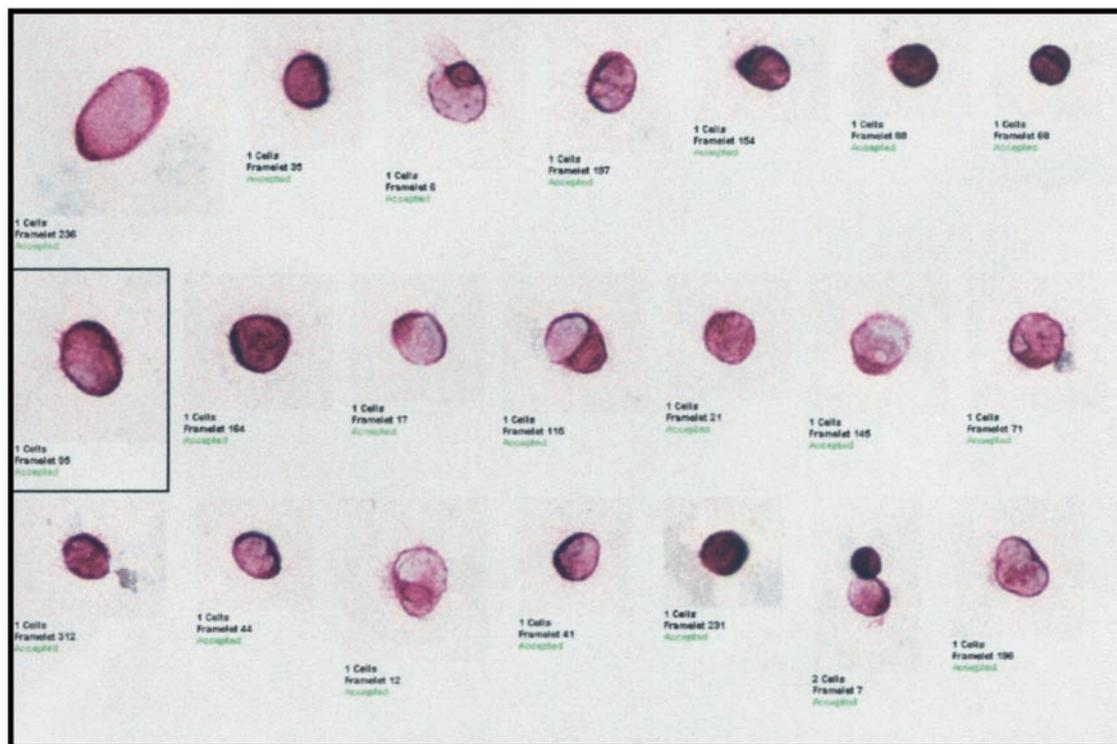
Fig. 1   Top panel, CK+ cell in a peripheral blood specimen from a breast carcinoma patient after anti-CK immunocytochemical staining in combination with the new fuchsin chromogen. The detected CK+ cell demonstrates *red* in cytoplasmic staining, with a *light blue* nucleus, reflecting hematoxylin counterstaining. This specimen was obtained after anti-EpCAM (positive selection) immunomagnetic enrichment. *Bottom panel,* montage of images containing CK+ cells detected by ACIS-assisted digital microscopy and assembled for review by laboratory professional. The cell enclosed in the box is the cell enlarged in the *top panel*.
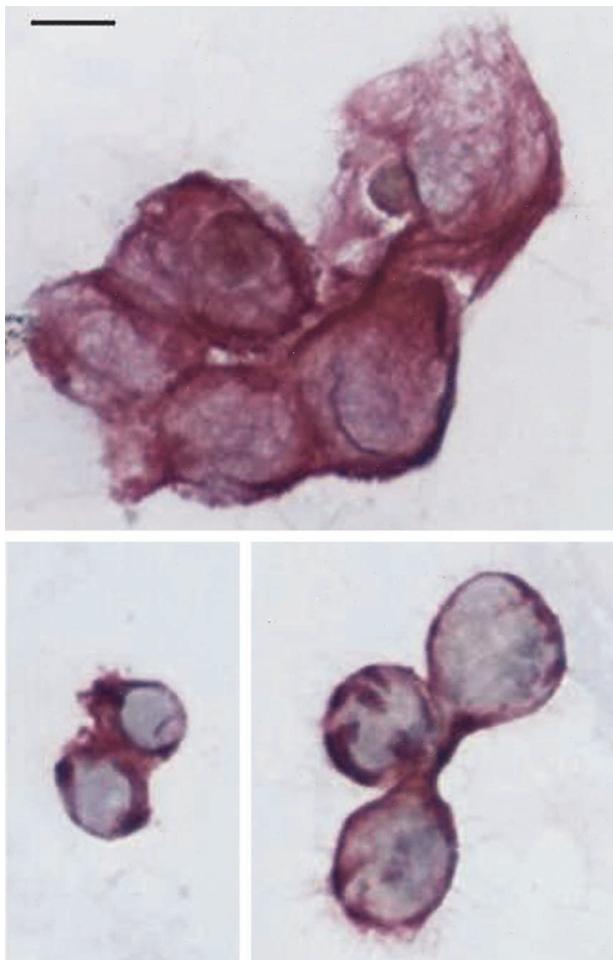
Fig. 2 Examples of clusters of CK+ cells from the peripheral blood of three patients with breast carcinoma.

Table 1 Digital microscopy-assisted analysis of CK+ cells from peripheral blood of normal donors and breast cancer patients

A series of 25 normal donor blood samples and 75 blood samples from patients diagnosed with breast cancer were analyzed without knowledge as to whether individual specimens were obtained from normal volunteers or breast cancer patients.

Diagnosis	Number of cases (*n*)	CK cell-positive cases[a]	Percentage of CK cell-positive cases[b] (95% CI)
Normal donors	25	0	0 (0–14)
Breast cancer (All cases)	75	21	28% (18–40)
N−	25	0	0 (0–14)
N+	25	2	8% (1–26)
Metastatic	25	19	76% (55–91)

[a] The number of cases demonstrating CK positive cells.
[b] Overall percentage CK+ cases for each category.

metastatic disease patients contained at least one cluster of CK+ cells containing two to five tumor cells (Table 2).

In 54 specimens (from 9 normal donors and 45 breast cancer patients), the effluent, which had passed through the

Table 2 Frequency of CK+ cells in the peripheral blood of metastatic breast cancer patients

Peripheral blood specimens from 25 metastatic breast cancer patients were processed for CK+ cell detection.

Case #	Number of detected CK+ cells[a]	Paired or clustered cells[b]
1	5	+
2	1	−
3	6	−
4	0	−
5	0	−
6	0	−
7	10	+
8	61	+
9	0	−
10	40	−
11	10	−
12	3	−
13	2	−
14	1	−
15	2	−
16	52	+
17	120	+
18	9	−
19	2	−
20	47	+
21	0	−
22	5	−
23	6	−
24	1	−
25	0	−
Mean (*n* = 25)	15	

[a] The number of CK+ cells detected by ACIS-assisted digital microscopy.
[b] The presence of paired or clustered CK+ cells is indicated by "+".

column used for immunomagnetic enrichment, was submitted to a second round of immunomagnetic enrichment. In these cases, "negative immunomagnetic selection" using the pan-leukocyte marker CD45 was used in an effort to deplete the residual specimens of the predominant leukocyte population. The resulting cell suspension was stained and analyzed for the presence of CK+ cells in parallel with the EpCAM-enriched fraction. No CK+ cell was identified in any (54 of 54) of these cases (including those from 45 of 45 breast cancer patients).

Studies on Control Blood Samples Spiked with BT474 Carcinoma Cells. To monitor the quality and the consistency of the CK cell detection method used for the breast cancer patient samples, blood samples were spiked with carcinoma cells and processed in the same manner as clinical specimens at various times over a period of 28 weeks. These control samples consisted of 20 ml of blood from normal donors spiked with a volume corresponding to 1–113 BT474 carcinoma cells (Table 3). CK+ cells were detected by ACIS analysis in 100% of the spiked positive control blood samples. The mean CK cell recovery was 86% with a range of 47%–100% (Table 3).

Reproducibility of Tumor Cell Detection in Peripheral Blood from Breast Cancer Patients Using ACIS. An additional study was performed to investigate the reproducibility of the ACIS for tumor cell detection. Four breast cancer patient specimens, including three CK+ and one CK−, were independ-

Table 3 Detection of CK+ cells (BT474-spiked peripheral blood specimens)

Peripheral blood specimens (20 ml each) were spiked with BT474 carcinoma cells and processed for CK cell detection by successive immunomagnetic selection, anti-CK immunocytochemistry, and ACIS-assisted digital microscopy analysis on 12 separate occasions over a period of 28 weeks.

Sample #	CK+ cells identified[a]	Percentage cells recovered after immunomagnetic enrichment[b]
1	1	100
2	19	90
3	19	100
4	9	69
5	21	84
6	21	100
7	20	100
8	53	93
9	17	47
10	8	80
11	77	68
12	18	100
Mean (n = 12)		86

[a] The number of recovered CK+ cells identified after immunomagnetic enrichment.

[b] The percentage of CK+ cells recovered after immunomagnetic enrichment, calculated relative to the mean of the three positive control slides. The mean percentage CK+ cell recovery for the 12 experiments is indicated on the bottom row.

Table 4 Reproducibility of CK+ cell detection after ACIS-assisted digital microscopy

Peripheral blood specimens from four breast cancer patients were analyzed three separate times on each of three ACIS instruments for the presence of CK+ cells. For each specimen, an identical number of CK+ cells was identified in every case.

Sample #	Tumor cells detected with ACIS #1 (3 runs)	Tumor cells detected with ACIS #2 (3 runs)	Tumor cells detected with ACIS #3 (3 runs)
1	1	1	1
	1	1	1
	1	1	1
2	6	6	6
	6	6	6
	6	6	6
3	49	49	49
	49	49	49
	49	49	49
4	0	0	0
	0	0	0
	0	0	0

ently analyzed using three separate ACIS instruments. Each specimen was scanned and analyzed on three separate occasions for each instrument. The results are presented in Table 4. Analysis of the four specimens revealed identical numbers of cells in all cases.

DISCUSSION

The finding of CK+ cells in the bone marrow of patients with breast cancer with light microscope-based immunohistochemistry methods has been reported to be an adverse prognostic factor (2, 17). The use of immunomagnetic particles to enrich samples for targeted tumor cell populations along with automated digital microscopy and RT-PCR has facilitated the study of blood for presence of malignant cells in patients with solid tumors. In this study we demonstrate that assisted digital microscopy can detect circulating CK+ cells. We found 28% (95% CI, 18–40%) of the patients in our study had at least one CK+ cell in the blood.

Other studies have also reported the detection of carcinoma cells in the blood of patients with solid tumors using various detection methods. Racila *et al.* (15) evaluated the peripheral blood of 30 patients with breast cancer, 3 patients with prostate cancer, and 13 controls using an immunomagnetic enrichment with flow cytometry using antibody to CK. They concluded that CK+ cells were present in 32 of 33 cancer patients. Martin *et al.* (9) evaluated peripheral blood specimens from patients with advanced carcinoma of the breast, prostate, colon, rectum, and lung, and detected CK+ cells in 21 of 34 patients using immunomagnetic enrichment and flow cytometry. In a study of the blood from 19 patients with breast cancer before and after surgery using immunomagnetic enrichment in a nonautomated slide-based light microscope system, Krag *et al.* (18) found that 95% (18 of 19) of patients had cells in the blood preoperative, and 30% were positive postoperative. Kraeft *et al.* (19) reported recently the detection of CK+ cells using an automated fluorescence microscope system. Their initial results showed CK+ cells in 17% of normal blood donors. They subsequently modified their procedure to use double-staining procedures, and this methodological change eliminated "positive" staining in normal donors and resulted in the detection of CK+ cells in 35% of peripheral blood specimens obtained from cancer patients without the requirement for immunomagnetic enrichment.

Interpretation of these results in some studies has been complicated by the finding in some studies of positive (*i.e.,* carcinoma cells) in the peripheral blood of "normal" donors. For example, Racila *et al.* (15) concluded that 7 of 13 peripheral blood specimens from healthy individuals included CK+ cells after immunomagnetic enrichment and flow cytometric analysis. Other reports using molecular methodologies have documented that false positive determinations can occur, which clearly impact in the interpretation of assay results (11).

None of the studies of circulating carcinoma cells in blood to date have analyzed the impact of this finding on disease-free survival and overall survival. The main reason for this is that it is important to first develop and rigorously test the performance of the technology for detecting these rare cells before large-scale clinical studies are undertaken. Because carcinoma cells are so rare in the blood, it is impractical to simply review slides with routine light microscopy. Key advantages of digital microscopy-based analysis are the opportunity for automated (unattended) scanning of specimen slides and the opportunity for visual review by the laboratory professional to assure that the collected events, in fact, represent CK+ carcinoma cells (Figs. 1 and 2).

The combination of immunomagnetic enrichment and digital microscopy as described in this study demonstrate that the technology is now available to be applied to blood samples from large groups of patients. Well-designed clinical studies should now provide the opportunity to learn whether the presence of

rare carcinoma cells is useful in the clinical management of patients.

Additional research is required to determine whether this test is of value in metastatic disease. In our study, none of the patients had a known objective status, and it is possible that the presence or absence of circulating cells might correlate with the effects of treatment. This would be a particularly important finding, because many patients do not have easily assessable metastatic disease.

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