Image Analysis Systems for the Detection of Disseminated Breast Cancer Cells on Bone-Marrow Cytospins

Sven Becker,* Graziella Becker-Pergola, Tanja Fehm, Robert Emig,
Diethelm Wallwiener, and Erich-Franz Solomayer

Department of Gynecology and Obstetrics, University of Tübingen, Tübingen, Germany

We assessed the accuracy of automated cell imaging systems when compared to manual evaluation of cytospin slides in determining the presence of cytokeratin-positive, disseminated breast cancer cells in bone marrow aspirates. A total of 298 cytospin slides of bone marrow aspirates were first evaluated by individual screening by one expert immunocytologist. Subsequently, all 298 slides were evaluated by the Automated Cell Imaging System (ACIS) by ChromaVision™. Two separate analysis cycles were performed using ACIS. The results of the two ACIS analysis cycles were almost identical: in 293 out of 298 samples (98.3%), identical numbers of

disseminated breast cancer cells were detected. In the remaining five samples (1.7%), the result of the two ACIS analysis cycles differed by only one tumor cell. By using the manual technique, 120 cytospin samples were found to be positive. ACIS was able to detect additional tumor cells in 64 cases. Not once did ACIS miss tumor cells when compared to the manual technique. Using ACIS, we were able to determine the bone marrow status of patients with nonmetastatic breast cancer faster, with greater accuracy, and with greater reproducibility than with the manual technique. *J. Clin. Lab. Anal.* 19:115–119, 2005. © 2005 Wiley-Liss, Inc.

Key words: minimal residual disease; micrometastases; immunocytology; immunohistochemistry; occult tumor cells

INTRODUCTION

Minimal residual disease (MRD) is an increasingly important aspect of the diagnosis and therapy of cancer. MRD is defined as the detection of microscopic and submicroscopic tumor residues in various body tissues and fluids (1). Other terms for MRD are: disseminated tumor cells, micrometastatic cells, and circulating tumor cells.

Disseminated tumor cells that do not qualify as clinically apparent metastases have been detected in different tissues of patients with breast cancer, prostate cancer, neuroblastoma, melanoma, and lung cancer, as well as gastrointestinal cancer (2–4). Subclinical tumor cell dissemination plays a crucial role in the outcome of the underlying malignancy. Numerous studies show that patients with positive tumor cell detection in the bone marrow have a worse prognosis when compared to patients with a negative aspirate (5–8). The prognostic relevance of micrometastatic tumor cell detection was further confirmed by meta-analysis (9).

Micrometastatic and circulating tumor cells can be detected in bone marrow using molecular and immunologic methods. At this time, immunocytology is the

method of choice for the detection of isolated cells (10). Although there are numerous immunocytologic studies described in the literature, there still is no broad consensus as to what constitutes the best methodologic approach. One explanation for this is the great variability in clinical situations described, i.e., patient populations differ with regard to the primary neoplasm (breast, lung, gastrointestinal, prostate) and stage of tumor (I, II, III, IV), as well as timing, location, number, and amount of the bone-marrow aspirates (11). Furthermore, the studies differ in number of patients and length of follow-up.

Methodologic differences abound regarding cell preparation methods (smear vs. cytospin), number of cells per slide, and particularly, choice of antibody. Many different antibodies have been used, such as: EMA,

*Correspondence to: Sven Becker, MD, University of Tübingen, Department of Obstetrics and Gynecology, Calwerstr.7, 72076 Tübingen, Germany. E-mail: sven.becker@med.uni-tuebingen.de

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CEA, 2E11, MFGM, HMFG (mucine) and CAM 5.2, AE1/AE3, CK2 (cytokeratin).

Manual screening of the bone marrow samples by microscopy, which is used by most research groups, has been criticized, since the method is complicated and results depend both on interexaminer and intraexaminer variability. Cells can be overlooked and the same stained cell can be interpreted in different ways (12).

The International Society for Cellular Therapy's European Work-Group for Standardization of Tumor Cell Detection was able to show that the same slides were evaluated with different results in different study centers. As a result, standardized diagnostic criteria have now been established (13).

Another common problem when evaluating slides is that questionable cells are often difficult to relocate for review by a second cytologist, making second opinions awkward and time consuming.

Automated microscope systems for the detection and analysis of potential tumor cells have been developed to solve these problems. Their main goal is to improve sensitivity of detection. In addition, by imaging and storing the pictures of suspicious cells, they should make review easier and increase reproducibility. The electronic storage of the slide allows for relocation of detected cells at any time (14).

In this study, we evaluated the Automated Cell Imaging System (ACIS; Chroma Vision Medical Systems, San Juan Capistrano, CA) with regard to accuracy and reproducibility when compared to manual evaluation by an expert immunocytologist.

MATERIALS AND METHODS

Sample Preparation

Bone marrow was aspirated from stage I-III breast cancer patients. None of these patients had any evidence of distant metastases on chest X-ray, bone scan, and abdominal and pelvic ultrasound. Bone marrow aspiration was performed under general anesthesia at the time of the initial breast surgery. All patients consented prior to the procedure. A total of 10 mL of bone marrow aspirate was collected from the anterior iliac crest and stored in heparinized Falcon tubes with Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, UK). Constituents of the aspirate were separated by density centrifugation with Ficoll-Hypaque (Biochrom Berlin, Berlin, Germany; density 1.077 g/mL). Cells in interphase ($4-5 \times 10^6$ cells) were washed twice, resuspended with DMEM, and then cytocentrifuged onto microscope slides (Model Universal 16A; Hettich, Tuttingen, Germany). The slides were stored at -20°C .

Immunocytochemical Staining

Immunocytochemical staining was performed using the murine monoclonal antibody 2E11 (ABC-method) as previously described (8).

The sensitivity of our method is about one tumor cell among 10^6 normal bone marrow cells. Membrane and cytoplasm of the tumor cells are stained bright red.

Traditional manual analysis was performed by one expert immunocytologist. This analysis was performed without prior knowledge of the surgical procedure, tumor stage, or other prognostic factors.

Analysis of Cytospins With ACIS

For automated analysis we used ACIS. Technical details about ACIS are presented in Ref. 12.

Briefly, the system consists of a microscope and an integrated computer with the image processing system. The microscope is attached to a 60 frames/sec 640×480 pixel three-chip camera (Sony DX9000; Sony, Tokyo, Japan). The computer consists of a dual 450 Mhz Pentium II computer (Pentium, Santa Clara, CA) running Microsoft Window's NT operating system (Microsoft, Redmond, WA). It has 512 megabytes of memory, 27 gigabytes of hard disk storage, and 25 gigabytes of tape backup, compressible to 50 gigabytes.

The system can successively analyze a maximum of 100 cytopins. The analysis of each cytopin requires two steps: 1) focusing and 2) scanning for potential tumor cells.

Focusing

Initially, ACIS looks for focal points. Focusing is done at low magnification ($10\times$). The number and location of focal points on the slide can be chosen individually; however, at least three points must be selected. If less than three focal points are found, no analysis is performed. In our study, we used five focal points. The quality and location of focal points can be reviewed using the montage tool available on the ACIS (Fig. 1). This step is crucial to rule out focusing difficulties with suboptimal specimens (e.g., air bubbles, cover slip unevenness).

Scanning for Potential Tumor Cells

After focusing, the slides are scanned at low magnification ($10\times$) for potential targets. In a second step, ACIS analyses selected targets at a higher magnification ($40\times$ or $60\times$). ACIS looks at each potential cell using color and morphology as selection criteria. For different staining techniques, target criteria can be changed and selected, looking at size, shape, color, and intensity of staining. With this approach,

Fig. 1. Focusing of the cytospins using three focus points. Staining was performed using the murine monoclonal antibody 2E11, ABC detection, and counterstained with hematoxylin.

Fig. 2. Slide report of analyzed cytospin samples. Potential tumor cells are listed by the computer for individual review. All cells displayed meet the criteria for identification of disseminated tumor cells established by ISHAGE and thus are considered positive.

potential tumor cells are separated from debris, clumps, and other atypical cells. These detected and then “discarded” targets are collected and can be reviewed at the end of the scanning process.

The location of all of the selected targets on the slide are stored using XY coordinates.

Selected potential tumor cells are listed by the computer for individual review in the “Slide Report” (Fig. 2). Each tumor cell can then be reanalyzed manually at optimal magnification. Potential tumor cells are categorized as accepted, rejected, or marked. Artifacts that are clearly not cells are deleted. At the

same time, the total number of mononuclear cells is automatically recorded by the computer.

An analysis protocol with pictures of the most important cells is created for each patient.

In this study, we initially looked at 298 cytopsin slides using the usual approach of expert immunocytologic review. These samples were analyzed manually with a Zeiss microscope (Zeiss, Oberkochen, Germany). Then, the same 298 cytopsin slides were analyzed using the ACIS setup as described above. Two independent analysis cycles were performed with each slide.

RESULTS

A total of 298 samples were analyzed once manually and twice using ACIS. Results are shown in Table 1.

By counting the samples manually, 178 cytopsin samples did not show any tumor cells. On 120 cytopsin samples at least one tumor cell was found. The median tumor cell number of the 120 positive cytopsin samples was two cells.

Using ACIS, the results were as follows. For analysis-cycle number one, 135 cytopsin samples were negative for tumor cells and 163 cytopsin samples were positive, with one or more tumor cells. The median detected tumor cell number was again two on the 163 positive samples. For analysis-cycle number two, again 135 cytopsin samples were categorized as negative and 163 cytopsin samples as positive. The median detected tumor cell number was again two for the 163 positive samples.

Identical numbers of tumor cells in both ACIS runs were found in 293 samples (98.3%). In five samples (2%) the results of the two ACIS runs differed from each other by only one tumor cell. All five slides were positive in both ACIS analysis, so the difference in counting did not affect the accuracy of the diagnosis.

By counting the samples manually, 178 cytopsin samples did not show any tumor cells at all, whereas ACIS was able to detect tumor cells in 43 of these cases (24%). In these 43 patients the tumor cell status had to be corrected from negative to positive.

By using the manual technique, 120 cytopsin samples were positive. By using ACIS, all these cases (n = 120) were

also positive and in comparison to the manual technique more tumor cells were detected in 64 out of 120 cases (21.5%). In the remaining 56 cases, ACIS detected the same number of tumor cells. In no case did ACIS miss tumor cells in comparison to the manual technique.

The manual evaluation took a median of 25 min per individual slide. The ACIS-reading time is about 20–40 min per slide. It then takes about 5 min to review and correct the readings. That means 50 min for 10 cytopsin. In contrast to that, it takes about 250 min for manual counts of 10 cytopsin. By using ACIS, the working time can be reduced by about 3 hr. While there is no absolute gain in time, ACIS can read numerous slides overnight, greatly reducing the manpower needed for evaluation.

DISCUSSION

While the prognostic impact of tumor cell detection in the bone marrow is well established, there are only a few studies with more than 300 patients (1) and with a median follow-up of over 5 years (6,15,16).

Many of the difficulties encountered are methodologic. As we further investigate micrometastatic dissemination, it is crucial to standardize methods of detection as well as methods of evaluation. Both are indispensable steps to make the results of different trials more comparable.

The evaluation of the presence or absence of micrometastatic cells in the bone marrow is a good example of this dilemma: competing staining techniques, different antibodies, and the considerable subjective influence of the immunocytologist have the potential to lead to quite variable results even within the same patient population.

With this article, we try to demonstrate that with regard to evaluation of cytopsin, standardization is possible using automated screening devices such as ACIS.

By using ACIS, we were clearly able to improve the quality of tumor cell detection in comparison to a manual technique. Furthermore, the required time period for reading a given number of slides was dramatically reduced, as the automated system can evaluate numerous cytopsin overnight.

The classification of suspected tumor cells into “real tumor cells” or artifacts can be done quickly and in a retrievable way; while the system preselects with great accuracy, all detected cells continue to be subjected to expert immunocytologist review. Reports can be prepared quickly and easily. These reports also contain individual pictures of the detected tumor cells, making further review and quality control easier.

Automated image systems can provide the basis for standardization and automation of cell detection in

TABLE 1. Study results*

Cells detected by both methods	% samples	Conclusion
ACIS + manual	24%	Status change to “positive”
ACIS < manual	10%	Differences, but status change
ACIS = manual	0%	
ACIS = manual	66%	No status change

*n = 185.

bone marrow or peripheral blood. Multicenter trials using this technology should be able to further delineate the clinical significance of disseminated cells.

REFERENCES

1. Diel IJ, Kaufmann M, Goerner R, Costa SD, Kaul S, Bastert G. Detection of tumor cells in bone marrow of patients with primary breast cancer: a prognostic factor for distant metastasis. *J Clin Oncol* 1992;10:1534–1539.
2. Braun S, Rosenberg R, Thorban S, Harbeck N. Implications of occult metastatic cells for systemic cancer treatment in patients with breast or gastrointestinal cancer. *Semin Surg Oncol* 2001;20:334–346.
3. Waldmann V, Deichmann M, Jackel A. Disseminated melanoma cells in blood and bone marrow. Significance and detection by potential tumor markers. *Hautarzt* 2001;52:298–303.
4. Heiss MM, Simon EH, Beyer BC, et al. Minimal residual disease in gastric cancer: evidence of an independent prognostic relevance of urokinase receptor expression by disseminated tumor cells in the bone marrow. *J Clin Oncol* 2002;20:2005–2016.
5. Mansi JL, Easton D, Berger U, et al. Bone marrow micrometastases in primary breast cancer: prognostic significance after 6 years' follow-up. *Eur J Cancer* 1991;27:1552–1555.
6. Braun S, Pantel K, Muller P, et al. Cytokeratin-positive cells in the bone marrow and survival of patients with stage I, II, or III breast cancer. *N Engl J Med* 2000;342:525–533.
7. Cote RJ, Beattie EJ, Chaiwun B, et al. Detection of occult bone marrow micrometastases in patients with operable lung carcinoma. *Ann Surg* 1995;222:415–423.
8. Solomayer EF, Diel IJ, Salanti G, et al. Time independence of the prognostic impact of tumor cell detection in the bone marrow of primary breast cancer patients. *Clin Cancer Res* 2001;7:4102–4108.
9. Funke I, Schraut W. Meta-analyses of studies on bone marrow micrometastases: an independent prognostic impact remains to be substantiated. *J Clin Oncol* 1998;16:557–566.
10. Pantel K, Cote RJ, Fodstad O. Detection and clinical importance of micrometastatic disease. *J Natl Cancer Inst* 1999;91:1113–1124.
11. Diel IJ, Cote RJ. Bone marrow and lymph node assessment for minimal residual disease in patients with breast cancer. *Cancer Treat Rev* 2000;26:53–65.
12. Bauer KD, de la Torre-Bueno J, Diel IJ, et al. Reliable and sensitive analysis of occult bone marrow metastases using automated cellular imaging. *Clin Cancer Res* 2000;6:3552–3559.
13. Borgen E, Naume B, Nesland JM, et al. The European ISHAGE Working Group for Standardization of Tumor Cell Detection: standardization of the immunocytochemical detection of cancer cells in BM and blood: I. Establishment of objective criteria for the evaluation of immunostained cells. *Cytotherapy* 1999;5:377–388.
14. Kraeft SK, Sutherland R, Gravelin L, et al. Detection and analysis of cancer cells in blood and bone marrow using a rare event imaging system. *Clin Cancer Res* 2000;6:434–442.
15. Mansi JL, Gogas H, Bliss JM, Gazet JC, Berger U, Coombes RC. Outcome of primary breast cancer patients with micrometastases: a long-term follow-up study. *Lancet* 1999;354:197–202.
16. Diel IJ, Kaufmann M, Costa SD, et al. Micrometastatic breast cancer cells in bone marrow at primary surgery—prognostic value in comparison with nodal status. *J Natl Cancer Inst* 1996;88:1652–1658.