

Reliable and Sensitive Analysis of Occult Bone Marrow Metastases Using Automated Cellular Imaging

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ABSTRACT

The presence of occult bone marrow metastases (OM) has been reported to represent an important prognostic indicator for patients with operable breast cancer and other malignancies. Assaying for OM most commonly involves labor-intensive manual microscopic analysis. The present report examines the performance of a recently developed automated cellular image analysis system (ACIS; ChromaVision Medical Systems, Inc.) for identifying and enumerating OM in human breast cancer specimens. OM analysis was performed after immunocytochemical staining. Specimens used in this study consisted of normal bone marrow ($n = 10$), bone marrow spiked with carcinoma cells ($n = 20$), and bone marrow obtained from breast cancer patients ($n = 39$). The reproducibility of ACIS-assisted analysis for tumor cell detection was examined by having a pathologist evaluate montage images generated from multiple ACIS runs of five specimens. Independent ACIS-assisted analysis resulted in the detection of an identical number of tumor cells for each specimen in all instrument runs. Additional studies were performed to analyze OM from 39 breast cancer patients with two pathologists performing parallel analysis using either manual microscopy or ACIS-assisted analysis. In 17 of the 39 cases (44%), specimens were classified by the pathologist as positive for tumor cells after ACIS-assisted analysis, whereas the same pathologist failed to identify tumor cells on the same slides after analysis by manual microscopy. These studies indicate that the ACIS-

assisted analysis provides excellent sensitivity and reproducibility for OM detection, relative to manual microscopy. Such performance may enable an improved approach for disease staging and stratifying patients for therapeutic intervention.

INTRODUCTION

A substantial body of literature exists evaluating the biological significance of OM² (1) from patients with operable breast cancer and a spectrum of other solid tumor types. Many studies have concluded that the presence of OM provides important prognostic information predictive of disease-free and overall survival in both locally recurring and advanced breast cancer (1–5). Similar conclusions have been made after the analysis of other human solid tumors including non-small cell lung carcinoma, colorectal carcinoma, and esophageal carcinoma (6–8).

By contrast, other reports have found no statistically significant relationship between OM and prognosis (9, 10). The issue of varying conclusions regarding the prognostic significance of OM was investigated recently by Funke and Schraut (11). These authors performed a meta-analysis of 20 published reports including the analysis of ~2500 patients. Although significant, the impact of this study was diminished by the fact that the authors compared a range of carcinoma types and included studies with highly variable staining and analysis methods, along with substantial variation in the duration of clinical follow-up. Despite these caveats, there is no doubt that a key conclusion of the authors is correct: there remains the need for improved standardization of OM assay methods before the prognostic significance of OM can be substantiated.

Surprisingly, large differences (>10-fold) in the number of bone marrow cells analyzed for OM classification are evident in reports published previously. Most studies evaluated between 10^5 and 10^6 normal bone marrow cells, but in some cases the number of cells analyzed was not specified. Such variation clearly affects the sensitivity of the OM assay and could impact on the prognostic significance of the results obtained (12). The importance of standardizing the number of cells assayed is further underscored from studies of Cote *et al.* (1). These studies suggest that the prognostic predictability of the OM assay is impacted by the number of tumor cells in the specimen, as opposed to simply whether a specimen is positive or negative for the presence of tumor cell(s).

The analytical requirement for identifying and enumerating very rare tumor cells in the OM assay is highly laborious, with the accuracy and sensitivity of the analysis result potentially

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² The abbreviations used are: OM, occult bone marrow metastasis; ACIS, automated cellular imaging system; RT-PCR, reverse transcription-PCR.

impacted by the fatigue of the reviewer. Variation in results between laboratories is further complicated by the use of varying criteria for the classification of cells as tumor or nontumor (13, 14). By providing objective computer-based analysis that can reduce the subjectivity inherent in manual microscopic interpretation, along with images of cells that are classified, automated cellular imaging holds considerable potential for improving both the sensitivity and interlaboratory consistency of the OM assay.

Previous reports (15–17) have provided initial proof-of-concept regarding the use of image analysis for rare tumor cell detection. Mansi *et al.* (15) analyzed bone marrow specimens that were spiked with human carcinoma cells. In this study, image analysis results agreed with those of manual microscopy in only 11 of 20 cases, and image analysis was reported to be considerably slower than analysis by manual microscopy. Mesker *et al.* (16) used a cell model system in which carcinoma cells were spiked into peripheral blood. The results of this study suggested that the image analysis-based detection of rare SKBR3 tumor cells could provide good correlation with results from manual microscopy when both specimen preparation and immunocytochemical staining were optimized. Kraeft *et al.* (17) described recently a fluorescence-based imaging system capable of identifying rare carcinoma cells in blood, bone marrow, and stem cell-enriched products. This system was reported to perform sensitive analysis of rare tumor cells from these specimens, although the consistency of tumor cell detection using the instrument was not addressed.

In the present study, the performance of a recently developed ACIS (ChromaVision Medical Systems, Inc.) as a tool for the evaluation of OM is explored. Results are described after the analysis of normal human bone marrow, bone marrow specimens spiked with breast carcinoma cells, and bone marrow specimens from 39 breast cancer patient specimens. OM assay sensitivity by ACIS-assisted analysis is compared with parallel analysis of the same specimens using manual microscopy. The performance of the imaging system in terms of OM assay reproducibility is also examined.

MATERIALS AND METHODS

Bone Marrow Specimens. Bone marrow specimens from breast cancer patients used in this study were provided by Dr. Ingo Diel. They were obtained from patients with primary, operable breast cancer stages T₁₋₄, N₀₋₂, and M₀ (International Union Against Cancer criteria; Ref. 18). Bone marrow puncture and aspiration were performed as detailed in Diel *et al.* (3). Ten to 12 ml of bone marrow aspirate were collected from two puncture sites on each anterior iliac crest (total, 40–50 ml) and stored in heparinized tubes with DMEM (Life Technologies, Inc., Paisley, United Kingdom). Components of the aspirate were separated by density centrifugation through Ficoll Hypaque (Biochrom, Berlin, Germany; density, 1.077 g/ml). After separation, cells were washed twice and resuspended with DMEM. Subsequently, the cell suspension was transferred onto microscope slides using a cytocentrifuge (Model Universal 16A; Hettich, Germany). The slides were suspended in a conventional freezing medium and stored in a freezer at –80°C, previous to immunocytochemical staining and analysis.

For normal human bone marrow or bone marrow spiked with carcinoma cells, the following procedure was used. Mononuclear cells were isolated from human bone marrow aspirates using standard protocols (Poietics; BioWhittaker, Gaithersburg, MD). Briefly, a nucleated cell count was performed on bone marrow aspirates using a Coulter counter with Zap-globin-II for red cell lysis, according to the manufacturer's directions. The bone marrow was diluted to 5 million nucleated cells/ml with HBSS containing 5 mM EDTA (pH 7.4; Sigma Chemical Co., St. Louis, MO). Fifteen ml of Ficoll-Paque Plus (Amersham Pharmacia, Piscataway, NJ) were overlaid with 35 ml of the diluted bone marrow in a 50-ml conical tube and centrifuged at 400 × g for 30 min at room temperature. The mononuclear cell layer was harvested from each tube, combined, diluted 1:4 with HBSS + EDTA, and centrifuged at 300 × g for 15 min at room temperature. The cells were resuspended in PBS (Dako, Carpinteria, CA), and an aliquot was counted. The cells were centrifuged at 250 × g for 10 min at room temperature. The mononuclear cells were resuspended in PBS at 5 million cells/ml and maintained on ice. Two hundred μl of the cell suspension (approximately one million cells) were cytocentrifuged onto silanized slides (Dako) at 500 rpm for 5 min at room temperature (Hettich Universal 16A cytocentrifuge). The supernatant was carefully removed from each slide after cytocentrifugation, and the slides were allowed to air dry overnight. For “spiked” slides, MDA-MB-468 breast carcinoma cells (American Type Culture Collection, Manassas, VA) were harvested, resuspended in PBS, and counted. The appropriate number of cells was added to the PBS resuspended bone marrow mononuclear cells to give a final count of 1.5 or 40 cells in 200 μl. Slides were prepared by cytocentrifugation as described above.

Immunocytochemical Staining. Details of the procedure used for the staining of bone marrow specimens from breast carcinoma patients are presented by Diel *et al.* (3). Briefly, the monoclonal antibody 2E11 (BM2), which recognizes a carcinoma-associated epithelial mucin MUC-1 (19–21), was used.

Before staining, the cells were fixed with 3.7% formalin in PBS for 15 min, rinsed three times with PBS, and postfixed with absolute methanol (–20°C) for 5 min. Endogenous alkaline phosphatase was next blocked with 20% acetic acid, 2.28% periodic acid, and 2% levamisole. After blocking, the slides were incubated with biotinylated BM2 antibody (2 μg/ml) in PBS that contained 1% BSA (Boehringer Mannheim, Mannheim, Germany) for 1 h at room temperature. Immune complexes were made visible by use of the avidin-biotin-alkaline phosphatase complexes (ABC test; Vectastain, Camon, Wiesbaden, Germany) and fast red as substrate. The cells were counterstained with 10% Gill 2 hematoxylin (Dako) in distilled water for 30 s. The specimen was next rinsed under running water for 2 min and then coverslipped using Aquatex mounting medium (Merck Darmstadt, Germany).

Normal marrow or bone marrow specimens spiked with human breast carcinoma cells were immunocytochemically stained using the EPiMET Epithelial Cell Detection kit (Baxter Europe, Micromet, Germany) with minor modifications to the manufacturer's recommendations. Briefly, slides were fixed in 0.5% neutral buffered formalin (Sigma) diluted in PBS (Dako) for 10 min at room temperature. Samples were gently washed

with PBS (Dako) and permeabilized according to the manufacturer's recommendations. Cytokeratins 8, 18, and 19 were stained with A45-B/B3 (conjugate of Fab-fragment of antibody A45-B/B3 with alkaline phosphatase), washed, and detected with chromogen (New Fuchsin) according to the manufacturer's recommendations. The slides were counterstained by incubating in undiluted hematoxylin stain (Dako) for 4 s and rinsed with deionized water. The slides were dried at 70°C in a drying oven for 20 min. The dried slides were coverslipped with a cellulose film using a Tissue-Tek SCA automated coverslipper (Sakura Finetek) and xylene.

ACIS System Overview. The ACIS (ChromaVision Medical Systems, Inc., San Juan Capistrano, CA) consists of two major subassemblies. The first is the microscope with its associated electromechanical hardware, and the second is a computer with a frame grabber and image processing system.

The microscope subsystem includes the components of a standard microscope (*e.g.*, lamp, condenser, and turret) mounted in a special shock-resistant frame with a video camera. The camera has a 60 frame/s 640 × 480 pixel three-chip camera (Sony DX9000). Also included in the microscope assembly is an infeed hopper; a stage and motors that provide X, Y, and Z (focus) translation; and an outfeed drawer. The system uses a carrier system, with four slides per carrier, and the input hopper capable of holding 25 carriers. The computer subsystem consists of a dual 450 MHz Pentium II computer running the Microsoft Windows NT operating system. It has 512 megabytes of memory, 27 gigabytes of hard disk storage, and 25 gigabytes of tape backup, compressible to 50 gigabytes.

For rare event detection, the ACIS makes use of proprietary software allowing for fast and highly sensitive color detection, along with the capability for the analysis of a variety of morphometric features. The application software available on the ACIS for OM detection involves first scanning a microscope slide at low magnification (×10). The system next returns to objects that were originally identified for a second analysis at higher magnification (×40 or ×60). In this case, more comprehensive 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 carcinoma cells. At the same time, 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. In the data file generated after specimen analysis, the X-Y coordinates of the object within a framelet are stored. The use of location data results in powerful sample navigation features. A "revisit" capability allows the user to double-click on framelets of interest to return to the proper location on the specimen slide for further review under manual control of the microscope. In this mode, it is possible to navigate across the slide, adjust focus, and change microscope objectives. Comparison of the montage images that result from each repeated run is another feature that uses location data. Tumor cells or cell clusters found multiple times by the system can be identified by highlighting framelets with proximate locations as "suspected duplicates."

RESULTS

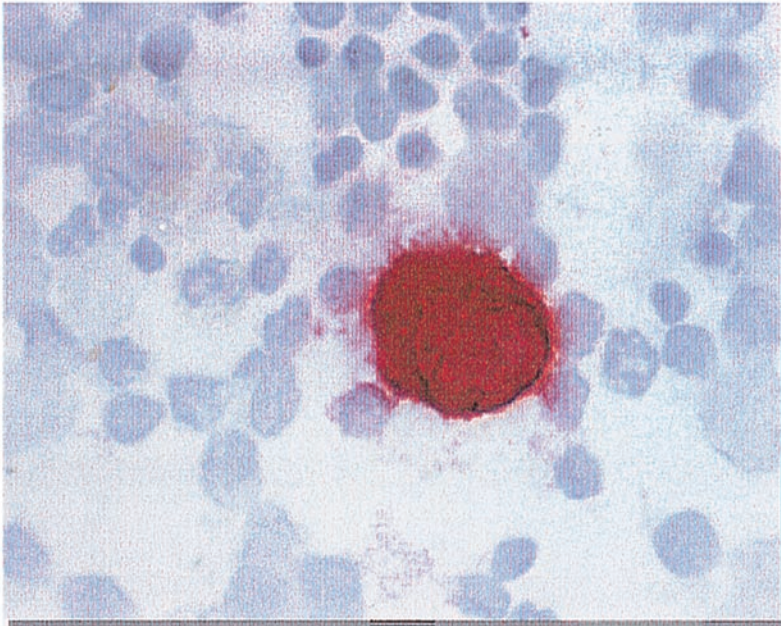
Fig. 1 illustrates an example of a carcinoma cell identified from a bone marrow preparation obtained from a breast cancer patient. The specimen was immunocytochemically stained using the BM2 monoclonal antibody and enzymatically visualized in combination with the fast red chromogen. Fig. 1A illustrates an example of positive immunocytochemical staining (red color) of a single tumor cell. Staining is evident on the plasma membrane and cytoplasm of the tumor cell but is absent in surrounding normal mononuclear bone marrow cells, which demonstrate only the blue hematoxylin nuclear counterstain. By using manual microscopy, OM analysis is conventionally performed after the laborious process of examining approximately 10^5 to 10^6 normal bone marrow mononuclear cells for the presence of one or more carcinoma cells.

In an effort to examine the feasibility for automating the OM assay, ACIS analysis was performed on the same bone marrow specimen. Fig. 1B illustrates the result of ACIS analysis using an application that detects likely tumor cells based on the combination of presence of the red chromogen immunocytochemical staining and preset morphological characteristics (*e.g.*, range of nuclear size and nuclear shape). A laboratory professional next reviews the collected framelets to classify them as tumor cells or non-tumor cells. In the case illustrated, eight collected objects were reviewed and classified as tumor cells, representing the result after analysis of the entire bone marrow specimen. In addition, the ACIS counted approximately 1.26×10^6 total mononuclear cells on the specimen slide. ACIS analysis of this specimen required ~18 min.

To evaluate the sensitivity and specificity of the ACIS for tumor cell detection, known control populations were next evaluated. Bone marrow specimens were obtained from normal individuals ($n = 10$), specimens into which one to seven *in vitro* human breast carcinoma cells (MDA-MB-468) were spiked ($n = 10$), and specimens spiked with 43–59 breast carcinoma cells ($n = 10$). Each of these specimens also included ~500,000 normal bone marrow mononuclear cells. The results of this study are shown on Table 1. No tumor cells were identified in any of the 10 normal bone marrow specimens analyzed. By contrast, one or more tumor cells were identified in each of the 20 specimens that were spiked with breast carcinoma cells.

Further studies were undertaken to evaluate and compare the OM review time as performed using the ACIS analysis *versus* manual microscopy using these same specimens. For ACIS-assisted analysis, two elements contribute to the total analysis time: ACIS image scan and collect (in which the instrument performs analysis in an unattended fashion); and the ACIS-assisted review of montage images, which is performed by a laboratory professional. The overall ACIS scan and collect time was an average of 17.75 min (2.69 SD), with a range of 14–25 min for the 30 specimens. The automated portion of the ACIS analysis for all specimens, therefore, was accomplished in a total of 8.9 h. The total pathologist-assisted ACIS review of all montage images from the 30 specimens was accomplished in slightly less than 1 h. This leads to a total ACIS analysis time (unattended scan and collect along with pathologist montage review) of 9.9 h. By contrast, manual microscopic evaluation of the 30 specimens required 11.9 h, or an average of 23.8 min for

A



B

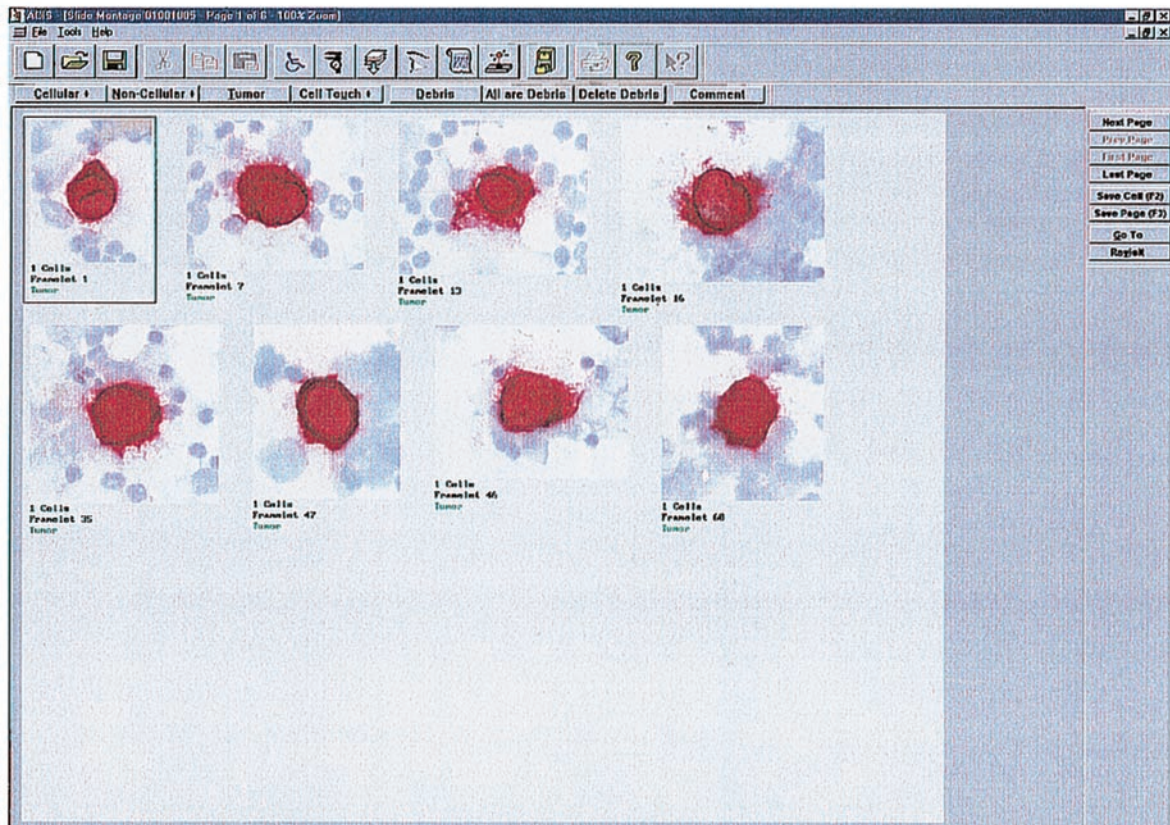


Fig. 1 A, example of a carcinoma cell in a bone marrow specimen obtained from a breast cancer patient. B, result obtained after ACIS analysis of the entire bone marrow specimen and review of collected images. Analysis of this specimen resulted in the classification of eight objects as tumor cells. ACIS analysis indicated a total mononuclear count of approximately 1.26×10^6 cells on the specimen slide.

Table 1 Specificity for tumor cell detection using ACIS-assisted analysis (normal bone marrow versus bone marrow “spiked” with MDA-MB-468 breast carcinoma cells)

	Tumor cells		Total cases
	Positive cases	Negative cases	
Bone marrow spiked with 4 ± 3 breast carcinoma cells (n = 10)	10	0	10
Bone marrow spiked with 51 ± 8 breast carcinoma cells (n = 10)	10	0	10
Normal bone marrow (n = 10)	0	10	10
Totals	20	10	30

Mononuclear cell preparations from normal bone marrow specimens and bone marrow to which varying numbers (range, 1–59) of MDA-MB-468 breast carcinoma cells were added were immunocytochemically stained using an alkaline phosphatase-conjugated anti-pan-cytokeratin antibody (A45-B/B3). Positive cells were visualized after incubation with the new fuchsin chromogen. The slides, each consisting of ~500,000 cells, were then analyzed using the ACIS. Specimens with one or more carcinoma cells were classified as tumor cell positive.

Table 2 Reproducibility within/between ACIS instrument(s) for tumor enumeration (bone marrow specimens from breast cancer patients)

Run no.	No. of positive tumor cells	
	Specimen A	Specimen B
Instrument I		
1	7	18
2	7	18
3	7	18
4	7	18
5	7	18
Instrument II		
1	7	18
2	7	18
3	7	18
4	7	18
5	7	18
Instrument III		
1	7	18
2	7	18
3	7	18
4	7	18
5	7	18

Bone marrow mononuclear cell preparations from two breast cancer patients were fixed and immunoenzymatically stained using the BM2 monoclonal antibody and the fast red chromogen. To examine the consistency of tumor cell detection and enumeration, each specimen was evaluated after analysis in five separate runs on each of three different ACIS instruments for a total of 15 independent analyses/specimen.

each of the 30 specimens. Thus, using ACIS-assisted analysis, a reduction of ~11.9-fold in pathologist review time was accomplished, relative to analysis using manual microscopy.

Bone marrow specimens obtained from five breast cancer patients were subsequently analyzed in an effort to assess the reproducibility of ACIS-assisted analysis results. Table 2 illustrates the result of this analysis for two specimens in which 15 separate analyses were performed for each specimen. Each of three separate instruments were used to analyze each specimen, with five separate analyses per instrument. The data indicate that the ACIS identifies tumor cells in a highly reproducible fashion. Exactly the same number of tumor cells (as classified by the pathologist after the review of montage images) was identified for each specimen.

Three additional breast cancer bone marrow specimens were next analyzed on three separate ACIS instruments, with

Table 3 Tumor cell detection: ACIS-assisted analysis versus manual microscopy (specimens from breast cancer patients, n = 39)

ACIS-assisted analysis	Manual microscopy	
	Tumor cell positive cases	Tumor cell negative cases
Tumor cell positive cases	9	17
Tumor cell negative cases	3	10
Total	12	27

Bone marrow specimens from 39 breast cancer patients were evaluated by both manual microscopy and ACIS-assisted analysis by two pathologists after immunoenzymatic staining with the BM2 monoclonal antibody and the fast red chromogen. Manual microscopy and ACIS-assisted analysis agreed on 9 positive and 10 negative cases. In 20 cases, manual microscopy and ACIS-assisted analysis disagreed. In 17 of these cases, the specimen was classified as “positive for tumor cells” after ACIS-assisted analysis but “negative for tumor cells” by manual microscopy. (These discrepant cases were further investigated as described in Table 4).

three independent runs per instrument. This analysis (data not shown) again revealed that exactly the same number of tumor cells was identified for each specimen in each run, with 23, 7, and 50 cells, respectively, consistently identified from each of three specimens.

The “montage compare” feature within the ACIS instrument displays multiple montage images of collected cells from separate instrument runs for review and comparison. Using this analysis tool, the reviewer determines whether identical cells are detected in different instrument runs. Reviewing the individual framelet images of all of the collected tumor cells from these three specimens revealed that in all cases, not only the same number of tumor cells but also exactly the same tumor cells were collected.

Additional studies were next performed using bone marrow specimens obtained from 39 breast cancer patients to further examine the performance of ACIS-assisted OM analysis versus OM analysis by manual microscopy. For these studies, the specimens were evaluated by either of two pathologists with independent evaluation performed using manual microscopy and ACIS-assisted analysis. The results of this study are shown in Table 3. In 17 of the 39 specimens (44%), the pathologist detected one or more tumor cells after ACIS-assisted analysis, which were not detected by the same pathologist using manual microscopy.

Table 4 Tumor cell detection reproducibility: ACIS-assisted analysis versus manual microscopy (specimens from breast cancer patients, $n = 21$)

Specimen	No. of tumor cells			
	Initial analysis		Second review	
	Manual microscopy	ACIS-assisted analysis	ACIS manual analysis	ACIS-assisted analysis
1	0	0	0	0
2	0	2	2	2
3	0	1	1	1
4	0	2	2	2
5	0	1	1	1
6	0	4	4	4
7	0	1	1	1
8	0	2	2	2
9	0	0	0	0
10	0	1	2	1
11	0	1	1	1
12	0	3	3	3
13	0	2	2	2
14	0	0	0	0
15	0	5	5	5
16	0	1	1	1
17	0	2	2	2
18	0	0	0	0
19	0	4	4	4
20	0	2	2	2
21	0	3	3	3

The number of tumor cells counted from a total of 21 bone marrow specimens from breast cancer patients that had initially been analyzed by both ACIS-assisted analysis and manual microscopy were reevaluated. For 17 specimens that had originally been classified as tumor cell negative by manual microscopy, a total of 37 objects were originally classified as tumor cells after ACIS-assisted analysis. These cells, along with 74 nontumor cells from the same 17 slides and five cells from each of four additional specimens (for a total of 131 cells) were recalled and reexamined by a pathologist, blinded to the original ACIS-assisted cellular classification. Finally, the pathologist reviewed ACIS montage images a second time, blinded to the original classification.

On the basis of the fact that the ACIS data file includes x-y coordinates of each object that was classified in the original ACIS-assisted analysis, it was possible for the pathologist to recall all tumor cells for further studies. Each of the tumor cells from the 17 disparate specimens (above) were manually reevaluated using the binoculars available on the ACIS. To ensure objectivity, additional non-tumor cells were also examined with all determinations blinded to the original cellular classification. The results of these studies are shown in Table 4. For all but one cell (specimen 10), manual microscopic review led to findings that agreed with those obtained after the original ACIS-assisted analysis. For the one cell where a disparity was noted, the pathologist again reviewed both the original ACIS montage image and the specimen slide. The pathologist's conclusion, consistent with the original ACIS-assisted analysis, was that the disparate cell was not a tumor cell.

Finally, to verify the consistency of the ACIS-assisted analysis, the pathologist independently reviewed all ACIS montage images a second time, blinded to the original classification. The second ACIS-assisted analysis led to results that were identical to the original ACIS-assisted analysis (Table 4). From these findings, we conclude that the original discrepancy be-

tween ACIS-assisted analysis and manual microscopy reflected tumor cells that were not detected (*i.e.*, "false negatives") by manual microscopy.

In three specimens, the original manual microscopic analysis (Table 3) led to the conclusion that tumor cells were present, whereas ACIS-assisted automated analysis revealed no detectable tumor cells. At a later date, the pathologist reanalyzed the specimen, blinded to the original interpretation. In an effort to optimize the reliability of the second read, the pathologist further reviewed the specimen slide for possible tumor cells at both conventional ($\times 200$) and higher ($\times 400$) resolution. After reanalysis, the pathologist concluded that none of the specimens included even one tumor cell, consistent with the original ACIS analysis but contrasting with the original analysis by manual microscopy. These findings indicate that the original manual microscopic analysis resulted in false-positive findings that were not observed after ACIS-assisted analysis.

DISCUSSION

This study demonstrates that analysis of OM using immunocytochemical staining and analysis by the ACIS provides a sensitive, highly reproducible, and efficient means for OM detection and enumeration. Superior sensitivity for OM detection was demonstrated using ACIS-assisted analysis, relative to manual microscopy. Specifically, in bone marrow specimens from 17 of 39 breast cancer patients in which the pathologist identified the presence of OM after ACIS-assisted analysis, the same pathologist incorrectly classified the specimen as negative after initial analysis by manual microscopy (Table 4). Analysis of the same specimen from breast cancer patients on multiple occasions across multiple ACIS instruments revealed excellent assay consistency (Table 2). Finally, an ~ 11.9 -fold reduction in pathologist review time was achieved for analysis of 30 bone marrow specimens for OM using ACIS-assisted analysis, relative to manual microscopy.

In addition to analysis by manual microscopy, two other methods are commonly used for the analysis of OM today, flow cytometry and nucleic acid-based methods including RT-PCR. Flow cytometry represents an alternative technology that allows for the rapid analysis of tens of thousands of cells. The detection sensitivity of this methodology varies in published reports but most commonly is reported to be in the range of approximately 1 cell in 10,000 (22). Other reports suggest a theoretical detection sensitivity in the range of approximately one cell in 200,000 or even rarer (23, 24). The results of Gross *et al.* (24), however, suggest that flow cytometric analysis of rare tumor cells (< 1 cell in 100,000) leads to a detection sensitivity of only 10–40%. One key advantage for analysis by automated cellular imaging as opposed to flow cytometry is the fact that the analysis result is a cellular image. The use of a conventional counterstain (*e.g.*, hematoxylin) facilitates interpretation by a pathologist or other laboratory professional, leading to enhanced diagnostic certainty of the assay. Only with the use of tedious cell sorting methods (available on flow cytometry research instruments) can this type of interpretation be made using flow cytometry.

DNA-based nucleic acid methods for detecting OM analyze specific gene mutations, sequences of carcinogenic viruses, or other alterations specific to neoplastic cells (25). This method

holds considerable potential, but its true clinical relevance awaits confirmation in larger prospective studies. An alternative strategy for detecting OM in bone marrow specimens involves the use of RT-PCR to amplify specific mRNA molecules, which are expressed in carcinoma cells but absent in normal cells. Although this approach holds considerable promise, it suffers from the fact that, for most cancer types, true tumor-specific mRNA molecules have not yet been identified. Inherent biological factors including the presence of pseudogenes or low level expression of the targeted mRNA in normal cells can lead to false-positive test results using this method (26, 27). For example, "illegitimate expression" of cytokeratin 19 mRNA has been reported in peripheral blood (28). In another study, seven epithelial- and tumor-associated markers, including cytokeratin 18 mRNA, were amplified by RT-PCR in a significant percentage of bone marrow samples from non-cancer patients (28), illustrating the risk of false-positive results by this method. On the other hand, down-regulation of a more specific carcinoma cell marker, such as prostate-specific antigen, in tumor cells may limit the sensitivity of RT-PCR in bone marrow from breast cancer patients (29), possibly leading to false-negative interpretations.

One assay component of critical importance for the immunocytochemical detection of OM is the antibody reagent used for immunocytochemical staining. To date, a variety of antibodies have been used for the OM assay, which vary substantially in terms of sensitivity and specificity for carcinoma cell detection (12). Antibodies used for OM detection have been reported to bind both specifically (*e.g.*, Fc-receptor-bearing leukocytes, "illegitimate" expression of epithelial antigens in normal hematopoietic cells) or nonspecifically in cells including macrophages, plasma cells and nucleated erythroid precursors (30–33). The use of antibodies with well-documented specificity for carcinoma cells, as opposed to normal hematological mononuclear cells (*e.g.*, A45-B/B3, 5), is highly advantageous for a robust OM assay. In addition, careful morphological assessment, along with proper interpretation of immunocytochemical staining, appear imperative to assure that immunocytochemically stained cells are, in fact, tumor cells (34), as opposed to leukocytes and other nonneoplastic cells. By providing cellular images with counterstains conventionally used for cellular diagnostic purposes, along with the capability to "revisit" the cell on the microscope slide, the ACIS provides the opportunity for cellular classification with increased diagnostic certainty, relative to the alternative methods discussed above.

In conclusion, these studies demonstrate that ACIS-assisted analysis combined with immunocytochemical staining offer the possibility of sensitive and reliable assessment of OM. Prospective studies now appear important to further explore the true clinical significance of this assay.

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