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REVIEW

Micrometastatic bone marrow involvement: detection and prognostic significance

S Braun[1] and K Pantel[2]*

[1]*Frauenklinik, Klinikum Innenstadt, Ludwig-Maximilians-Universität, Maistrasse 11, D-80337 München, Germany; and;* [2]*Molekulare Diagnostik & Therapie, Universitäts-Frauenklinik, Universitätsklinikum Eppendorf, Martinistrasse 52, D-20246 Hamburg, Germany*

The present review focuses on the methodology and clinical significance of new diagnostic approaches to identify individual cancer cells present in bone marrow, both as a frequent site of metastasis formation and an indicator organ for hematogenous tumor cell dissemination. The steadily increasing number of studies on this issue is characterized by considerable methodological variations of important variables, such as the size of the study population, and the reliability of monoclonal antibodies used for tumor cell detection. Emerging data indicate that this disturbing heterogeneity might be overcome by the use of reliable and specific anti-cytokeratin antibodies (for example, A45-B/B3) as, for the time, standard markers for the detection of micrometastatic tumor cells in bone marrow. Prospective clinical studies have shown that immunoassays based on anti-CK antibodies identify patients' subgroups with a poor clinical prognosis with regard to early metastasis manifestation and reduced overall survival in various epithelial tumor entities, including breast, colon, rectum, stomach, esophagous, prostate, renal, bladder, and non-small cell lung cancer. The immunocytochemical assays may be therefore used to improve tumor staging with potential consequences for adjuvant therapy, because disseminated cells appeared to be dormant, non-cycling (for example Ki-67 antigen-negative) cells, suggesting a resistence to cell-cycle dependent therapy, such as chemotherapy. Therefore, cell-cycle independent antibody-based immunotherapy might be an interesting option to complement chemotherapy. Another promising clinical application is monitoring the response of micrometastatic cells to adjuvant therapies, which, at present, can only be assessed retrospectively after an extended period of clinical follow-up. The outlined current strategies for detection and characterization of cancer micrometastasis might help to design and control new therapeutic strategies for secondary prevention of metastatic relapse in patients with operable primary carcinomas.

Keywords: solid tumors; micrometastases; bone marrow; tumor staging; adjuvant therapy

*Correspondence: Professor Dr. Klaus Pantel, Universitäts-Frauenklinik, Universitätsklinikum Eppendorf, Martinistr. 52, D-20246 Hamburg, Germany.
E-mail: pantel@uke.uni.hamburg.de
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Introduction

In the European Union, the estimated number of new cases of cancer in 1990 were approximately 1 500 000; approximately 500 000 men and 400 000 women died of cancer in that year.[1] Despite recent progress in early detection and surgical therapy, this mortality remained unchanged over the past decades. The major reason for this disturbing discrepancy is that occult dissemination of viable cancer cells can occur at an early stage of tumorigenesis.[2] This implies that the acquisition of at least some characteristics of metastatic behavior can occur prior to attainment of the unrestrained growth observed in fully developed tumors forming clinically detectable metastasis. In this context, it is important to consider that tumorigenesis and metastasis development are not necessarily the result of the same genetic changes.[3,4]

Occult dissemination of tumor cells in patients with operable cancer may be considered a determinant of subsequent metastasis formation, yet is usually missed by conventional tumor staging. Several groups (including ours) have therefore designed immunocytochemical and molecular assays to identify such minimal amounts of residual tumor cells that have successfully invaded secondary organs (Table 1). Among the various organs investigated, bone marrow played a prominant role as determinant for such micrometastatic organ involvement due to easy accessibility and physiological absence of epithelial cells. In addition, bone marrow represents a relevant site of distant metastasis in breast cancer. The development of antibodies to epithelial differentiation antigens, such as cytokeratins, as essential constituents of the epithelial cytoskeleton, and tumor-associated cell membrane glycoproteins has opened a diagnostic window to detect such disseminated tumor cells as early as at primary diagnosis.[5,6]

Along with emerging data in support of the prognostic relevance of this phenomenon,[7] there is an urgent need for appropriate therapeutic approaches directed against micrometastatic cells. It is known from the clinical practice that both locoregional and distant tumor recurrences occurred in patients treated with curative intent, for example complete tumor resection (R_0) in patients without distant metastasis (M_0)—even if systemic cytotoxic chemotherapy was applied, which pointed to the existence of at least some resistent tumor cells. Although various mechanisms may be contribute to this apparent chemo-resistence, the latter assumption could be supported by the absence of proliferation-associated markers on disseminated tumor cells in bone marrow.[8] In this view, cell-cycle independent treatment strategies, such as antibody-based immunotherapy, which have been recently shown to be active in breast[9,10] and colorectal cancer,[11] might gain increased interest for the design of future clinical trials.

The present review focuses on the methodology and the clinical relevance of new diagnostic approaches with their impact on identification, characterization and treatment of minimal residual cancer cells detected in bone marrow.

Diagnostic approaches of micrometastasis detection

Immunocytochemistry

So far, data on bone marrow screening for cancer micrometastasis are almost exclusively based on immunocytochemical analyses. Bone marrow is an easily accessible site for the aspiration needle, and the mesenchymal organ is physiologically devoid of epithelial cells. Therefore, extrinsic epithelial cells can be discriminated from autochthonous bone marrow cells using monoclonal antibodies directed against epithelial differention markers. Far diverging detection rates comparing similar study populations,[12–22] for example in breast cancer as summarized in Table 1, however, made a systematic analysis of critical variables rather advisable in order to avoid the discussion on discrepant results of clinical follow-up studies caused by methodological variations, for example small study populations, use of unspecific detection antibodies.[23–25]

Since the specificity of the immunocytochemical assay for single tumor cells is one of the major concerns, alkaline phosphatase-based staining techniques being now preferred over immunoperoxidase methods recently applied for the detection of disseminated tumor cells in blood and bone marrow[15,26] because hematopoietic bone marrow cells produce endogenous peroxidase. Furthermore, most investigators decided to use monoclonal antibodies rather than polyclonal antisera for tumor cell detection. Most of the monoclonal antibodies applied for epithelial tumor cell detection are directed to either cytokeratins (CK) as major

Table 1 Immunocytochemical studies on the detection of micrometastatic BM involvement in breast cancer patients

Marker	*Antibody*	*Disease stages*	*Detection rate*	*Tissue preparation*	*Staining technique*	*Reference*
Mucin	MBr1	I-III	20/121 (17%)	biopsy	IF	12
Mucin	LICR-LON-M8	I-III	4/50 (8%)	biopsy	POX	13
CK, EMA	KL1	I-III	1/93 (1%)	biopsy	POX	14
EMA	E29	I-III	89/350 (25%)	cell smears	POX	15, 16
Mucin	LICR-LON-M8	I, II	12/25 (48%)	cell smears	POX	17
EMA, CK	E29, CK8/18/19	I-III	38/100 (38%)	cell smears	AP	18
TAG12	2E11	I-III	315/727 (43%)	cell smears	AP	19
CK, TAG12	AE1, C26, T16	I-III	18/49 (37%)	cell smears	IF	20
CK & anti-epithelial	AE1, AE3, cocktail	IV	27/71 (38%)	cell smears	AP	21
CK	CK2	I-III	84/349 (24%)	cytospins	AP	22

IF, immunofluorescence; POX, immunoperoxidase; AP, alkaline phosphatase; BM, bone marrow.

constituents of the epithelial cytoskeleton, or membrane-bound mucins, such as epithelial membrane antigen (EMA), human milk fat globules (HMFG), human epithelial antigen-125 (HEA-125) or tumor-associated glycoprotein-12 (TAG-12).

Analysis of a large series of non-carcinoma control patients,[23,27,28] however, revealed that the specificity of monoclonal antibodies to CK is superior to that of monoclonal antibodies against mucins (Table 2). Therefore, the crossreactivity of anti-mucin monoclonal antibodies with normal bone marrow cells might limit the reproducibility of mucin-based immunoassays for tumor cell detection in bone marrow. Moreover, the epithelial nature of CK-positive cells in bone marrow was supported by double labeling analyses: to resolve concerns that single CK-positive cells are rare hematopoietic cells with aberrant CK expression, we demonstrated that neither the leukocyte common antigen CD45 nor the mesenchymal intermediate filament protein vimentin are coexpressed by CK-positive cells.[5,25,29] In prostate cancer patients, disseminated CK-positive tumor cells exhibited coexpression of the prostate-specific antigen (PSA) in about 40% of cases, an incidence being consistent with the rate of PSA coexpression in primary carcinomas and the LNCap prostate cancer cell line.[30] Taken together, these findings suggest that disseminated CK-positive cells are descendants of the epithelial neoplasia.

Table 2 Immunocytochemical staining of bone marrow from non-carcinoma control patients

Antibody	*Antigen*	*Fraction of bone marrow samples with immuno-reactive cells (%)*
CK2[a]	CK18	6/215 (3%)
A45-B/B[b]	pan-CK	2/165 (1%)
E29[c]	epithelial membrane antigen, EMA	20/75 (27%)[d]
2E11[b]	tumor-associated glycoprotein-12, TAG-12	66/105 (63%)[d]
HMFG1[c]	human milk fat globule, HMFG	32/75 (43%)[d]

[a]Per bone marrow sample 4×10^5 mononucleated cells were stained (5, 25).
[b]Per bone marrow sample 2×10^6 mononucleated cells were stained (23).
[c]Per bone marrow sample 1.5×10^5 mononucleated cells were stained (5).
[d]$P < 0.001$ compared to CK2 or A45-B/B3 immunostaining (χ^2 test).

To estimate the sensitivity of the immunocytochemical assay, previous methodological studies have used surrogate model systems of bone marrow samples spiked with cancer cells from cell lines, demonstrating that the technique can detect 2–4 cells at a concentration of 10 per 10^6 and, by extrapolation, a 95% chance of detecting one cancer cell at a concentration of two per 10^6.[31,32] The clinical relevance of these evaluations remain however disputed, because tumor cells selected *in vitro* may display different characteristics as compared to cancer cells *in vivo*. Based on the extensive experience derived from immunocytochemical analysis of bone marrow samples, the representative example for the detection rate of tumor cells using our quantitative bone marrow assay (antibody A45-B/B3, alkaline phosphatase anti-alkaline phosphotase (APAAP) staining technique) indicated an actual assay sensitivity to detect one carcinoma cell in 2×10^6 mononucleated bone marrow cells (Figure 1).

Recent studies implied that a certain minimal amount of tumor cells might be required to initiate subsequent overt metastasis.[20,33] Determination of such a minimal residual tumor load required an exact quantification of the residual tumor burden which so far has

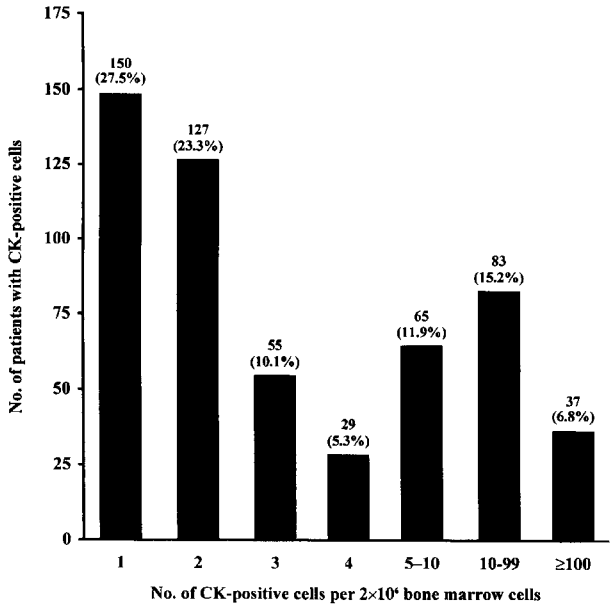

Figure 1 *Frequency distribution of micrometastatic carcinoma cells detected in bone marrow of 546 patients with various epithelial cancers including malignancies of the breast (n = 226), prostate (n = 117), cervix uteri (n = 36), ovary (n = 28), colon/rectum (n = 29), lung (n = 22), skin (spinicellular carcinoma; n = 15), esophagus (n = 13), kidney (n = 12), stomach (n = 8), and other organs (n = 40). Per patient, 2×10^6 mononucleated bone marrow cells were analyzed using antibody A45-B/B3 and alkaline phosphatase anti-alkaline phosphatase (APAAP) staining technique.*

not been performed in most studies due to the use of cell smears,[18–20,34] a technique not permitting a reproducible quantitative transfer of cells to the slide surface. Therefore, prognostic assessments relying on the number of detected cells are jeopardized by the applied smear technique. More reproducible results can be obtained by cytocentrifugation, allowing the transfer of a well-defined cell number to slides.[25,35]

However, even if anti-CK monoclonal antibodies on cytospin preparations are used, the detection rate is still affected by blood contamination of the bone marrow specimen, the number of aspirates analyzed, and the number of mononucleated bone marrow cells screened per aspiration site.[25] Therefore, the result of any immunocytochemical screening for isolated carcinoma cells in bone marrow largely depends on the applied method which underscores the urgent need for an internationally standardized protocol in order to foster its implementation into clinical practice.

Molecular approaches

Polymerase chain reaction (PCR)-based assays should be the most sensitive methods for the detection of minimal residual disease. Thus far, this approach has only proven successful in lymphoma patients who showed a prolonged survival after receiving tumor-free marrow transplants as defined by PCR analysis.[36] The successful application of the PCR method for the detection of occult metastatic disease in lymphoma patients was supported by the fact that lymphoma cells have unique genomic characteristics such as certain chromosomal translocations or idiotypic rearrangements of the immunoglobulin locus.

In contrast, the genomic characteristics of epithelial cancer cells are more heterogenous. Among the most common changes are mutations in the K-*ras* and the *p53* tumor suppressor gene.[37,38] More recently, Hayashi *et al*[39] described an elegant approach, called the mutant allele-specific amplification (MASA) method, which is capable of detecting one tumor cell in thousands of lymph node cells by the assessment of K-*ras* and *p53* mutations. The prognostic relevance of this approach has been so far demonstrated in lymph nodes excised from colorectal cancer patients which were judged negative by routine histopathological examination.[40] Although the assay sensitivity needs to be improved to identify bone marrow micrometastasis usually occuring at frequencies of 10^{-5} to 10^{-6}, it appears to be a promising approach. Nevertheless, screening for genomic changes is very tedious requiring molecular analysis of every individual primary tumor to determine whether the tumor cells of this individual patient carry the respective alteration.

More frequently, histogenetic mRNA marker expression has been investigated by development of reverse transcriptase (RT)-PCR assays for the detection of epithelial cells in samples from mesenchymal organs such as bone marrow, peripheral blood, and lymph nodes of various carcinomas[41–45] The specificity of this approach might not be absolute but rather reflect quantitiative differences in the expression level of malignant cells and the surrounding autochthonous cells. As limitating factors in the detection of micrometastatic cells by RT-PCR we and others recently described the illegitimate transcription of tumor-associated or epthelial-specific genes in hematopoietic cells, and the deficient expression of the marker gene in micrometastatic tumor cells.[43,46–49]

This problem has been well addressed by Schoenfeld *et al*,[48] who used RT-PCR for CK19 to detect breast cancer micrometastasis in axillary lymph nodes. However, despite an optimal cut-off point to distinguish between involved and metastasis-free lymph nodes, the CK19-mRNA product was also found in normal lymph nodes from patients without epithelial cancer, if the sensitivity was increased by a two-stage amplification using nested primers.[48] Several studies have recently applied RT-PCR for the detection of CK19-mRNA in bone marrow, lymph nodes and peripheral blood from breast cancer patients.[41,43,47,49,50] Among the latter, the study by Datta *et al*[41] claimed a sensitivity of 10 cancer cells per 10^6 hematopoietic cells. However, the specificity of this finding remains obscure, since hematopoietic cells are known to express low levels of CK-mRNA that are detectable by PCR technology.[43,47,49]

The majority of genes encoding for tumor-associated molecules are not uniquely expressed in carcinoma cells, but also exert some expression in certain benign tissues. The organ in which the disseminated tumor cells should be detected thus needs to be carefully evaluated for such expression. Gerhard *et al*[45] have recently applied RT-PCR to screen for the expression of carcinomembryonic antigen (CEA)-mRNA in bone marrow obtained from breast cancer patients. These authors did not find CEA-mRNA in bone marrow and peripheral blood samples from non-carcinoma patients, which is in contrast to our own results, demonstrating CEA-mRNA in bone marrow from comparable control patients.[43] Even mRNA of the oncogenes erb-B2 and c-erb-B3 were found to be expressed at very low levels in normal bone marrow cells.[43]

To overcome the problem that a specific marker gene is downregulated or completely suppressed multimarker PCR assays may provide improved sensitivity over single marker-based approaches, unless the gain of sensitivity is paid for by loss of specificity. The identification of marker genes exclusively expressed in tumor cells is of utmost importance before the standard immunocytochemical assay is replaced by the promising new RT-PCR approach. Although several PCR studies claim improved sensitivity of their assay over immunocytochemistry, they usually avoid comparison with a true immunocytochemical benchmark method consisting of a standardized assay with a specificity proven monoclonal antibody (for example, A45-B/B3) and sufficient sample size (for example, 2×10^6 mononucleated cells). At present, we have initiated ring experiments comparing different tumor cell detection assays within the European Working group sponsored by the International Society of Hematotherapy and Graft Engineering.

Clinical relevance of bone marrow micrometastasis

So far, several groups including ours demonstrated the prognostic relevance of disseminated tumor cells for breast, colon, rectum, stomach, esophagous, prostate, renal, bladder, and non-small cell lung cancer.[12,14–21,26,27,33,51–59] An overview of the currently available results is given in Table 3. To exemplify that the phenomen of tumor cell dissemination—which appeared to be common for tumor entities as diverse as ones listed above—may have a prognostic impact independently from the manifestation of bone or bone marrow metastasis, we selected breast, colorectal and ovarian cancer for an in-depth description.

Breast cancer

Although less than 10% of women with primary breast cancer present with clinicopathologic signs of overt metastasis, metastatic relapse occurs in about half of the cases with apparently localized tumors within five years after surgery. At first relapse, bone marrow metastasis are detectable in 23% of patients by conventional diagnostic techniques, and this rate increases up to 80% in necropsy studies of patients with metastatic breast cancer.[60]

An important question was whether the incidence of epithelial antigen-positive cells was correlated to established risk factors, such as lymph node involvement indicating tumor cell dissemination at the regional level. Yet, the results on this interesting issue obtained by various studies are discrepant: some found significant correlations between bone marrow positivity and nodal status,[19,55,61] while others failed to assess such an association[18] or merely found a tendency towards correlations between both parameters.[5,62] A common characteristic of these studies might have been a variation of an important methodological variable, namely the detection antibody: both use of antibodies directed against membrane-bound mucins (for example, TAG-12, EMA) which are known for cross-reactivity with

Table 3 Immunocytochemical studies on the detection of micrometastatic bone marrow involvement in patients with various tumor entities

Tumor origin	*Marker*	*Prognostic value*	*References*
Breast	Mucin	none	12
	CK, EMA	none	14
	EMA	DFS[a]	15, 16
	Mucin	none	17
	EMA, CK	DFS, OS	18
	TAG12	DFS, OS	19
	CK, TAG12	DFS, OS	20
	CK/anti-epithelial	none	21
	CK	DDFS	51
Colon/Rectum	CK	DFS, OS	52
	Ca19-9	not determined	53
Stomach	CK18	DFS, OS	33
	CK18	DFS, OS	54
	CK18	not determined	55
Pancreas	CK/Ca19-9	not determined	53
Esophagus	CK	DFS, OS	56
Lung	CK	DFS, OS	27
Prostate	CK	not determined	57
	CK	not determined	58
	CK/PSA/EMA	not determined	26
Bladder	CK	not determined	57
Kidney	CK	not determined	57
Ovary	CK	DDFS	59

DFS, disease-free survival; OS, overall survival; DDFS, distant disease-free survival, EMA; epithelial membrane, CK; cytokeratins, PSA; prostate specific antigens, TAG; tumor associated glycoproteins.
[a]Prognostic value supported by multivariate analysis.

bone marrow cells,[23,25,63–65] and the monospecific CK2 antibody directed against CK18 which is less sensitive than a broad-spectrum antibody[23] might explain these discrepancies. Applying the broad-spectrum anti-CK monoclonal antibody A45-B/B3 for tumor cell detection we found a significant association of bone marrow micrometastasis with diagnosis of inflammatory breast cancer ($P = 0.006$), distant metastasis ($P < 0.0001$), and extensive lymph node metastasis (≥ 10 nodes involved; $P = 0.009$).[51] An interesting report derived from Fox *et al*[66] described that an assessment of tumor angiogenesis and vascular invasion gives a reliable indication of the probability of the presence of EMA-positive cells in bone marrow from breast cancer patients, and that both processes contribute to metastasis formation.

In order to assess the significance of isolated tumor cells in bone marrow, clinical follow-up studies were initiated. Some of the most recent reports on the immunocytochemical evaluation of bone marrow from breast cancer patients are summarized in Table 3. A follow-up examination of 727 primary breast cancer patients without manifest distant metastasis after a median follow-up time of 36 months (3–108 months) reported that the presence of TAG-12-positive cells identified patients with reduced metastasis-free ($P < 0.001$) and overall survival ($P < 0.001$).[19] The detection of TAG-12-positive cells was described as an independent prognostic indicator for both metastasis-free and overall survival being superior to axillary lymph node status, tumor stage, and tumor grade. Harbeck *et al*[18] confirmed these results which examined bone marrow aspirates from 100 patients with primary breast cancer. Isolated tumor cells were detected in 38% of the patients using a cocktail of monoclonal antibodies to EMA, TAG-12 and CK. After a median follow-up of 34 months (7–64 months) multivariate analysis using the Cox proportional-hazard model revealed that bone marrow positivity was a strong, significant prognostic indicator for relapse-free and overall survival.

The prognostic relevance of tumor cells in bone marrow identified with antibodies directed only against CK proteins has been demonstrated in numerous clinical studies on patients with various forms of epithelial tumors.[20,33,52,55,56] Yet, the only study available for breast cancer has been published by Cote *et al*[20] and was based on the analysis of 49 patients. Using a cocktail of monoclonal antibodies to cell-surface antigens (C26 and T16) and CKs (AE-1), they demonstrated that the tumor burden in bone marrow was an important risk factor.[20] In their analysis of bone marrow cell smears, the number of isolated tumor cells per sample (0 or < 10 v ≥ 10 cells) was the only independent predictor of early recurrence ($P < 0.003$).

Colorectal cancer

In contrast to breast cancer with its propensity for bone metastasis, it may surprise that disseminated tumor cells are detected in bone marrow from patients with tumor entities that rarely form skeletal metastasis, such as colorectal and ovarian cancer (see below). Clinically manifest metastasis are described in 1–4% of cases with colorectal cancer,[67] although this rate increases to 6–12% in autopsy studies.[68,69] While the tendency for dissemination during early stage disease (for example, M_0) was identical to that observed in breast cancer

patients (Table 4), bone marrow positivity was significantly reduced in M_1 colorectal cancer patients as compared to M_1 breast cancer patients.[8] This discrepancy for advanced tumor stages might be explained by a specific growth or survival advantage of breast cancer cells in bone marrow. Besides the interaction of tumor cells with surrounding parenchymal and stromal cells, hemodynamic aspects might play a relevant role for the dissemination of tumor cells. In case of colorectal cancer, tumor cells have to pass the hepatic capillary bed which might enhance the manifestation of liver metastasis.

In their study on 88 patients with colorectal cancer, Lindemann *et al*,[55] found 28 (32%) of cases with disseminated tumor cells in bone marrow. After a median observation time of 35 (12–58) months, patients with a positive bone marrow finding showed a significantly shorter disease-free survival than those without such a finding ($P = 0.008$); multivariate analysis confirmed bone marrow positivity as the strongest independent determinant of relapse (relative risk 2.98; $P = 0.004$). Since bone marrow was not the preferred site of tumor relapse, the detection of disseminated tumor cells was interpreted as the evidence for the disseminative capacity of an individual tumor.[52] This interpretation was supported by the observation that patients with CK-positive cells in bone marrow more frequently succumbed with distant metastasis, predominantly in the liver.

Ovarian cancer

Epithelial ovarian cancer—the major cause of death from malignancies of the gynecological tract in the U.S.[70] as well as in Europe[1]—is characterized by lethal effects of local progression rather than of manifestation of overt distant metastasis. Autopsy studies, however, indicate a considerable frequency of occult hematogenous metastasis at distant sites, such as liver, lungs, bone, and bone marrow.[71,72] These observations already suggested that hematogenous dissemination of malignant cells is more frequent, as recently shown,[59,73,74] than can be expected from the clinically observed pattern of relapses.

In the largest prospective study on ovarian cancer patients, we were able to detect disseminated tumor cells in 28 (30%) of 95 patients, indicating the capacity of the individual tumor for hematogenous dissemination.[59] Interestingly, hematogenous dissemination was identified only in cases with tumors that extended beyond the ovarian parenchyma (stages IcIV). Although no correlation was found between bone marrow positivity and established risk parameters, including tumor grading, residual tumor, increased serum levels of the CA-125 tumor marker, and retroperitoneal lymph node involvement, evaluation of the clinical followup after a median observation time of 18 months revealed a significant correlation with early distant metastasis formation, predominantly in liver and lungs.[59] In multivariate Cox's regression analysis, bone marrow positivity turned out to be the only independent predictor for a distant metastatic relapse.[59] These data challenge the dogma that hematogenous spread of tumor cells is only a prerequisite of advanced tumor stages when tumor cells get access to blood vessels by continuous shedding. Hematogenous tumor cell dissemination was found in a considerable number of patients as early as at the time of first diagnosis of the tumor, and bone marrow appeared to represent a relevant reservoir for viable tumor cells. In view of the increasingly better control of the local tumor growth by aggressive chemotherapy, the detection of disseminated and potentially therapy-resistent tumor residues in bone marrow might emerge as a clinically relevant prognosticator.

Table 4 Frequency of CK18-positive tumor cells in bone marrow of patients with breast and colorectal cancer

Tumor entity	*No. of patients*	*No. of patients with CK18-positive tumor cells*[a]
Breast cancer	**135**	**49 (36.3%)**
M_0	116	35 (30.2%)
M_1	19	14 (73.7%)[b]
Colorectal cancer	**277**	**85 (30.7%)**
M_0	195	53 (27.2%)
M_1	82	32 (39.0%)[c,d]
Controls[e]	**215**	**6 (2.8%)**

[a]Per bone marrow sample 4×10^5 mononucleated cells were stained with antibody CK2.[8]
[b]$P < 0.001$ compared to M_0 breast cancer patients (χ^2 test).
[c]$P = 0.005$ compared to M_1 breast cancer patients (χ^2 test).
[d]$P = 0.05$ compared to M_0 colorectal cancer patients (χ^2 test).
[e]Non-carcinoma control patients.

Monitoring of therapeutic efficacy

The efficacy of adjuvant therapy can thus far be only assessed retrospectively in large scale clinical trials following an observation period of at least five years.

Consequently, progress in this form of therapy is extremely slow and cumbersome and, in addition, therapy is difficult to tailor to the special need of an individual patient. The importance of a surrogate marker assay that would permit the immediate assessment of therapy-induced cytotoxic effects on residual cancer cells is therefore obvious.

The feasibility of follow-up bone marrow aspirations during anti-cancer therapy, has been recently investigated in a prospective study on prostate cancer patients (stage C) treated with androgen-deprivation.[75] The number of tumor cells determined before and after androgen-deprivation was compared to the standard serum tumor marker prostate-specific antigen (PSA), and the clinical follow-up. After androgen-deprivation 20 out of 21 previously CK-positive bone marrow aspirates revealed a reduced or no detectable tumor cell load. Since seven patients with persistently high tumor cell counts had no detectable serum-PSA titers, the study further demonstrated that serum-PSA is an unsuitable marker to indicate the presence of disseminated tumor cells, and, therefore, permits no conclusions on the therapeutic elimination of tumor cells under androgen-deprivation.[75]

Monoclonal antibodies respresent another promising therapeutic option for the specific treatment of cancer residues.[76] To maintain the efficacy of this tumor-specific approach, it will be expedient to determine the individual expression pattern of tumor-associated cell-surface targets on disseminated tumor cells,[77] since this pattern may be rather heterogeneous due to the known genetic instability of epithelial cancers. Using double marker immunoassays together with the choice of appropriate tumor-specific targets, it may become possible to establish a surrogate assay for therapeutic efficacy as demonstrated by the specific elimination of target-positive tumor cells. In a pilot study on eight breast cancer patients with advanced tumor stages,[78] we have been able to show the feasibility of such an approach. Follow-up bone marrow aspirations before and after the administration of a single dose of 500 mg edrecolomab (17-1A antibody) revealed both the reduction of CK-positive and EpCAM-positive/CK-positive tumor cells in all cases examined. To exclude the possibility of any antitumor activity other than that evoked by the applied antibody, we both determined the tumor cell number after 5–7 d post treatment and excluded patients with concomitant antitumor treatment. Therefore, the notion is likely that the observed reduction or eradication of CK-positive cells was an effect of the infused antibody.

In another pilot study by Schlimok *et al*[79] 40 patients with breast and colorectal cancer were treated in a randomized fashion with 6×100 mg antibody ABL 364 which is directed to the Lewis Y (Le^Y) blood group precursor carbohydrate antigen[80] *vs* placebo infusion; CK-positive cells in bone marrow were monitored on day 15 and 60 after initiation of treatment. Even in patients with an extremely low number of CK-positive cells ($1-11/4\times10^5$ MNC), a tendency for reduction of CK-positive cells was seen after antibody therapy. Significant data, however, were only obtained from the ten breast cancer patients who displayed an initial cell count of more than 20 CK-positive cells per 4×10^5 MNC. Of the seven patients treated with antibody, five showed a distinct reduction or eradication of CK-positive/Le^Y-positive cells (96–100%), while in two patients with CK-positive but Le^Y-negative cells no response was registered. Similarly, in the three patients receiving human serum albumin no significant tumor cell reduction was observed. Because of the marked antibody dependent cellular cytotoxicity and complement dependent cytotoxicity that the antibody ABL 364 exhibits in *ex vivo* experiments with serum of treated patients,[80] Schlimok *et al*[79] postulated that the observed disappearance of tumor cells from bone marrow is due to the action of the administered antibody.

Despite the preliminary character of these studies, they exemplify a new approach towards a more rational selection of antibodies for adjuvant studies in minimal residual disease. The proposed use of CK-positive cells as surrogate markers for the prediction of therapeutic response may benefit from the recent improvements of the cytokeratin assay[25] which allows a more precise quantitation of the individual tumor load. Clinical studies are now required to evaluate whether the eradication of CK-positive cells translates into a longer disease-free and overall survival. Availability of such a surrogate marker would considerably enhance our abilities to rationally design new therapies directed towards minimal residual disease.

Current cytotoxic chemotherapy regimens might fail to eliminate dormant, non-proliferating tumor cells, which may explain metastatic relapse even after high-dose chemotherapy. Two pilot studies on breast cancer patients undergoing either ifosfamide-carboplatin-

epirubicin ($n = 18$) or vinblastin-ifosfamide-carboplatin ($n = 10$) high-dose (HD) chemotherapy with autologous stem cell transplantation described the presence of CK-positive cells in 15 (83%) and 3 (30%) bone marrow specimens obtained after completion of treatment with the majority of patients being in complete remission.[81,82] Therefore, complementary strategies, such as antibody-based immunotherapy, need to be considered. Hempel *et al*[81] who offered additional 17-1A antibody (edrecolomab) therapy to patients with disseminated CK-positive cells resistent to HD chemotherapy succeeded to eliminate these cells, and avoid early metastatic relapse in 2 out of 3 individuals. Interestingly, residual CK-positive cells in both patients yielded coexpression of EpCAM, while the respective cells of the third patient were EpCAM-negative.[81]

Baselga *et al*[10] and, more recently, Slamon *et al*[9] reported on clinical trials demonstrating the successful treatment of metastatic breast cancer with a humanized monoclonal antibody directed against the $p185^{erb\text{-}B2}$ growth factor receptor in combination with chemotherapy. The importance of these trials is that they are among the first studies in breast cancer patients displaying a biological effect of unconjugated recombinant antibody against established solid tumors. However, the relatively low objective response rates clearly pointed out that other aspects need to be taken into account. Jain *et al*[83] previously demonstrated that the relatively high intratumoral oncotic pressure represents a physiological barrier to deliver monoclonal antibodies and other macromolecules to solid tumors. Therefore, it is clear that a major consideration for the successful application of antibody therapy is the choice of the appropriate disease stage in which the tumor cells are accessible for intravenously administered immunoglobulins.[84]

In addition, the well known genomic instability of neoplastic cells may lead to a considerable heterogeneity in the expression pattern of potential immunotherapeutic target antigens.[76] In a pilot study, we recently investigated the pattern of tumor-associated antigens, including EpCAM and $p185^{erb\text{-}B2}$, expressed on bone marrow micrometasases in breast cancer patients.[77] Our analysis revealed that despite a relatively high incidence of antigen coexpression, the number of cells with antigen coexpression per total number of detectable tumor cells varied considerably, indicating a heterogenous expression pattern of the investigated antigens. To cope with this antigen heterogeneity a combination of antibodies directed to independently expressed antigens should be more efficient than a single agent.[77] Since considerable recent progress achieved translation of antibody-based immunological therapies from the laboratory to the clinic, the adjuvant trials initiated have supported the potential of the selective targeting approach for cancer therapy.[85] In this context, the possibility to perform follow-up bone marrow aspirations may facilitate the monitoring of the therapeutic efficacy against residual tumor cells.

Concluding remarks

Various immunocytochemical and molecular methods have been applied to detect disseminated carcinoma cells in mesenchymal organs, especially such as bone marrow. At present, we feel the need that international concerted activities rather than meta-analysis of extremely heterogeneous sets of data[86] are now required to develop standardized procedures that may then serve also as a gold standard for other diagnostic approaches, such as PCR-based methods.

Thus far, the biology of bone marrow micrometastases has remained poorly understood. This ignorance has been particularly disturbing in patients that remain free of cancer relapse despite the presence of tumor cells at the time of diagnosis. Our present results indicate that CK-positive micrometastatic tumor cells represent a dormant and selected population of cancer cells which, however, still express a considerable degree of heterogeneity.[8,77] With the development of new techniques like single-cell PCR and the *in vitro* expansion of micrometastatic cells,[87] it becomes possible to determine the characteristic genotypic features of those cells.[88,89]

The outlined current strategies for detection and characterization of cancer micrometastasis might help to design and control new therapeutic strategies for secondary prevention of metastatic relapse in patients with operable primary carcinomas. Minimal residual disease offers the advantage of a small burden of dispersed tumor cells which are more accessible to intravenously applied drugs than gross metastasis. In view of the dormant nature of micrometastatic cells in bone marrow,[8] therapies that are also directed against quiescent cells, such as antibody-based immunotherapy, might be complementary to chemotherapy.

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