Review

Detection of tumor cells in bone marrow, peripheral blood and lymph nodes by automated imaging devices [1]

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Abstract. The presence of tumor cells in bone marrow, peripheral blood and lymph nodes has proven its clinical and prognostic value. Since the frequency of these cells in bone marrow and blood is sometimes as low as 1 per million and due to the fact that for the analysis of lymph nodes many sectioning levels have to be analyzed, automated imaging devices have been suggested as an useful alternative to conventional manual screening of specimens. The aim of this paper is to review the performance of current equipment that is commercially available, based on literature published so far. Requirements for introducing this equipment for routine clinical practice are discussed.

Keywords: Disseminated tumor cells, micrometastases, immunohistochemistry, image analysis, automated devices (microscopy)

1. Introduction

Prognostication in case of newly diagnosed cancer is of pivotal importance. The presence of tumor cells outside the primary tumor is associated with a worse course of disease. Depending in which organ tumor cells are detected, different names have been coined for these cells. Generally one speaks about micrometastases when lymph nodes (LN) are considered, disseminated tumor cells (DTC) in case of bone marrow (BM) and circulating tumor cells (CTC) in case of peripheral blood (PB).

1. The search for metastases in lymph nodes (LN) involvement is an established prognostic factor for recurrence and survival [13,35,51]. The current routine method for the detection of metastases exists of surgery of the primary tumor and resection of the surrounding LN followed by pathological investigation of one routine heamatoxylin and eosin (H&E) stained section per node.

In case of epithelial tumors the application of immunohistochemistry (IHC) for cytokeratin (CK) specific antibodies can be applied to improve the ability to recognize smaller size metastasis and single disseminated epithelial cells. In combination with the analysis of multiple sections per node this results in the detection of up to 35% more positive nodes as compared to conventional histopathology [1,12,26]. This method is particularly applied for thorough examination of the sentinel lymph node (SLN) biopsy, which is the first node to which the tumor metastasizes. For breast cancer it has been shown that the SLN can predict axillary status in 95% of cases [24,46,49,53]. Further, in a significant percentage (18%) of patients the detection of micrometastases (<0.2 mm) is accompanied by second echelon axillary lymph node metastases [45,50].

2. The detection of disseminated tumor cells in BM is a new prognostic marker. Both in breast cancer as in colorectal cancer is the presence of tumor cells in BM a strong predictor for the development of overt

metastases. Although recent publications convincingly show that the presence of DTC in BM is an important prognostic marker with clinical impact, it has not become routine clinical practice [10,36,40,42,47,54]. To make this step, large randomized clinical trials have to be performed. This demands a standardized protocol, for both sample processing and staining as well as for specimen analysis and interpretation of the obtained results. Such protocols have to be robust and reliable but also cost effective.

3. The presence of CTC in PB appears to be an early marker for recurrence and relapse as has been demonstrated for breast cancer patients [14,15]. However for this new prognostic marker the level of evidence has not yet reached that as for BM.

In a consensus meeting held at the 5th International Symposium of Minimal Residual Cancer in San Francisco September 2005 a uniform protocol was discussed for the processing and analysis of BM samples to detect DTC. Also the use of an automated imaging device was suggested to detect low frequency DTCs and CTCs since many cells have to be analyzed to obtain statistical reliable quantitative results. Furthermore since investigation of multiple sections increases the chance for detecting CK positive cells in LN automated imaging devices also have advantages in case of LN section analysis.

Other methods to be considered for detection of rare cells are (RT) PCR and flow cytometry. PCR, when specific markers are available, theoretically offers unparalleled sensitivity (up to 1 tumor cell in 10^7 or better), but this method is hampered by high false positive determinations [17,19,30]. Furthermore no visual control of the detected events is possible. Flow cytometry is a powerful technique to analyze a large number of cells in a few minutes, but lacks the sensitivity needed for this purpose.

Manual screening of IHC stained slides is time consuming and is prone to human error particularly when multiple slides or sections have to be analyzed. In the past, automated microscopy using image analysis has been introduced for automated screening of large amounts of cells to detect one single occurring cell [6,41]. Proper interpretation of immunocytochemical (ICC) staining along with careful morphological assessment appears imperative to assure that (ICC) stained cells are, in fact, tumor cells, as opposed to leukocytes or other non-neoplastic cells [7].

At least two models, including variations, have been proposed for the process of metastases. According to

the first model the primary tumor is biologically heterogeneous and metastatic capacity is acquired late in tumorigenesis [22]. The second model supports the hypothesis that the capacity to metastasize is acquired early during tumorigenesis and is intrinsic to the malignancy. Consequently, metastases can be found very early from the onset of disease [4]. In the last model early detection of the disseminated cells is of pivotal importance for further patient treatment. Recently Kang et al report on a variant of these theories, still only proven in mouse models, that within the primary tumor subpopulations are present with a genetic signature which codes for the site of preferential homing [29]. One of the shortcomings of these tumor models is that they insufficiently take the various pathways of dissemination into account, such as haematogenic and lymphatic dissemination [42].

Current research finds new hypothesis for tumor models and metastatic spread. According to the latest theory only the stem cells are responsible for metastasis formation [21]. Analysis of these cells is important for the understanding of the tumor biology. Automated imaging in combination with laser-capture techniques can play an important role in the detection, identification and analysis of such selected cells using molecular techniques.

In this review article we have evaluated the performance of automated imaging devices for the detection of occult tumor cells in BM, PB and LN, its present status for implementation in routine pathology diagnostic and to some attend its implications for patient management.

2. Automated imaging devices

Commercially available systems typically consist of an automated microscope, a personal computer and a camera, mostly of the CCD type (charged coupled device), for acquiring image data. Most functions of the microscope such as filter selection, focusing, stage movement, selection of magnification and of the dichroic filter cubes in case of fluorescence are controlled by the PC. The PC also processes the images of the detected cells. These system parameters largely determine the performance of the automated devices. The CCD camera can be a color or black/white (b/w) model. In the b/w model artificial color images can be produced by combining sequentially recorded monochromatic red, green, blue (RGB) images using the proper filter settings. Devices are available for the au-

tomatic loading of slides on the microscope stage. This means that large batches of slides can be automatically processed and analyzed overnight. The capacity of these slide loaders varies from 8 to 100 slides.

Analysis can be performed using a conventional bright-field microscope or be based on fluorescence. Bright-field microscopy has the advantage over fluorescence that the image acquisition is faster. In addition slides stained immuno-enzymatically for bright-field analysis are stable over a long period and archival material can be used. A disadvantage is that the number of antigens that can be stained simultaneously is limited; in practice not more than two. Fluorescence microscopy offers the advantage of multi-marker assessment. However acquisition of images takes more time, since sufficient numbers of photons must be integrated to provide good quality images. In some cases photobleaching offers a problem and automated focusing in fluorescence is more complex and time consuming than in case of bright-field imaging.

According to Abbe's law the spatial resolution depends on the wavelength of the light and the numerical aperture of the objective lens. Optimal results are obtained if sampling occurs according to Nyquist, that is the sampling frequency should be at least twice the optical resolution. For example, when the spatial resolution is 0.5 μm sampling should be at least 0.25 μm. In case of digital image acquisition also the pixel size of the CCD camera is relevant, as this relates directly to the sampling frequency.

Obviously trade off's between resolution and speed can be made. For most systems switching between objectives is performed to increase efficiency. Scanning is then performed first on lower magnification to select regions of interest and subsequently only these regions are analyzed at a higher magnification for the detection of positive events.

A typical analysis procedure involves hands off analysis of a preset area. During the automated analysis the slide is moved by an automated scanning stage. Generally, stepping or DC motors are used that run at speeds varying between, for instance, 20–200 μm/sec depending on the screw spindle used. This implies that in principle a slide is scanned in several seconds. However, this is the movement only and does not take into account the start/stop commands, the recording of the image by the CCD camera and the time needed for automated focus. Generally, stage movement is not the speed limiting step in automated analysis. Autofocussing can be performed on regular fields e.g. every 6th field, or be based on bilinear interpolation between predefined focus points. Cells are usually selected on the basis of intensity and color of the applied ICC staining in combination with shape and size of the positive stained cells. Artifacts such as degenerated cells, clumps of cells and debris can be recognized as such by optimized image analysis algorithms based on shape and intensity parameters. Selected cells are stored in image memories for review by the pathologist; in addition, of all selected cells corresponding coordinates (x, y, z) are saved which allows for visual inspection using reallocation under the microscope and subsequent confirmation or revision of the selected tumor cells by the operator. Often an overall low magnification digital image is produced by stitching the individual recorded images together. Essential is that proper corrections are made for image overlap (typically, scanning is performed with some overlap in fields in order to prevent the missing of important events) and for imaging errors such as shading. Some commercial systems provide bright-field as well as fluorescence analysis. Typically IHC stained cells are detected on the basis of bright-field microscopy, but further analysis of selected events of multiple fluorescence markers (either FISH or ICC) is subsequently applied for confirmation or to obtain additional prognostic information [37].

Besides conventional microscopy (image plane scanners), also object plane scanners exist, often used in the fluorescence mode. Examples are laser scanning systems [11,25,28]. Krivacic et al. reported on a fiberoptic array scanning technology (FAST) which applies laser-scanning techniques in combination with automated digital microscopy offering a considerable advantage in terms of speed over conventional automated (fluorescence based) microscopy [34].

In conclusion, to assess the performance of an automated scanning system, key parameters are: (1) Speed: what is the estimated frequency of the rare event to be detected, and how many cells have therefore to be analyzed? Example: for a frequency of 1 in 10^5, at least 10^6 cells should be analyzed. (2) Image quality: determined by the optics [numerical aperture (NA)] and magnification of the objective and the type of CCD camera (number of pixels). Note that speed and image quality are related: a higher magnification leads to a smaller field of view and therefore to longer scanning and analysis time. Also, the higher the numerical aperture, the smaller the depth of focus, implying that autofocussing becomes more critical and may take more time. (3) Robustness: largely determined by the software, in particular in situations of unexpected events

in the sample such as large areas of dirt, air bubbles, empty fields, etc.

Other techniques for the detection of occult tumor cells include the enumeration of epithelial cells, which are separated from the blood by antibody-coated magnetic beads and identified with the use of fluorescently labelled antibodies against CK and with a fluorescent nuclear stain; a process that takes place in a specially designed cell chamber [15]. For slide based systems cells preferentially should be deposited on a fixed location of the glass slide, evenly distributed to avoid overlapping cells and preferentially as flat as possible for easy focusing. Staining of the cells of interest with specific monoclonal antibodies facilitates automated analysis since cells are selected on basis of the specific color of the immuno-enzymatic substrate. Regular staining and analysis of positive and negative control samples is essential to monitor possible variables both in staining and system performance. For most systems software has been optimized to not miss any positive cells, thereby generating an affordable number of artifacts.

2.1. Types of automated systems

Two commercial systems are available based on bright-field microscopy and single marker detection for cells in BM, PB and LN; the ACIS system from ChromaVision Medical Systems, Inc (San Juan Capistrano, CA) and the ARIOL-SL50 system (formerly MDS system) from Applied Imaging Corp. (Santa Clara, CA) [2,8].

Furthermore the Metafer 3.0 (MetaSystems GmbH, Altlussheim, Germany) represents an example of an automated microscope system based on fluorescence imaging, that has been successfully used to detect small numbers of tumor cells in PB and BM [37]. Finally, there exists a system named REIS frequently reported in the literature for the detection of tumor cells in PB, which however is not commercially available [31–34]. As previously described fluorescence-based analysis allows combining multiple markers to characterize the DTC. The ARIOL and Metafer system therefore have incorporated the possibility to combine the scanning process in absorption with re-analysis of selected events in fluorescence at higher microscopic magnification.

The CellSearch System (Veridex) has a somewhat different set up than the other systems. It consists of a semi-automated system for the preparation of a sample and a semi automated fluorescence-based microscopy

system but cells remain in a chamber while analyzed using the microscope [14,15]. See also Table 1.

Next to the above mentioned systems, other slide scanning systems (e.g. Aperio) are available but these lack the dedicated application for the detection of disseminated cells.

3. Detection of disseminated tumor cells in bone marrow

In a large meta-analysis of 4703 patients with stage I, II, or III breast cancer, the prognostic significance of the presence of DTC in the BM at the time of diagnosis was evaluated and found to be strongly associated with a poor prognosis [9]. The results of this study are of significant clinical value and would considerably impact nowadays patient management in case all breast cancer patients would be subjected to a BM aspirate to search for DTC. Therefore attempts have been made to analyze BM samples automatically.

In a recent, study Bauer et al. evaluated the use of the ACIS system for identifying DTCs in human breast cancer BM specimens in a study of normal BM, spiked BM samples and BM samples obtained from breast cancer patients [2]. Cells were stained using ICC for cytokeratin with New Fuchsin as a substrate for alkaline phosphatase and hematoxylin as a nuclear counterstain. The spiked BM samples were all found positive whereas for the 39 originally reported negative breast cancer patient samples 17 (44%) were scored positive for the presence of CK positive cells by the pathologist after automated analysis. Furthermore this study reports a $\sim$11.9-fold reduction in pathologist review time for automated microscopy using ACIS-assisted analysis, relative to manual microscopy.

3.1. Performance of automated analysis compared to conventional manual analysis

In a paper by Borgen et al. the detection sensitivity of automated imaging using the MDS/ARIOL system was compared to conventional manual screening [8]. Two pathologists analyzed clinical BM slides from patients with breast cancer. Slides were stained for CK using IHC. The automated procedure detected 50 out of 52 (96%) positive samples whereas only 32 (62%) were found by pathological examination. The two missed samples contained abnormal cells that were located outside the screening area defined for the automated analysis.

Table 1

	ACIS	ARIOL	REIS	Metafer 3.0	CellSearch System
Detection system	Absorption	Absorption and fluorescence	Fluorescence	Fluorescence and absorption	Fluorescence
Microscope	Standard bright field. Type not known	Olympus BX61	Nikon Eclipse 1000	Axioplan 2 (Zeiss)	Cell Tracks Analyzer II
Camera	3-chip Sony DX 9000	CCD Cohu	CCD b/w Sensicam	CCD	CCD
Software	Own design Color detection	Own design Color, morphology and fluorescence based detection	Image-Pro Plus 4.5	Own design	Own design. Fluorescence detection
Time to analyze 1×10^6 cells	14 min	15 min	20 min	**	Contents of 7.5 ml blood in 7 min
Time for reviewing*	2 min/slide	1–2 min/slide	2 min/slide	data not given	Related tumor cell number and tumor cell debris
Capacity	100 slides/run	50 slides/run	1 slide/run	8 slides/run	9 cartridges/hour
Magnification	10×*	10×*	4×*	variable	10×
Evaluation of detected cells at higher magnification	Second scan: ×40, ×60	Second scan: ×20, ×40, ×60	Second scan: ×10, ×20, ×40, ×60	Second scan: ×63, ×100	
Application	BM, PB, LN	BM, PB, LN	PB	BM	BM, PB
Target staining (enzyme or direct label)	Cytokeratin (alkaline phosphatase)	Cytokeratin (alkaline phosphatase)	Cytokeratin (rhodamine)	Cytokeratin (FITC)	EpCAM (ferrofluid)
Additional features		Reanalysis of selected objects using fluorescence mode	Total cell count based on DAPI image	Total cell count based on DAPI image	Confirmation selected cells: Cytokeratin, CD45, DAPI

*Evaluation at higher magnification with interactive artifact rejection software based on shape and size.
**According to information from brochure; acquisition up to 7000 cells/second, analysis time is unknown.

Kraeft et al. found in a study of 39 BM samples from breast cancer patients, fluorescently stained for cytokeratin, that 44% of the positive samples were missed by manual screening but positively identified using the ACIS system [2].

Becker et al. analyzed 298 BM slides from patients with breast cancer, stained for bright-field using the 2E11 monoclonal antibody. Manual analysis of 178 cytospin samples did not show any tumor cells, whereas the ACIS system was able to detect tumor cells in 43 (24%) of these cases [3].

The results of these studies show that automated imaging devices are able to detect DTC in the mononuclear cell fraction with a sensitivity that is at least equal but often better than manual screening.

The reliability of the automated screening procedure has been tested by Borgen et al. for the MDS/ARIOL system in a series of repeated measurements using slides stained for CK using IHC [8]. They found that the screening analysis was stable from day to day for a single machine and from one machine to the other. The variation of the system in the detection of the number of objects to be verified by the operator was 8–9%; the reproducibility in detecting true positive cells was 100% over a period of 5 days [2].

4. Detection of circulating tumor cells in peripheral blood

Since tumor cell dissemination into the BM takes place via blood circulation, it should be possible to detect these cells in a PB sample. A simple blood test would, in contrast to BM aspiration, allow for the analysis to be frequently repeated and be used for staging at diagnosis as well as for therapy monitoring and long-term patient management. Circulating tumor cells (CTC) have been successfully detected and isolated from blood but not yet with an unequivocal consensus [20,27,43,44], although recently a large technical progress in this field has been made by Veridex [14,15]. The frequency of positive cells in the different published studies varies considerably. These variations can be due to the analysis of different tumor types but can also be a result of a limited sensitivity of the available assays. The biology and clinical importance of CTC's in PB is far from understood.

Kraeft et al. used the REIS system and investigated its sensitivity for the analysis of PB samples from cancer patients [33]. Data from 80 slides obtained from breast and lung cancer revealed that as many as 14 of 35 positive slides (40%) were missed by manual screening but positively identified by REIS.

Witzig et al. examined the feasibility for identifying and enumerating CK+ cells in the PB of breast cancer patients using anti-EpCAM-conjugated immunomagnetic beads for carcinoma cell enrichment by automated detection using the ACIS system [55]. Twenty-eight percent of the breast cancer patients demonstrated CK+ cells including 76% of patients with metastatic disease, 8% with N+ disease and none of the patients with N− disease. A further study should demonstrate a possible correlation with prognosis [55]. This group performed an additional study to investigate the reproducibility of the ACIS system for tumor cell detection. Four slides were independently analyzed using 3 separate systems. All four specimens revealed identification of identical numbers (reproducibility 100%) of cells (range 1–49) for three systems [55].

Till now only two studies have analyzed the impact of finding CTC on disease free and overall survival. The main reason is that it is important to first develop and rigorously test the performance of isolation and detection of these cells before large-scale clinical studies are undertaken. Pierga et al. report a study on the comparison of blood and BM samples from 114 breast cancer patients at different stages of disease (I–IV) [43].

Cytospin Ficoll fractions of mononuclear cells were analyzed from both PB and BM, stained for the cytokeratin monoclonal antibody A45B/B3 and automatically analyzed with ACIS. CK+ cells were detected in 28 (24.5%) patients in blood and in 67 (59%) patients in bone marrow. The presence of CK+ cells in blood strongly correlated with the presence of CK+ cells in BM. Twenty-six (93%) patients with CK+ cells in blood also had positive BM ($p < 0.001$). Positive cells were detected in the peripheral blood from 7.5% of stage I/II patients, 36% in stage III and 41% patients with stage IV ($P = 0.017$). The presence of circulating CK+ cells in PB did not statistically correlate with disease-free survival. However, the authors conclude that this method of detection may be useful to monitor the efficacy of treatment in advanced metastatic disease.

Recently, Cristofanilli et al. published two papers in which the presence of CTC was evaluated in a prospective study for patients with metastatic breast cancer. Patients were also monitored for 6 months after treatment initiation. Detection of CTCs before and after initiation of first-line therapy in patients with metastatic breast cancer were found highly predictive factors for progression-free ($P = 0.0014$) and overall survival ($P = 0.0048$) [14,15].

5. Detection of micrometastases in lymph nodes

Strategies for investigating SLN were designed in a consensus meeting of the College of American Pathologists primarily aiming to detect metastases of size 2 mm or larger, since only the presence of metastasis of this size had been shown to convincingly correlate with survival [23]. It was furthermore recommended to apply multiple sectioning of whole SLN at 2 mm intervals in order to increase detection sensitivity.

Despite the increased workload many centers apply multiple sectioning at even smaller intervals, since it has been shown that the sensitivity for detecting occult cells and micrometastasis <0.2 mm increases up to a certain level with the number of sections investigated [50]. Although they may not have prognostic impact with respect to overall survival, such micrometastases or even single cells are in a significant percentage of patients accompanied by second echelon metastases, thereby being clinically relevant as they indicate the need for further axillary dissection [16,45,50].

IHC staining using cytokeratin specific antibodies is applied to improve the recognition rate of smaller size

metastasis and single tumor cells in particular. As an example, the application of IHC in combination with the analysis of multiple sections results in the detection of up to 35% more positive nodes as compared to conventional histopathology [1,12,26].

Basically, micrometastasis have been further defined with a lower limit and are designated pN1mi for metastases >0.2 mm but not >2 mm. The category of metastases that are <0.2 mm have been designated isolated tumor cells and are classified pN0 with modifiers to indicate method of detection as follows: pN0(I−) IHC performed but negative; pN0(I+), IHC positive but no clusters larger than 0.2 mm; pN0(mol− and +) identical for PCR" [18,48].

One of the key questions will be, if manual methods provide sufficient sensitivity and if examination of multiple sections can be performed in a cost-effective way. The rate of missed metastasis (micro or macro) in routine diagnostic pathology ranges from 2% to 9% [48]. Metastases may also be missed even when IHC staining is applied, which is ascribed to human failure and fatigue; problems that can be circumvented to a large extent when automated detection is applied.

5.1. Performance of automated analysis compared to conventional manual analysis

Recent studies have indicated that automated imaging devices are potentially more sensitive than manual microscopy [38,52]. In a study by Weaver et al. 100 IHC-negative SLN biopsies were re-analyzed by automated imaging. Additional micrometastases, were detected in 10.4% of the cases that were originally negative classified as node negative using conventional manual microscopy, and revealed the presence of single tumor cells and groups up to 30 μm in size [52].

In a recent published study our group has reported results on the use of an automated image device to re-analyze sections from SLN that had been IHC stained as part of routine common practice in two clinical centers in the Netherlands [39]. Automated imaging did not misclassify one single positive node. However, in a significant percentage (5.9% and 12.5%) of nodes manually classified as negative, tumor cells and even groups of cells were detected and confirmed by the pathologist. Moreover, it was shown that due to a high degree of automation the pathologist's operational time at the instrument was kept within constraints, which enables the introduction of this new technology in routine clinical pathology.

6. Conclusions and future prospects

Based on studies reported so far it is justified to conclude that automated imaging devices are potentially more sensitive, reproducible, and accurate than routine pathological evaluation for rare events like disseminated tumor cells in bone marrow, peripheral blood and lymph nodes. Automated analysis is also faster due to the possibility to analyze large sets of slides overnight. Thereby it offers the possibility to analyze a larger part of the patient sample (more LN sections, more BM and PB slides from one patient), which is essential in situations where the disseminated cells are at very low frequency. Concluding one can state that (1) the methodology is ready, (2) the clinical impact is proven, (3) thus it is time for implementation. The first question that will rise is, if these systems are ready for routine use in pathology laboratories. As far as their performance is concerned, most likely yes. Whether this can be done in a cost effective way largely depends on the workload c.q. number of patients for which this type of analysis is meaningful. The problem is that most hospitals will not have enough patient material to effectively utilize the capacity that is provided by the 24 hour analysis capability of some machines. That is, to say not within the current clinical practice. However if for all major cancers, for which PB, BM and LN examinations are considered meaningful, automated analysis would be applied, then one machine for an average size hospital probably would be enough. This workload strongly depends on the embedding of above mentioned PB, BM and LN analysis in the clinic when clinicians are enthusiastic to change the current concept and request for these examinations. Clinical implementation will also depend on the fact to what extent a positive result of these assays will have therapeutic consequences. As a result the operational costs of these systems can then be reimbursed by regular health insurance.

When applying an automated system to detect CTCs, DTCs or micrometastasis in case of different tumor types, it is strongly recommended to fine tune the software algorithm for cell selections for each type of cancer on a representative set of tumor cases (learning set) and subsequently test these (test set) prior to clinical applications. Besides tumor type, the variability of staining protocols is a potential performance determining factor that should be controlled in a similar way.

The second question is whether detection (and enumeration) of IHC positive cells is enough for proper patient management. Current research focuses on the

development of new markers that further characterize these cells [5]. Such markers may provide information about their proliferative activity, their metastatic potential, or the predictive value for therapy outcome. By the improvement of the array techniques the analysis of single cells will become a realistic option. Automated imaging devices can assist in this technology. There is the feasibility to search for target cells on basis of cellular characteristics (CK, EpCAM, e.g.) and transform the coordinates of the found objects to laser microdissection systems (LMD). Once specific markers are found, this can be translated to FISH probes and reanalyze the slides by the same system for further characterization which will aid in specific and accurate analysis of these single cells. This may lead to new markers that can be used to help in therapy selection and predict outcome and as such contribute to patient management.

References

[1] H. Altinyollar, N. Kapucuoglu, I. Pak and U. Berberoglu, Lymphatic mapping and sentinel lymphadenectomy in early stage breast carcinoma, *J. Exp. Clin. Cancer Res.* **19** (2000), 141–144.

[2] K.D. Bauer, J. Torre-Bueno, I.J. Diel, D. Hawes, W.J. Decker, C. Priddy, B. Bossy, S. Ludmann, K. Yamamoto, A.S. Masih, F.P. Espinoza and D.S. Harrington, Reliable and sensitive analysis of occult bone marrow metastases using automated cellular imaging, *Clin. Cancer Res.* **6** (2000), 3552–3559.

[3] S. Becker, G. Becker-Pergola, T. Fehm, R. Emig, D. Wallwiener and E.F. Solomayer, Image analysis systems for the detection of disseminated breast cancer cells on bone-marrow cytospins, *J. Clin. Lab. Anal.* **19** (2005), 115–119.

[4] R. Bernards and R.A. Weinberg, A progression puzzle, *Nature* **418** (2002), 823.

[5] A. Bocking, J. Stockhausen and D. Meyer-Ebrecht, Towards a single cell cancer diagnosis. Multimodal and monocellular measurements of markers and morphology (5M), *Cell. Oncol.* **26** (2004), 73–79.

[6] G.J. Boland, W.E. Mesker, R. Doorn, J.J. Ploem-Zaaijer, H.J. Tank and G.C. de Gast, Detection improvement of cytomegalovirus antigen in human peripheral blood using monoclonal antibodies and automated reading of cell preparations, *Eur. J. Clin. Invest.* **25** (1995), 639–646.

[7] E. Borgen, K. Beiske, S. Trachsel, J.M. Nesland, G. Kvalheim, T.K. Herstad, E. Schlichting, H. Qvist and B. Naume, Immunocytochemical detection of isolated epithelial cells in bone marrow: non-specific staining and contribution by plasma cells directly reactive to alkaline phosphatase, *J. Pathol.* **185** (1998), 427–434.

[8] E. Borgen, B. Naume, J.M. Nesland, K.W. Nowels, N. Pavlak, I. Ravkin and S. Goldbard, Use of automated microscopy for the detection of disseminated tumor cells in bone marrow samples, *Cytometry* **46** (2001), 215–221.

[9] S. Braun, K. Pantel, P. Muller, W. Janni, F. Hepp, C.R. Kentenich, S. Gastroph, A. Wischnik, T. Dimpfl, G. Kindermann, G. Riethmuller and G. Schlimok, Cytokeratin-positive cells in the bone marrow and survival of patients with stage I, II, or III breast cancer, *N. Engl. J. Med.* **342** (2000), 525–533.

[10] S. Braun, F.D. Vogl, B. Naume, W. Janni, M.P. Osborne, R.C. Coombes, G. Schlimok, I.J. Diel, B. Gerber, G. Gebauer, J.Y. Pierga, C. Marth, D. Oruzio, G. Wiedswang, E.F. Solomayer, G. Kundt, B. Strobl, T. Fehm, G.Y. Wong, J. Bliss, A. Vincent-Salomon and K. Pantel, A pooled analysis of bone marrow micrometastasis in breast cancer, *N. Engl. J. Med.* **353** (2005), 793–802.

[11] R.J. Clatch, J.L. Walloch, J.R. Foreman and L.A. Kamentsky, Multiparameter analysis of DNA content and cytokeratin expression in breast carcinoma by laser scanning cytometry, *Arch. Pathol. Lab. Med.* **121** (1997), 585–592.

[12] R.J. Cote, H.F. Peterson, B. Chaiwun, R.D. Gelber, A. Goldhirsch, M. Castiglione-Gertsch, B. Gusterson and A.M. Neville, Role of immunohistochemical detection of lymph-node metastases in management of breast cancer. International Breast Cancer Study Group, *Lancet* **354** (1999), 896–900.

[13] R.J. Cote, H.F. Peterson, B. Chaiwun, R.D. Gelber, A. Goldhirsch, M. Castiglione-Gertsch, B. Gusterson and A.M. Neville, Role of immunohistochemical detection of lymph-node metastases in management of breast cancer. International Breast Cancer Study Group, *Lancet* **354** (1999), 896–900.

[14] M. Cristofanilli, G.T. Budd, M.J. Ellis, A. Stopeck, J. Matera, M.C. Miller, J.M. Reuben, G.V. Doyle, W.J. Allard, L.W. Terstappen and D.F. Hayes, Circulating tumor cells, disease progression, and survival in metastatic breast cancer, *N. Engl. J. Med.* **351** (2004), 781–791.

[15] M. Cristofanilli, D.F. Hayes, G.T. Budd, M.J. Ellis, A. Stopeck, J.M. Reuben, G.V. Doyle, J. Matera, W.J. Allard, M.C. Miller, H.A. Fritsche, G.N. Hortobagyi and L.W. Terstappen, Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer, *J. Clin. Oncol.* **23** (2005), 1420–1430.

[16] G. Cserni, Complete sectioning of axillary sentinel nodes in patients with breast cancer. Analysis of two different step sectioning and immunohistochemistry protocols in 246 patients, *J. Clin. Pathol.* **55** (2002), 926–931.

[17] A. Dimmler, R. Gerhards, C. Betz, K. Gunther, B. Reingruber, T. Horbach, I. Baumann, T. Kirchner, W. Hohenberger and T. Papadopoulos, Transcription of cytokeratins 8, 18, and 19 in bone marrow and limited expression of cytokeratins 7 and 20 by carcinoma cells: inherent limitations for RT-PCR in the detection of isolated tumor cells, *Lab. Invest.* **81** (2001), 1351–1361.

[18] K. Dowlatshahi, M. Fan, J.M. Anderson and K.J. Bloom, Occult metastases in sentinel nodes of 200 patients with operable breast cancer, *Ann. Surg. Oncol.* **8** (2001), 675–681.

[19] T.A. Fava, R. Desnoyers, S. Schulz, J. Park, D. Weinberg, E. Mitchell and S.A. Waldman, Ectopic expression of guanylyl cyclase C in CD34+ progenitor cells in peripheral blood, *J. Clin. Oncol.* **19** (2001), 3951–3959.

[20] R.S. Faye, S. Aamdal, H.K. Hoifodt, E. Jacobsen, L. Holstad, E. Skovlund and O. Fodstad, Immunomagnetic detection and clinical significance of micrometastatic tumor cells in malignant melanoma patients, *Clin. Cancer Res.* **10** (2004), 4134–4139.

[21] A.P. Feinberg, R. Ohlsson and S. Henikoff, The epigenetic progenitor origin of human cancer, *Nat. Rev. Genet.* **7** (2006), 21–33.

[22] I.J. Fidler and M.L. Kripke, Metastasis results from preexisting variant cells within a malignant tumor, *Science* **197** (1977), 893–895.

[23] P.L. Fitzgibbons, D.L. Page, D. Weaver, A.D. Thor, D.C. Allred, G.M. Clark, S.G. Ruby, F. O'Malley, J.F. Simpson, J.L. Connolly, D.F. Hayes, S.B. Edge, A. Lichter and S.J. Schnitt, Prognostic factors in breast cancer. College of American Pathologists Consensus Statement 1999, *Arch. Pathol. Lab. Med.* **124** (2000), 966–978.

[24] M. Fraile, M. Rull, F.J. Julian, F. Fuste, A. Barnadas, M. Llatjos, E. Castella, J.R. Gonzalez, V. Vallejos, A. Alastrue and M.A. Broggi, Sentinel node biopsy as a practical alternative to axillary lymph node dissection in breast cancer patients: an approach to its validity, *Ann. Oncol.* **11** (2000), 701–705.

[25] A. Gerger, P. Bergthaler and J. Smolle, An automated method for the quantification and fractal analysis of immunostaining, *Cell. Oncol.* **26** (2004), 125–134.

[26] A.E. Giuliano, P.I. Haigh, M.B. Brennan, N.M. Hansen, M.C. Kelley, W. Ye, E.C. Glass and R.R. Turner, Prospective observational study of sentinel lymphadenectomy without further axillary dissection in patients with sentinel node-negative breast cancer, *J. Clin. Oncol.* **18** (2000), 2553–2559.

[27] H.J. Kahn, A. Presta, L.Y. Yang, J. Blondal, M. Trudeau, L. Lickley, C. Holloway, D.R. McCready, D. Maclean and A. Marks, Enumeration of circulating tumor cells in the blood of breast cancer patients after filtration enrichment: correlation with disease stage, *Breast Cancer Res. Treat.* **86** (2004), 237–247.

[28] L.A. Kamentsky, D.E. Burger, R.J. Gershman, L.D. Kamentsky and E. Luther, Slide-based laser scanning cytometry, *Acta Cytol.* **41** (1997), 123–143.

[29] Y. Kang, P.M. Siegel, W. Shu, M. Drobnjak, S.M. Kakonen, C. Cordon-Cardo, T.A. Guise and J. Massague, A multigenic program mediating breast cancer metastasis to bone, *Cancer Cell* **3** (2003), 537–549.

[30] Y. Ko, E. Grunewald, G. Totzke, M. Klinz, S. Fronhoffs, I. Gouni-Berthold, A. Sachinidis and H. Vetter, High percentage of false-positive results of cytokeratin 19 RT-PCR in blood: a model for the analysis of illegitimate gene expression, *Oncology* **59** (2000), 81–88.

[31] S.K. Kraeft, A. Ladanyi, K. Galiger, A. Herlitz, A.C. Sher, D.E. Bergsrud, G. Even, S. Brunelle, L. Harris, R. Salgia, T. Dahl, J. Kesterson and L.B. Chen, Reliable and sensitive identification of occult tumor cells using the improved rare event imaging system, *Clin. Cancer Res.* **10** (2004), 3020–3028.

[32] S.K. Kraeft, R. Salgia and L.B. Chen, Detection and analysis of lung cancer cells from body fluids using a rare event imaging system, *Methods Mol. Med.* **75** (2003), 423–430.

[33] S.K. Kraeft, R. Sutherland, L. Gravelin, G.H. Hu, L.H. Ferland, P. Richardson, A. Elias and L.B. Chen, Detection and analysis of cancer cells in blood and bone marrow using a rare event imaging system, *Clin. Cancer Res.* **6** (2000), 434–442.

[34] R.T. Krivacic, A. Ladanyi, D.N. Curry, H.B. Hsieh, P. Kuhn, D.E. Bergsrud, J.F. Kepros, T. Barbera, M.Y. Ho, L.B. Chen, R.A. Lerner and R.H. Bruce, A rare-cell detector for cancer, *Proc. Natl. Acad. Sci. USA* **101** (2004), 10501–10504.

[35] G.J. Liefers, A.M. Cleton-Jansen, C.J. van de Velde, J. Hermans, J.H. van Krieken, C.J. Cornelisse and R.A. Tollenaar, Micrometastases and survival in stage II colorectal cancer, *N. Engl. J. Med.* **339** (1998), 223–228.

[36] F. Lindemann, G. Schlimok, P. Dirschedl, J. Witte and G. Riethmuller, Prognostic significance of micrometastatic tumour cells in bone marrow of colorectal cancer patients, *Lancet* **340** (1992), 685–689.

[37] G. Mehes, A. Luegmayr, I.M. Ambros, R. Ladenstein and P.F. Ambros, Combined automatic immunological and molecular cytogenetic analysis allows exact identification and quantification of tumor cells in the bone marrow, *Clin. Cancer Res.* **7** (2001), 1969–1975.

[38] W.E. Mesker, F.S. Doekhie, H. Vrolijk, R. Keyzer, W.C. Sloos, H. Morreau, P.S. O'Kelly, G.H. de Bock, R.A. Tollenaar and H.J. Tanke, Automated analysis of multiple sections for the detection of occult cells in lymph nodes, *Clin. Cancer Res.* **9** (2003), 4826–4834.

[39] W.E. Mesker, H. Torrenga, W.C. Sloos, H. Vrolijk, R.A. Tollenaar, P.C. de Bruin, P.J. van Diest and H.J. Tanke, Supervised automated microscopy increases sensitivity and efficiency of detection of sentinel node micrometastases in patients with breast cancer, *J. Clin. Pathol.* **57** (2004), 960–964.

[40] B. Naume, G. Wiedswang, E. Borgen, G. Kvalheim, R. Karesen, H. Qvist, J. Janbu, T. Harbitz and J.M. Nesland, The prognostic value of isolated tumor cells in bone marrow in breast cancer patients: evaluation of morphological categories and the number of clinically significant cells, *Clin. Cancer Res.* **10** (2004), 3091–3097.

[41] J.C. Oosterwijk, C.F. Knepfle, W.E. Mesker, H. Vrolijk, W.C. Sloos, H. Pattenier, I. Ravkin, G.J. Van Ommen, H.H. Kanhai and H.J. Tanke, Strategies for rare-event detection: an approach for automated fetal cell detection in maternal blood, *Am. J. Hum. Genet.* **63** (1998), 1783–1792.

[42] K. Pantel and R.H. Brakenhoff, Dissecting the metastatic cascade, *Nat. Rev. Cancer* **4** (2004), 448–456.

[43] J.Y. Pierga, C. Bonneton, A. Vincent-Salomon, P. de Cremoux, C. Nos, N. Blin, P. Pouillart, J.P. Thiery and H. Magdelenat, Clinical significance of immunocytochemical detection of tumor cells using digital microscopy in peripheral blood and bone marrow of breast cancer patients, *Clin. Cancer Res.* **10** (2004), 1392–1400.

[44] E. Racila, D. Euhus, A.J. Weiss, C. Rao, J. McConnell, L.W. Terstappen and J.W. Uhr, Detection and characterization of carcinoma cells in the blood, *Proc. Natl. Acad. Sci. USA* **95** (1998), 4589–4594.

[45] F.D. Rahusen, S. Meijer and P.J. van Diest, Re: Chu et al. "Do all patients with sentinel node metastasis from breast carcinoma need complete axillary node dissection?", *Ann. Surg.* **231** (2000), 615–616.

[46] S. Sandrucci, P.S. Casalegno, P. Percivale, M. Mistrangelo, E. Bombardieri and S. Bertoglio, Sentinel lymph node mapping and biopsy for breast cancer: a review of the literature relative to 4791 procedures, *Tumori* **85** (1999), 425–434.

[47] G. Schlimok, I. Funke, B. Holzmann, G. Gottlinger, G. Schmidt, H. Hauser, S. Swierkot, H.H. Warnecke, B. Schneider and H. Koprowski, Micrometastatic cancer cells in bone marrow: in vitro detection with anti-cytokeratin and in vivo labeling with anti-17-1A monoclonal antibodies, *Proc. Natl. Acad. Sci. USA* **84** (1987), 8672–8676.

[48] S.E. Singletary, C. Allred, P. Ashley, L.W. Bassett, D. Berry, K.I. Bland, P.I. Borgen, G.M. Clark, S.B. Edge, D.F. Hayes, L.L. Hughes, R.V. Hutter, M. Morrow, D.L. Page, A. Recht, R.L. Theriault, A. Thor, D.L. Weaver, H.S. Wieand and F.L. Greene, Staging system for breast cancer: revisions for the 6th edition of the AJCC Cancer Staging Manual, *Surg. Clin. North Am.* **83** (2003), 803–819.

[49] V.C. Tjan-Heijnen, P. Buit, L.M. Widt-Evert, T.J. Ruers and L.V. Beex, Micro-metastases in axillary lymph nodes: an increasing classification and treatment dilemma in breast cancer due to the introduction of the sentinel lymph node procedure, *Breast Cancer Res. Treat.* **70** (2001), 81–88.

[50] H. Torrenga, F.D. Rahusen, S. Meijer, P.J. Borgstein and P.J. van Diest, Sentinel node investigation in breast cancer: detailed analysis of the yield from step sectioning and immunohisto-chemistry, *J. Clin. Pathol.* **54** (2001), 550–552.

[51] R.R. Turner, D.T. Nora, S.D. Trocha and A.J. Bilchik, Colorectal carcinoma nodal staging. Frequency and nature of cytokeratin-positive cells in sentinel and nonsentinel lymph nodes, *Arch. Pathol. Lab. Med.* **127** (2003), 673–679.

[52] D.L. Weaver, D.N. Krag, T. Ashikaga, S.P. Harlow and M. O'Connell, Pathologic analysis of sentinel and nonsentinel lymph nodes in breast carcinoma: a multicenter study, *Cancer* **88** (2000), 1099–1107.

[53] L. Widt-Levert, V. Tjan-Heijnen, P. Bult, T. Ruers and T. Wobbes, Stage migration in breast cancer: surgical decisions concerning isolated tumour cells and micro-metastases in the sentinel lymph node, *Eur. J. Surg. Oncol.* **29** (2003), 216–220.

[54] G. Wiedswang, E. Borgen, R. Karesen, H. Qvist, J. Janbu, G. Kvalheim, J.M. Nesland and B. Naume, Isolated tumor cells in bone marrow three years after diagnosis in disease-free breast cancer patients predict unfavorable clinical outcome, *Clin. Cancer Res.* **10** (2004), 5342–5348.

[55] T.E. Witzig, B. Bossy, T. Kimlinger, P.C. Roche, J.N. Ingle, C. Grant, J. Donohue, V.J. Suman, D. Harrington, J. Torre-Bueno and K.D. Bauer, Detection of circulating cytokeratin-positive cells in the blood of breast cancer patients using immunomagnetic enrichment and digital microscopy, *Clin. Cancer Res.* **8** (2002), 1085–1091.