Spectrum of Cytokeratin-positive Cells in the Bone Marrows of Colorectal Carcinoma Patients

JAYANT D. SHETYE1, MARIA L. LILJEFORS1, STEFAN O. EMILIAN2, JAN-ERIK FRÖDIN1, KARIN STRIGARD2, HAKAN T. MELLSTEDT1 and ANJA PORWIT1

1Department of Oncology/Pathology (Radihemmet), Karolinska, University Hospital Solna, SE-171 76 Stockholm; 2Department of Surgery, Norrlands University Hospital, SE–901 85 Umeå; and 3Department of Surgery, Karolinska University Hospital Huddinge; SE–141 86 Stockholm, Sweden

Abstract. Background: Bone marrow micrometastases (BMM) is considered to be of interest as a prognostic marker in solid tumors. The use of density-gradient separated bone marrow (BM) aspirates does not allow proper morphological characterization of the cells. An alternative approach, using routinely processed clots of BM aspirates, is presented. Materials and Methods: BM clots from 56 colorectal carcinoma patients were stained for cytokeratin (CK), p53 and Ki67 by double immunohistochemistry. Cytokeratin-positive (CK+) cells were immunohistochemically divided into three groups, viz. Group A (CK+ probably malignant epithelial cells), Group B (CK+ morphologically non-epithelial cells) and Group C (CK+ contaminating cells). Results: Thirty-three patients (59%) had CK+ cells, of which 19 (58%) had Group A cells and 14 (42%) had Group B cells. Fourteen of the 56 patients had reactive BM, eight of these had Group A cells and 3 had Group B cells. Group B cells and Group C cells did not express p53. Group A cells were noted in 35% of patients with carcinomas of Dukes’ stage C and in 41% of patients with metastatic disease. Conclusion: Double immunohistochemical staining of routinely processed BM clot, for p53 and Ki67 along with CK, allows the sub-classification of CK+ cells.

Sloane et al. (1) were the first to describe bone marrow (BM) micrometastases (BMM) in patients with primary breast carcinoma. The phenomenon was defined as metastases seen at microscopic level, i.e. not overtly present. The definition was based on the expression of cytokeratin (CK) or other epithelial markers in non-haematogenous cells of the BM. Subsequently, there have been several reports describing BMM in patients with carcinoma of the prostate, lung, stomach, pancreas, colon and squamous cell carcinomas of the head and neck region (2-6).

The clinical significance of BMM is not well established, particularly as the incidence of BMM does not seem to reflect the incidence of overt BM metastases in solid tumors (7-9). Moreover, the malignant propensity of cytokeratin-positive (CK+) cells in BM is also unclear. Only 4-8% of the colorectal carcinoma patients have overt BM metastases, while BM micrometastases have been reported in 12-60% of colorectal carcinoma patients (10-12).

Apart from a few reports, using flow cytometry and RT-PCR methods (13, 14), most investigators have identified epithelial cells on cyospins from BM aspirates enriched for mononuclear cells (MNC) (2, 5, 6, 9, 11, 12, 15, 16). Initially, cyospins were immunostained with monoclonal antibody (Mab) against epithelial membrane antigen (EMA) (3). As EMA was found to be non-specific and to circumvent the unpredictability of detecting tumor cells by using various tumor-associated antigens, the use of Mabs against CKs has become the accepted method for detection of BMM (17, 18). Density gradient separation of BM aspirates for enrichment of epithelial cells is cumbersome and tumor cells with a density different from MNC may be lost. Up to 60% of MNC have been reported to be lost during the gradient separation of BM (19). The morphology of CK+ cells may also be distorted due to centrifugation. Furthermore, the intercellular relationship and distribution of cells in the BM cannot be assessed. The advantage offered by the flow cytometric analyses of CK+ cells, which is also carried out on density gradient-separated BM, is a rapid enumeration of the CK+ cells, while the absence of morphological assessment and lack of sensitivity are a disadvantage (20).

To increase the sensitivity of detecting BMM, PCR-based methods have been tried. However, the main disadvantage of these methods is the difficulty in discriminating between the various types of CK+ cells possibly present in the BM.

Correspondence to: Jayant Shetye, Cancer Center Karolinska, R08:01, Karolinska Hospital, Stockholm, SE-171 76 Sweden. Tel: 46-8-51774308, Fax: 46-8-318327, e-mail: jayant.shetye @ckk.ki.se

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Furthermore, cytokeratin 18 pseudogene in BM cells of healthy donors has been demonstrated and the expression of CK in non-epithelial cells has also been shown (13, 21-23). Funaki et al. (23) reported a sensitive RT-PCR assay for the detection of cytokeratin 20 in peripheral blood cells of colorectal carcinoma patients. However, a higher detection rate of CK 20+ cells was seen in the BM than in peripheral blood in another study (24).

To improve the evaluation of epithelial tumor cells in the BM, we used immunohistochemistry on sections of routinely processed, formalin-fixed BM clots, which preserves the intercellular relationship and the cytomorphology of BM cells including CK+ cells. In addition, to assess a possible malignant predilection (i.e. expression of p53) and the proliferative propensity (i.e. expression of Ki67) of CK+ cells, we used a sequential double immunohistochemistry technique.

Materials and Methods

Patients. Fifty-six patients with resected colorectal carcinoma were included in the study. Thirty-four patients had no overt evidence of disease (NED) at the time of BM aspiration while twenty-two patients had distant metastases, i.e. advanced disease (AD). BM aspiration was performed between one to three months after resection of the primary tumor and before administration of medical treatment.

Preparation of bone marrow clot. With the aid of a 16-GA, disposable 'T' type, BM aspiration needle (MANAN Medical Products Inc., Northbrook, IL, USA), 5 ml of BM was aspirated under local anaesthetic (Xylocaine, AstraZeneca, Södertälje, Sweden) from bilateral posterior superior iliac spines. The BM aspirate was layered along the circumference of a concave viewing glass. When the blood had flowed towards the centre of the dish, BM fragments left along the circumference of the glass were collected together to form a clot. If a prolonged clotting time was observed, a few granules of coagulant (Topostasin, Roche, Basel, Switzerland) were added. The clot was then fixed in 4% neutral buffered formalin for 4 h and processed for paraffin embedding. Only rarely, the BM aspirates contained a few, tiny bone fragments which did not require decalcification.

Routine staining. Eight-μ sections were deparaffinized and rehydrated in decreasing grades of alcohol, then stained routinely with H&E, Prussian blue, Gordon Sweet (silver impregnation of reticulin) and periodic acid Shiff's reagent (PAS), respectively.

Immunohistochemistry. For detection of p53 and Ki67, a peroxidase-labeled avidin-biotin complex (ABC) method was performed with an elite ABC kit (Vector Lab., Burlingame, CA, USA) (step 1) followed by an alkaline phosphatase-labeled streptavidin-biotin complex (Dakopatts, Copenhagen, Denmark) staining of CK (step 2) (25). A minimum of five randomly selected 8-μ-thick sections on superfrost glass, from a series of 15 consecutive sections, from each paraffin block were deparaffinized, rehydrated and unimmunostained, three out of 5 sections for p53 and two out of 5 sections for Ki67. Before immunostaining, the sections were boiled in citrate buffer (pH 6) in a microwave oven (Philips 602, 900 Watts) (26). For Mab p53 (Do 1, wild-type p53), (1:100), (Santa Cruz Biotechnology, CA, USA), 3 sections were boiled for 5 min and for Mab MIB-1 (Ki67), (1:150), (a gift from Dr Johannes Gredes, Borstel Institute of Research, Borstel, Germany), 2 sections were boiled for 7 min. During the boiling procedure, when necessary, the Hellendahl’s staining jars were topped up with distilled water. After cooling (15 min), the sections were washed in TBS (3x5 min). Endogenous peroxidase was inhibited by incubating with 3% H2O2 in distilled water (1:3) overnight at room temperature in a dark cupboard. The sections were then washed in TBS (3x5 min) and incubated with a blocking serum (normal horse serum) (30min). Excess serum was drained off and the sections were incubated with the primary MAb (p53 or MIB-1) at 4°C, overnight. After washing (3x10min), the sections were incubated at room temperature with biotinylated antimouse IgG (Vector Lab.) followed by incubation (1h) with prediluted and premixed ABC elite complex (1 h) at room temperature with washings (TBS) (3x5 min) in between. Bound peroxidase was visualised after heavy metal (cobalt) intensification of the DAB/H2O2 reaction (27). Following the DAB reaction (step 1), the slides were washed in TBS (3x5 min). Subsequently (step 2), the same procedure as described above was repeated with the anti-pancytokeratin Mab AE1/AE3 (1:100) (Boehringer Mannheim), except that an alkaline phosphatase conjugated streptavidin biotin complex (1h) (Dakopatts) was used as a chromogen. The bound alkaline phosphatase was visualised using the substrate kit Vector Red (Vector Lab.). The sections were then rinsed in TBS and washed in running tap water, lightly counterstained with Mayer’s Haematoxylin, air dried and mounted directly in Mountex (Histolab Products AB, Gothenburg, Sweden).

Controls

a.) Tissue controls. Positive control: Immunohistochemically-proven p53+ and MIB-1+ colorectal carcinoma with adjacent normal mucosa as well as overt BM metastases from patients with colorectal carcinoma and breast carcinoma were used. In addition, the haematopoietic cells of the test marrow served as internal positive control for Mab MIB-1.

Negative control: Reactive BM from patients with non-neoplastic disease (n=8) and BM infiltrated with various lymphoproliferative diseases (n=7) as well as normal BM from patients undergoing thoracotomy (n=5) were used. The megakaryocytes and the megakaryoblasts of the test marrow served as an internal negative control for Mab AE1/AE3 (CK).

b.) Controls for the staining procedure. Six controls for the staining procedure were used. Two positive controls for Mabs p53 and MIB-1 were used in the peroxidase conjugated ABC elite method (step1) followed by Mab AE1/AE3 used in the alkaline phosphatase conjugated streptavidin biotin method (step2) and 4 negative controls (28). In addition, contaminating desquamated superficial squamous cells served as an internal control for the alkaline phosphatase staining of CK.

Evaluation of immunohistochemistry. Cytokeratin positivity in the cells was inferred from the alkaline phosphatase positive pink color in the cytoplasm while p53 and Ki67 positivity were construed from the black color of the nucleus. The aim was to get at least one of each CK+ and p53+- or CK-+ and Ki67-+ positive sections. In the
A. Sub-classification of CK+ cells

1. A probably proliferating mesenchymal cell was diagnosed as a types were seen in groups (Table I).

Four distinct types of cells could be identified. None of these cell

I) Group A Cells (CK+ probably malignant epithelial cells)

if both

BM expressed either p53 or Ki67 in conjunction with CK, then a

eventuality of a reactive marrow or marrow showing alkaline

phosphatase-positive cellular debris not showing any CK+ cells,

then an extra set of five sections were stained before accepting a

negative result.

The pattern of cytoplasmic expression of CK in the cells of the

BM was made the basis for a sub-classification of CK of positive

cells (Table I). Primarily three patterns of CK expression were

seen: i) diffuse web-like, ii) diffuse homogenous and iii) punctate
dot-like or globular in the Golgi area. Diffuse web-like expression

of CK, characteristically seen in the desquamated squamous cells

(Figure 1), was raison d’être for classifying cells as epithelial cells.

If a cell expressed any CK pattern, the reactivity of the same

cells against Mabs p53 and Ki67 was evaluated. In single- or
double-stained cells, the size and shape of the CK+ cell, as well as

the nuclear to cytoplasmic (N:C) ratio, and presence of nucleolus,

were considered for the classification of the CK+ cells. If cells in a

BM expressed either p53 or Ki67 in conjunction with CK, then a

few more sections were immunostained in order to detect if both

the phenotypes (CK+ p53+ and CK+ Ki67+) were present.

A. Sub-classification of CK+ cells

I) Group A Cells (CK+ probably malignant epithelial cells)

Cells with features consistent with malignant cells had a

characteristic diffuse web-like cytoplasmic expression of CK and

expressed in their nuclei either p53+ or Ki67+ (Table I). These

cells were large (approx. 15-20 μ), polygonal-shaped with a high

N:C ratio, and with an occasional prominent nucleolus or a mitotic

figure. (Figure 2, Figure 3). When a CK+ cell had a p53+ nucleus,

the CK was stained weakly. In a few cases (n=10), cells similar to

the above with p53+ as well as Ki67+ nuclei were present in

consecutive sections but not in the same cell. These cells were seen

in groups of 3-4 and were considered as a probable group of cancer

cells (Figure 4).

II) Group B cells (CK+ non-epithelial cells)

Four distinct types of cells could be identified. None of these cell
types were seen in groups (Table I).

1. A probably proliferating mesenchymal cell was diagnosed as a

cell showing a diffuse homogenous cytoplasmic expression of

CK, the size was similar to that of haematopoietic cells, polygonal,

high or normal N:C ratio, Ki67+ and p 53- condensed

nucleus with no nucleoli (29) (Figure 5).

2. A cell with similar features to the above but with p53- and Ki67-
nucleus was diagnosed as a probably non-proliferating

mesenchymal cell (Figure 6).

3. A spindle-shaped cell, probably a smooth muscle cell or an

endothelial cell, was diagnosed when the size of the cell was

similar to or smaller than the haematopoietic BM cells and with a

diffuse homogenous cytoplasmic expression of CK, Ki67+ and p53-
nucleus, normal N:C ratio and no nucleoli (30-32) (Figure 7).

4. A probable neuroendocrine cell was a distinct cell with expression

of CK either in a punctate dot-like or globular pattern adjacent to

the nucleus, a p53+ and Ki67+ nuclei with a normal N: C ratio

and a finely granular chromatix (33) (Figure 8).

III) Group C (CK+ contaminating cells)

Two types of CK+, p53- and Ki67- contaminating cells, probably

introduced by the aspiration procedure, were identified (Table I). Cells

of this type were disregarded when enumerating the frequency of BMM.

1. Desquamated superficial squamous epithelial cells from the skin

were larger than megakaryocytes and showed the characteristic

web-like diffuse expression of CK associated with epithelial cells.

The cytoplasm was occasionally thrown in folds with an

eosinophilic punctate nucleus (Figure 1).

2. Dermal sweat gland cells appeared as a synciatial mass of cells

with a web-like diffuse expression of CK and a normal N:C ratio,

with a vesicular nucleus and no mitoses (Figure 9).

Plasma cells. Plasma cells showed a diffuse homogenous/vacuolated

CK expression and Ki67 as well as p53+ nucleus, with an occasional

cartwheel pattern of nuclear chromatin (34) (Figure 10). Such CK+

plasma cells were seen throughout the various processed test and

control marrows and were disregarded while enumerating the

frequency of all types of CK+ cells and therefore not included

when referring to CK+ non-epithelial cells.

Results

The evaluation of slides for routine histopathology and

immunohistochemistry was done blinded with regard to the

clinical status of the patients. All the H&E- and

immunohistochemistry-stained slides were independently

reviewed by two pathologists (JDS, AP).

Routine histopathology. On H&E-stained sections, the BMs

were either hypercellular or normocellular. There was no

morphological evidence of abnormal cells. Haematopoietic

cells were morphologically unremarkable and eosinophilia

was not observed in any patient. Except for plasma cells and

macrophages, no other PAS-positive cells were noticed. The

reticulin pattern was unremarkable and the iron content was

within normal limits. BMs from 6 out of 32 patients in the

NED group and from 8 out of 18 patients in the AD group

had an activated myelopoiesis with small lymphoid follicles

and occasionally mild diffuse infiltration of lymphocytes

(reactive changes) (Figure 11). Eleven of the 14 patients

with a reactive BM had CK+ cells in their BM (5 patients

in the NED group and 6 patients in the AD group). Eight of

these 11 patients had CK+ cells (type A) with a malignant

phenotype (CK+ cells expressing p53+ and/or Ki67+ and an

atyypical cellular morphology).
Figure 1. The distinctive diffuse web-like expression of cytokeratin (CK), characteristically seen in a desquamated squamous epithelial cell (Group C) BM clot double-stained with Mab AE1/AE3 and Mab p53. (x100)

Figure 2. A Group A cell double-stained with Mab AE1/AE3 and Mab p53. Note the cell has an abnormal nucleus and a high nuclear:cytoplasmic (N:C) ratio. The cytoplasm shows a web-like diffuse expression of cytokeratin (CK) and the nucleus appears black. The adjacent haematopoietic cells are negative for p53. (x100)

Figure 3. BM clot double-stained with Mab AE1/AE3 and Mab MIB-1. Note a Group A cell along with MIB-1-positive haematopoietic cells. The adjacent haematopoietic cells, which are Ki67+, served as an internal control. (x100)

Figure 4. A group of abnormal cells (Group A) not detected in an H&E-stained slide was unveiled by double-staining with Mab AE1/AE3 and Mab p53. Note the abnormal N:C ratio (arrow) and the presence of nucleoli in one of the abnormal cells (arrow head). (x100)

Figure 5. A double-stained cell (Mab AE1/AE3 and Mab MIB-1) with a normal N:C ratio, probably a proliferating mesenchymal (Group B) cell. (x100)

Figure 6. A polygonal CK+ cell with a p53-negative nucleus, but normal N:C ratio and normal nuclear characteristics (arrow), probably a non-proliferating mesenchymal cell of Group B type. Note an adjacent, lightly-stained CK+ desquamated squamous epithelial cell of Group C type. (arrowhead). (x100)
Figure 7. Mab p53- and Mab AE1/AE3- stained BM clot demonstrating CK+ spindle-shaped (Group B) cell with a diffuse homogenous expression of CK+. (x100)

Figure 8. BM clot double-stained with Mab MIB-1 and Mab AE1/AE3. Note the globular pattern of CK positivity adjacent to the Ki67+ nucleus with a finely granular chromatin of probably a neuroendocrine (Group B) cell. The nuclei of the haematopoetic cells are black (Mab MIB-1 positive). (x100)

Figure 9. CK+ Ki67-, syncytial mass of cells, probably from the sweat gland of a patient's skin. Note the Ki67+ haematopoetic cells of the marrow. (x140)

Figure 10. Mab MIB-1- and Mab AE1/AE3-stained BM clot. A binucleated plasma cell showing a vacuolated, diffuse CK+ positivity with a Ki67-negative nucleus. While the haematopoetic cells are Ki67+ (x100)

Figure 11. MIB-1-stained BM clot demonstrating a hypercellular marrow with a lymphoid follicle, a reactive BM. (x20)

Figure 12. A hypercellular BM clot showing the presence of alkaline phosphatase-stained cellular debris in a slide double-stained with Mab AE1/AE3 and Mab p53. (x40)
A) Immunohistochemistry of BMs with Groups A, B and C CK+ cells. Thirty-three of the 56 patients (59%) had Group A and Group B cells in their BM. Twenty of the 33 patients (61%) were from the NED group and 13 out of 22 (59%) patients were from the AD group. Group A cells. Nineteen out of the 56 patients (34%) had Group A cells, 10 in the NED group and 9 in the AD group (Table II). Nine of the 10 patients from the NED group had Dukes’ stage C while one patient had a Dukes’ stage B tumor. Only one out of 10 patients had a Ki67+ nucleus (Table III). None of the 4 patients in the AD group had a Ki67+ nucleus in the CK+ cells (Table III).

Group C cells. Thirty-two out of the total 56 patients had superficial squamous epithelial cells and one patient had a group of sweat gland cells along with a neuroendocrine cell.

B) Immunohistochemistry of BM without cytokeratin-positive cells. Twenty-three out of 56 marrows did not have any Group A and Group B cells in the BM. However, an occasional plasma cell was positive for CK in 5 out of 23 patients. In contrast to marrows with Group A cells, the marrows in these patients did not show any alkaline phosphatase-stained cellular debris.

C) Immunohistochemistry of controls BMs. Apart from an occasional plasma cell stained in 15 of the 20 BM aspirates used as controls (non-neoplastic reactive, lymphoproliferative diseases and normal BMs), no other cell type stained for CK and no alkaline phosphatase reactive cellular debris was observed in these cases.

Discussion

To our knowledge, this is the first documented study of routinely processed, formalin-fixed clots of BM aspirates in
a sequential double-immunostaining for simultaneous detection of CK, p53 and Ki67. The clear advantage of this method is the preservation of the cytological morphology with a preserved intercellular relationship of marrow cells. The main disadvantages of the method are the loss of histological morphology and the more extended time required to stain and evaluate the sections. However, the increased use of automated cellular imaging systems should considerably shorten the time required to enumerate CK+ cells and increase the reproducibility of quantifying the tumor burden in the BM (35).

To increase the specificity of CK+ cells in the marrow, Risenberg et al. used prostate specific antigen (PSA) together with CK on BM aspirates in patients with prostatic carcinoma (36). Pantel et al. used CK and leukocyte common antigen (CD45) in a double-staining technique to identify false CK+ cells (37). To improve the characterization of CK+ cells, we studied the simultaneous expression of p53 or Ki67 in addition to CK. This allowed the discrimination of various types of CK+ cells, conferring an advantage compared to single staining for CK. Furthermore, contrary to what has hitherto been reported by Schlimok et al. (38), the morphology of the CK+ cells in the clots was characteristic and enabled us to divide these cells into three distinct groups. Group A cells (CK+ probably malignant epithelial cells) most probably represent BMM and most probably are of clinical importance. The significance of Group B cells (CK+ non-epithelial cells) has yet to be established, while Group C cells (CK+ contaminating cells) are without any clinicopathological importance. In patients with Group A cells in the BM, alkaline phosphatase-stained cellular debris was occasionally noted in sections. This suggests ongoing necrosis of cells of Group A type.

Previously in the literature, a sine qua non of BMM in cancer patients was the immunohistochemical evidence of CK+ cells. This has resulted in a discrepancy between the higher incidence of BMM compared to overt BM metastases, particularly in colorectal carcinoma, and various theories have been advocated to explain this discrepancy (39, 16, 40). Except for reports of CK+ plasma cells in BMs, so far no studies have focused on CK+ cells with a non-epithelial cytology (Group B cells) in marrows. This is probably due to the common use of density gradient separation of marrow and centrifugation-induced distortion of the morphology (cytospin preparations) along with the absence of pre-fixation, which makes it difficult to delineate the various types of CK+ cells. Enumeration of CK+ non-epithelial cells (Group B+ cells) presumably contributes to the previously reported high incidence of BMM as compared to overt BM metastases.

The expression of CK in cells other than epithelial cells has been well-documented in normal tissues and in tissues from inflammatory and neoplastic diseases (29-34). In vitro, conversion of mesenchymal cells to epithelial cells has also been observed (40, 41). They further showed that epithelial cancer cells can transform to various other cell types and that this is associated with the maintenance of CK expression. However, the pattern of CK expression in these morphologically-transformed cells was diffuse homogeneous contrary to the diffuse web-like seen in an epithelial cancer cell (41, 42). Our study is the first emphasizing the presence of CK+ non-epithelial (Group B) cells in the BM of cancer patients. Such B-type of cells was observed together with CK+ epithelial (type A) cells in three out of 56 marrows examined. In two other patients, sequential marrow biopsies showed initially only CK+ epithelial (type A) cells while later only CK+ non-epithelial (type B) cells were seen. In two other patients, first only CK+ non-epithelial (type B) cells were recorded while later only CK+ epithelial (type A) cells were seen. Furthermore, control (non-cancer) BMs did not show the presence of CK+ epithelial (type A) cells or CK+ non-epithelial (type B) cells, suggesting that Group B did not develop in BMs without cancer and that these Group B cells may have been derived from epithelial cancer (GroupA) cells. These results might reflect the in vitro observations of Boyer et al. and Boyer and Thiery (41, 42).

Most previous investigators have presented results exclusively as CK+ cells in the BMs of various types of cancer patients. To compare our findings with those reported in the literature, we should evaluate Group A and Group B cells as one group. Thus, 33 out of 56 patients (59%) had Group A and Group B cells in their BMs. These results are comparable to those reported by Broll et al. where 50-60 % of colorectal carcinoma patients had BMM (11). However, if strict immunocyto-morphological criteria (excluding the Group B cells) are used, only 19 out of 56 (35%) colorectal carcinoma patients in our study had Group A cells and thus have BMM. This incidence is similar to that of overt BM metastases in a large autopsy study reported by Wises et al. (43). However, the ante-mortem incidence of overt BM metastases in colorectal carcinoma is only 4-8% (10), which is a figure closer to the frequency of our patients, 8 out of 56 (14%), with Group A and a simultaneously reactive BM.

On routine H&E staining, there were no signs of gland formations, nor were there any tell-tale signs of metastases i.e. no necrosis or eosinophilia was observed even after extensive deeper sectioning for immunohistochemistry. Although "consecutive" sections were selected for staining, the choice of individual sections used for staining of Mab CK and Ki 76/p53 was randomly made. On an average only 3 out of 5 sections from each BM clot demonstrated CK+ cells. Furthermore, only rarely could we identify the same cell in adjacent sections. Therefore, confirmatory staining on the Group B cells was not feasible. Moreover, the numbers of CK+
cells, of both Group A and Group B type, are usually so low precluding microdissecting these cells for further assays.

Primary tumors from our patients were not stained for p53 and Ki67, since it was demonstrated in a study by Maehara et al. that the expression of p53 and Ki67 in the primary tumor did not correlate with the presence or absence of CK+ cells in the marrow (44). The discordance between p53 and Ki67 expression in our study is possibly due to limitations of the staining protocol. Random sections were selected for staining of p53 (n=3) and Ki67 (n=2), which could account for the discrepancy. Another explanation is the possibility of dissociation between the expression of Ki67 and p53, as demonstrated by Kressner et al. in colorectal carcinomas (45).

In summary, the cellular morphology of BM is well preserved in BM clots and the double-immunohistochemical staining technique allows discrimination of various types of CK+ cells including CK+ non-epithelial (Group B) cells, which probably account for the previously reported high incidence of BMM. The presence of CK+ epithelial cells together with a reactive BM appears to be an indication of incipient overt metastasis but not necessarily of osteolytic metastases. Thus, in the present study, only two out of eight patients with a reactive marrow have developed overt BM metastases after a follow-up period of 3 years (unpublished data). Furthermore, the presence of Group A cells in BM may represent dissemination of the disease but the clinical significance remains to be evaluated. However, an analysis of BMM by double-immunostains is warranted in monitoring the course of the disease and for early evaluation of the effect of adjuvant treatment.

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The study was approved by the Ethical Committee of the Karolinska Hospital and Karolinska Institute.

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