Clinical Significance of Immunocytochemical Detection of Tumor Cells Using Digital Microscopy in Peripheral Blood and Bone Marrow of Breast Cancer Patients

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ABSTRACT

Purpose: **The presence of tumor cells in bone marrow has been reported to represent an important prognostic indicator in breast cancer, but the clinical significance of circulating cells in peripheral blood is less well known. The aim of this study was to evaluate the feasibility of identifying cytokeratin (CK)-expressing cells in peripheral blood with an automat-assisted immunohistochemical detection system and to compare it with detection of tumor cells in bone marrow samples.**

Experimental Design: **Cytospun Ficoll fractions of peripheral blood and bone marrow were obtained simultaneously in 114 breast cancer patients at different stages of the disease (I to IV) before treatment with chemotherapy. The pancytokeratin (CK) monoclonal antibody A45-B/B3 (anti-CKs 8, 18, and 19) was used for epithelial cell detection. Immunostained cells were detected by an automated cellular imaging system (ChromaVision Medical System).**

Results: **CK+ cells were detected in 28 (24.5%) patients in blood and in 67 (59%) patients in bone marrow. Twenty-six (93%) patients with CK-positive cells in blood also had positive bone marrow ($P < 0.001$). Positive cells were detected in peripheral blood in 3/39 (7.5%) operable breast cancers (stage I/II), 9 of 36 (25%) locally advanced breast cancers (stage III), and 16 of 39 (41%) patients with metastatic disease (stage IV; $P = 0.017$). In the subgroup of nonmetastatic patients ($n = 75$), prognostic factors for poor disease-free survival were: absence of estrogen receptor; presence of CK+ cells in bone marrow ($P = 0.012$); clinical nodal involvement; large tumor size (T4); and presence of tumor emboli. Presence of circulating CK+ cells in the peripheral blood was not statistically correlated with disease-free survival. On multivariate analysis, independent indicators for disease-free survival were: absence of estrogen receptor ($P = 0.043$) and presence of CK+ cells in bone marrow ($P = 0.076$).**

Conclusions: **The clinical relevance of circulating epithelial cells as a prognostic factor is not supported by the present data, especially in comparison with tumor cells in the bone marrow. However, this method of detection may be useful to monitor the efficacy of treatment in advanced or metastatic breast cancer.**

INTRODUCTION

At the time of primary diagnosis of breast carcinoma, several clinicopathological parameters such as tumor size, involvement of axillary lymph nodes, tumor grade, and hormone receptor status determine the prognosis and indications of adjuvant systemic treatment (1, 2). Breast cancer is considered to be a systemic disease because early tumor cell dissemination may occur even with small tumors. The survival benefit associated with adjuvant treatment almost certainly results from the eradication of preexisting micrometastatic disease (3, 4). Several investigators have shown that epithelial cells can be identified in bone marrow aspirates or peripheral blood of otherwise metastasis-free patients (5, 6). Immunocytochemical (ICC) detection of epithelial cells in bone marrow (BM) of breast cancer patients has been reported with various antibodies directed against epithelial membrane antigen (7) or other cellular mucins, including tumor-associated glycoproteine-12 (8). The specificity and clinical relevance of the markers used to characterize epithelial cells remain controversial as they have been shown to cross-react with hematopoietic cells (9). Large prospective studies using a more specific antibody directed against CKs have demonstrated the clinical importance of occult tumor cells in BM representing an independent predictive and prognostic factor for distant relapse and overall survival in nonmetastatic breast cancer patients (10–12). The confirmed predictive and prognostic value of occult tumor cells in BM, independent of axillary nodal status, may modify individual treatment decisions (13). The presence of tumor cells after adjuvant chemotherapy was associated with poor prognostic and BM monitoring could help predict the response to systemic treatment (14). However, repeated and frequent BM aspirations looking for occult tumor cells may not be easily accepted by breast cancer patients, especially when they are in clinical remission. Because of the acceptability of repeated sampling, blood-borne tumor cells would constitute an alternative to BM surveillance for longitudinal investigations. In contrast with BM, the prognostic value of detection of tumor cells in peripheral blood has not been

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clearly established. Recent studies using reverse transcription-PCR (RT-PCR) demonstrated that detection of circulating CK 19 mRNA-positive cells in peripheral blood of operable breast cancer patients before or after adjuvant therapy had independent prognostic value as a marker of poor clinical outcome (15, 16). Despite a high sensitivity, RT-PCR techniques are controversial because of high false-positive rates due to background signal (17–21).

The aim of the present study was to evaluate the feasibility of identifying CK-expressing cells in peripheral blood with an automated-assisted immunohistochemical detection system and to compare the results with the same detection method in BM samples. We conducted this study on blood and BM samples from breast cancer patients with stage I to IV disease before chemotherapy.

PATIENTS AND METHODS

Patients. A prospective trial of the feasibility and evaluation of the prognostic value of micrometastatic disease in the BM of breast cancer patients has been underway at the Institut Curie since November 1998. BM aspirations were performed and analyzed in >600 breast cancer patients at various clinical stages and included in various therapeutic trials. Among these patients, from May 1999 to January 2001, peripheral blood samples were obtained from 114 patients before chemotherapy at the same time as BM aspiration. Before starting chemotherapy (neoadjuvant/adjuvant or for metastatic disease), a single BM aspiration was performed under local anesthesia from the posterior iliac crest (71 patients) or by sternal tap (43 patients). Written informed consent was obtained from 75 patients with localized breast cancer before neoadjuvant or adjuvant chemotherapy and from 39 stage IV patients. Patient characteristics are shown in Table 1. The median age was 51 years (range: 31–78 years). Forty-five of the 75 nonmetastatic patients received neoadjuvant chemotherapy and 30 received adjuvant chemotherapy based on 4–6 cycles of docetaxel-doxorubicin or epirubicin, cyclophosphamide +/− 5-fluorouracil. Twelve patients who received neoadjuvant chemotherapy were treated for inflammatory breast cancer (T4d). Twenty-four patients received repeated high-dose chemotherapy with peripheral blood stem cell reinfusion: 10 for inflammatory breast cancer and 14 for high axillary nodal involvement (>8N+). All patients with positive hormone receptors received adjuvant tamoxifen. Thirty-nine metastatic patients received chemotherapy: as first-line

Table 1 Patient characteristics and correlation with cytokeratin-positive (CK+) cell detection in bone marrow and peripheral blood in 75 nonmetastatic patients

	n (%)	CK+ cells in bone marrow (%)	*P*	CK+ cells in peripheral blood (%)	*P*
Menopausal status					
Pre	43 (57)	26 (60.5)		8 (19)	
Post	32 (43)	11 (34)	0.024	4 (12.5)	0.471
Clinical tumor size					
T1/T2	39 (52)	13 (33)		3 (7.5)	
T3	20 (27)	12 (60)		5 (25)	
T4	16 (21)	12 (75)	0.025	4 (25)	0.35
Clinical nodal status					
N0	36 (48)	16 (44.5)		6 (17)	
N1	32 (43)	16 (50)		4 (15)	
N2	7 (9)	5 (71.5)	0.331	2 (28.5)	0.879
Hormone receptors					
Estrogen receptor+	44 (59)	17 (38.6)		4 (9)	
Estrogen receptor−	31 (41)	20 (64.5)	0.026	8 (26)	0.053
Progesterone receptor+	14 (19)	6 (43)		1 (7)	
Progesterone receptor−	45 (60)	26 (58)	0.328	11 (24.5)	0.126
Missing	16 (21)				
Tumor grade					
I	12 (16)	5 (42)		0 (0)	
II	27 (36)	10 (37)		3 (11)	
III	34 (45)	22 (65)	0.079	9 (26.5)	0.066
Missing	2 (3)				
Tumor emboli					
Negative	40 (53)	16 (40)		4 (10)	
Positive	33 (44)	21 (64)	0.043	8 (24)	0.101
Missing	2 (3)				
Histological nodal status					
pN0	23 (31)	15 (65)		7 (30)	
pN1	48 (64)	21 (44)	0.088	5 (10)	0.041
Missing	4 (5)				
pN <3N+	38 (51)	19 (50)		9 (24)	
pN >3N+	33 (44)	17 (52)	0.898	3 (9)	0.094
Missing	4 (5)				
Histology					
Ductal	65 (87)	33 (51)		11 (17)	
Lobular	9 (12)	4 (44)		1 (11)	
Other	1 (1)	0 (0)	0.573	0 (0)	0.822

treatment in 85% (33 patients) or second- or third-line treatment in 15% (6 patients). Twenty-five stage IV patients (64%) had clinically documented bone metastasis.

BM and Blood Specimens. Three to 5 ml of BM aspirate were collected on EDTA (Vacutainer; Becton Dickinson) and 7–14 ml of blood were obtained by venipuncture. All blood samples for the study were obtained after discarding the first 5 ml of blood to avoid contamination of blood with epidermal cells. Components of the BM aspirate or blood were processed separately under sterile conditions using the same procedure. Each sample was diluted in half the volume of Hanks solution (Life Technologies, Inc.) under laminar flow. They were separated by Ficoll/Hypaque density centrifugation (density, 1.077 g/ml; Sigma) in Leucosep tubes (830 g, 15 min, 20°C; Polylabo). The mononuclear cell (MNC) layer was harvested in each tube, combined, diluted in 50 ml of Hanks, and centrifuged at 360 rpm for 5 min at 20°C in a 50-ml conical tube. Cells were resuspended in PBS solution +0.1% of BSA. An aliquot was counted after dilution to 3% in acetic acid for red cell lysis. The percentage of cell recovery was 50−80% as evaluated before the study with normal BM and blood samples spiked with tumors cell from breast cancer cell lines (data not shown). The MNCs were resuspended in PBS solution +0.1% of BSA at 1.10^6/ml. One ml of the cell suspension (~1 million cells) was cytocentrifuged onto polylysine- coated slides at 580 × *g* twice for 3 min (Hettich Universal 16A cytocentrifuge; Ref. 22). The supernatant was carefully removed from each slide after the first cytocentrifugation, and the slides were allowed to dry in air overnight. Slides were stored at room temperature before staining or were stored at −20°C and then at −80°C until staining. A median number of 10×10^6 MNCs (range: 5–35) and 40×10^6 MNCs (range: 5–530) were recovered after Ficoll in blood and BM samples, respectively.

ICC Staining. The pancytokeratin (CK) monoclonal antibody A45-B/B3 (Micromet, Munich, Germany and ChromaVision, San Juan Capistrano, CA), which recognizes several CK epitopes CK 8, CK 18, and CK 19, was applied for cell detection (23). The immunostaining procedure was standardized by using a Cadenza automat (Shandon). Before staining, cytospots were fixed with 4% paraformaldehyde for 5 min, then dried for 15 min at room temperature. Endogenous alkaline phosphatase was then blocked with Tris-buffered saline solution (Sigma) with 2% AB serum (15 min; Sanofi Diagnostics Pasteur). This solution was used to dilute primary and secondary antibodies. After blocking, the slides were incubated with the primary antibody A45 B/B3 for 40 min (2 μg/ml). Control slides were incubated under the same conditions with a mouse monoclonal anti-FITC IgG1 (1/1250; Sigma Immuno Chemicals). Slides were incubated for 20 min with secondary polyclonal rabbit antimouse antibody (Dako). After each step, the slides were rinsed for 5 min in Tris-buffered saline 1× solution. Immune complexes were revealed by the alkaline phosphatase-antialkaline phosphatase technique (1/50;Dako) for 25 min (24). The chromogen reaction was performed for 20 min with a colorimetric substrate of fuchsin solution (2.5% in 2 N HCl; New Fuchsin, Sigma) with 4% $NaNO_2$, 8% β-naphthol (Sigma), and 2% levamisole (Dako). Cells were counterstained with Mayer hematoxylin (1 min; Sigma) and diluted to one-third in distilled water. The specimen was then rinsed under running water for 5 min and then in Tris-buffered saline. Slides were coverslipped using Faramount mounting medium (Dako). A total of 3×10^6 mononuclear cells (three slides) was evaluated for each patient and for each sample (BM and blood sample). The number of MNCs evaluated was based on a statistical model established on the hypothetical assumption that a highly sensitive antibody recognizes all epithelial cells in a pure BM sample examination of 3×10^6 BM-derived cells to provide a probability of detecting one epithelial cell/10^6 MNC with 95% confidence interval (6, 25). Negative controls, stained with anti-FITC monoclonal mouse antibody, were performed on an equivalent number of cells (*i.e.*, three slides, 3×10^6 mononuclear cells) for each patient.

Positive controls were obtained with BM from normal donors undergoing orthopedic surgery (Cochin Hospital), spiked with SKBR3 or MCF7 cell lines, 10 to 10^2 for 10^6 mononuclear cells/cytospot. One positive control slide and one negative control slide were added to each series of 20 stained slides in the automated device.

CK+ Cell Detection by Digital Microscopy. The automated cellular imaging system (ChromaVision Medical Systems, Inc., San Juan Capistrano, CA) is a computerized microscope, which includes an image processing system that has been optimized for the detection of rare carcinoma cells in specimens (26). The application software supplied with the instrument starts by scanning a microscope slide at low magnification (×10). The instrument then returns to objects originally identi-

Table 2 Categorization of immunostained cells[a]

Category		Characteristics	Definition
Positive case	A	Tumor cells	Typical tumor cell morphology. Cell cluster, obviously enlarged or atypical nucleus. Never found in false positive.
Positive case	B	Probable tumor cells	No convincing hematopoietic cell characteristics. Classified as positive case only if no immunostained cells are present in the corresponding negative control specimen.
Negative case	C	Hematopoietic cells, squamous skin epithelial cells	Immunostained cells with hematopoietic cell-like morphology. Presence of immunostained cells on corresponding negative control specimen. Contaminating squamous skin epithelial cells.
Negative case	D	No cell detected	

[a] Criteria for evaluation of immunostained cells in bone marrow were adapted from Borgen *et al.* (27) based on the results of the European ISHAGE Working Group for standardization of tumor cell detection.

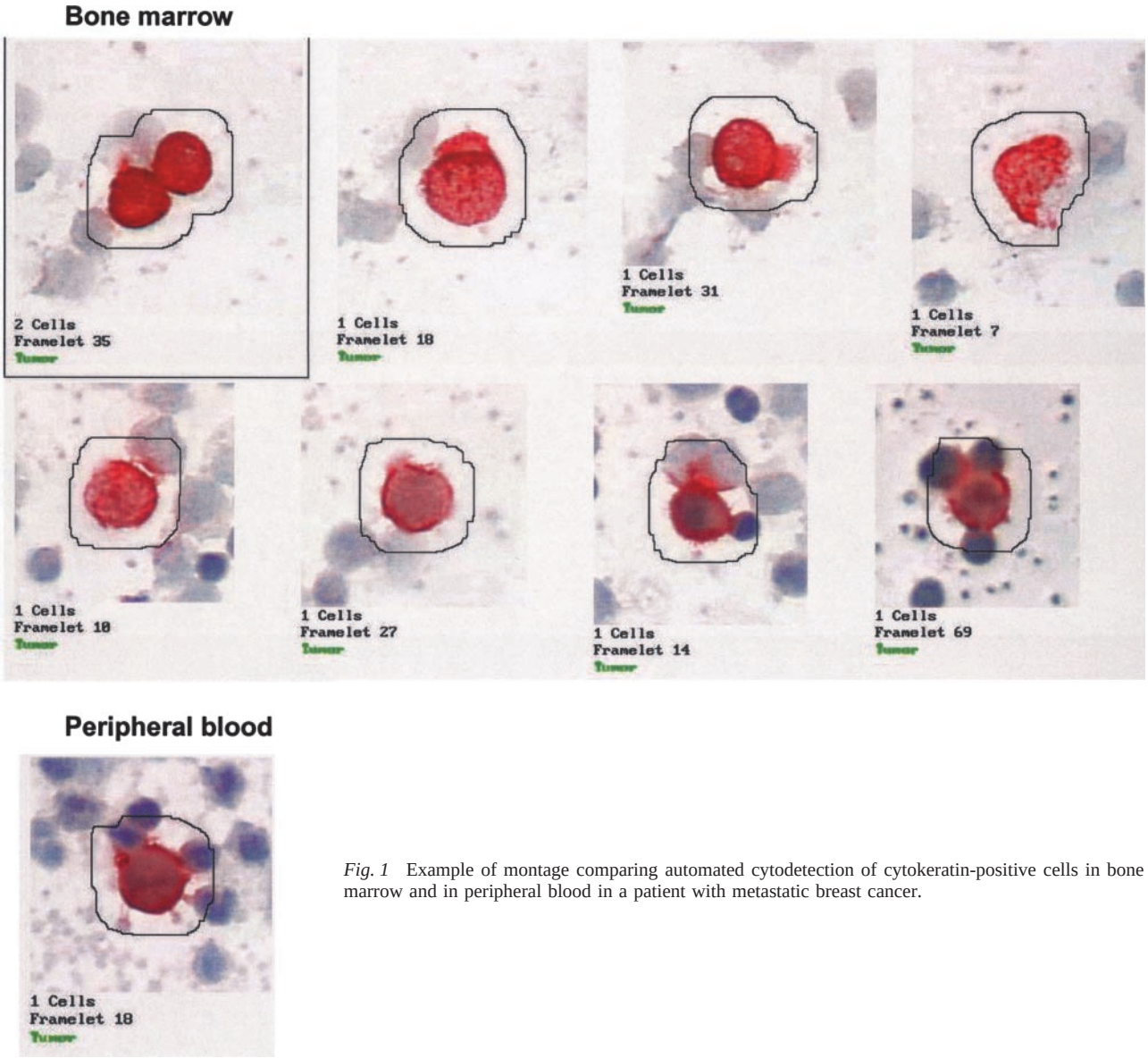

Fig. 1 Example of montage comparing automated cytodetection of cytokeratin-positive cells in bone marrow and in peripheral blood in a patient with metastatic breast cancer.

fied by their stain for a second analysis at higher magnification (×40 or ×60). In this case, more sophisticated image analysis of color and morphometric characteristics is performed to exclude cellular debris, large clumps, and cells with morphological features typical of normal hematological mononuclear cells as opposed to CK+ carcinoma cells. Cellular objects that meet color- and morphometry-based criteria for probable tumor cells are collected and presented as montage images for review and classification by a pathologist or another investigator (J-Y. P.). Independently, slides of BM specimens were manually blind reviewed by a pathologist. Pictures of CK+ cells detected manually by the pathologist were digitized on his own microscope and stored. After comparison of the results of automated cellular imaging system and the pathologist's examination (A. V-S.), in the case of discrepancy (15% of the cases), a consensus was established between the observers based on joint evaluation. Criteria for evaluation of immunostained cells in BM were adapted from Borgen *et al.* (27) based on the results of the European ISHAGE Working Group for standardization of tumor cell detection. These criteria are summarized in Table 2. Blood specimens were analyzed with the automated cellular imaging system and were reviewed by a single investigator (J-Y. P.), blinded to the patient characteristics.

Statistical Methods. Patient characteristics were prospectively recorded on the Institut Curie medical files. Differences between treatment groups were analyzed by χ^2 tests for categorical variables and *t* tests for continuous variables (or Mann-Whitney test for nonparametric variables). Survival time and disease-free survival time were measured from the date of BM aspiration until the date of death or last follow-up. Survival curves were determined using a Kaplan-Meier product-limit method (28). Statistical significance between groups was as-

sessed using the log-rank test. Multivariate analysis was carried out to assess the relative influence of prognostic factors on disease-free survival, using the Cox proportional hazards model in a forward stepwise procedure (29). Statistical analyses were performed by Statview software (SAS Institute, Inc.).

RESULTS

Detection. CK+ cells were detected in the blood of 28 (24.5%) patients and in the BM of 67 (59%) patients. Twenty-six (93%) patients with positive blood also had positive BM ($P < 0.001$), whereas only 26 of 67 (39%) patients with positive BM also had CK+ cells in blood. An example of CK+ cells in both BM and blood is shown in Fig. 1. Only 2 patients had positive blood with negative BM (Table 3). CK+ cells were detected in peripheral blood in 3 of 39 (7.5%) patients with operable breast cancer (stage I/II), 9 of 36 (25%) patients with locally advanced breast cancer (stage III), and 16 of 39 (41%) patients with metastatic disease (stage IV; Table 4). In four blood samples, 1 or 2 positive immunostained cells were detected on control slides and were therefore classified as negative cases. In BM, the percentage of positive cases ranged from 33% in stage I and II to 77% in stage IV (Table 4). Thirty seven (49%) nonmetastatic patients were considered to be positive for BM micrometastatic cell detection, according to our classification adapted from Borgen *et al.* (27): 11 were classified as A; 26 as B; 16 as C; and 22 as D. The BM classification for the 114 patients was 30 as A, 37 as B, 18 as C, and 29 as D.

The mean and median number of CK+ cells detected in blood-positive patients were 20 and 3 (range: 1–400), respectively. The mean and median number of CK+ cells detected in BM-positive patients were 70 and 6 (range: 1 to >1000), respectively. In the subgroup of nonmetastatic patients, the mean number of cells in blood-positive patients was 4.2, and the median was 2 (range: 1–35). For BM-positive patients, these values were 10.8 and 4 (range: 1–104), respectively (Fig. 2). The mean and median number of CK+ cells in BM-positive patients with positive blood were 129 and 23, respectively, *versus* 49 and 5 in BM-positive patients with negative blood ($P = 0.05$, Mann-Whitney).

The presence of CK+ cells in blood in nonmetastatic patients was correlated with negative estrogen receptor (ER)

Table 3 Comparison of tumor cell detection in blood samples and in bone marrow

		Bone Marrow		
		Positive	Negative	Total
Blood	Positive	26 (93%)	2 (7%)	28 (24.5%)
	Negative	41	45	86 (76.5%)
$P < 0.0001$	Total	67 (59%)	47 (41%)	114

Table 4 Patients clinical stage and correlation with cytokeratin-positive (CK+) cell detection in bone marrow and peripheral blood in 114 patients

114 patients	No. of patients	CK+ in bone marrow (%)	CK+ in peripheral blood (%)
Stage I/II	39	13 (33)	3 (7.6)
Stage III	36	24 (66)	9 (25)
Stage IV	39	30 (77)	16 (41)
		$P = 0.001$	$P = 0.017$

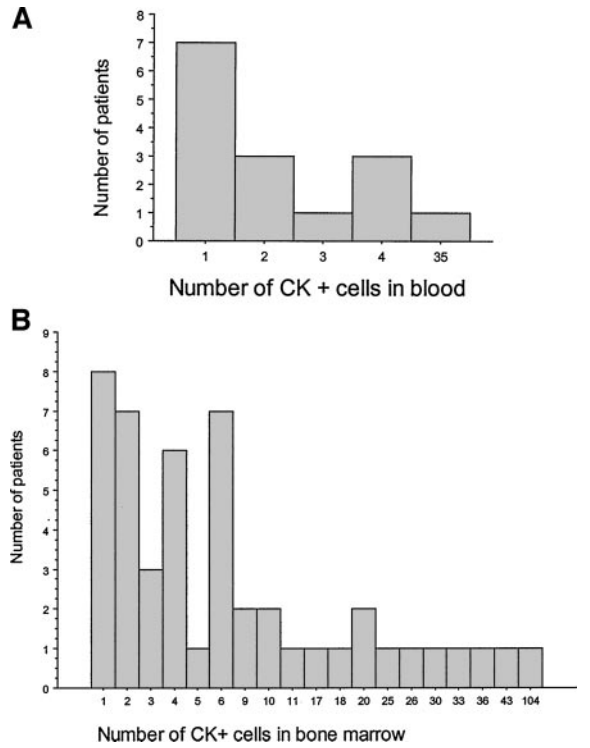

Fig. 2 Comparison of the number of cells detected per case in peripheral blood and in bone marrow in 75 nonmetastatic patients.

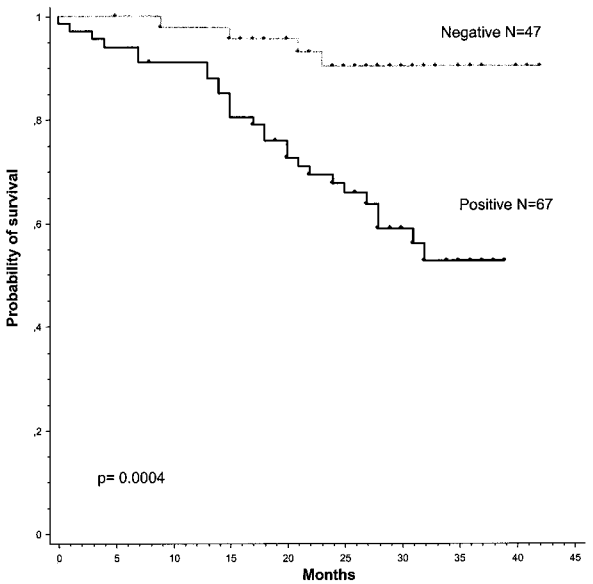

Fig. 3 Overall survival in the 114 patients according to the presence of cytokeratin-positive cells in bone marrow.

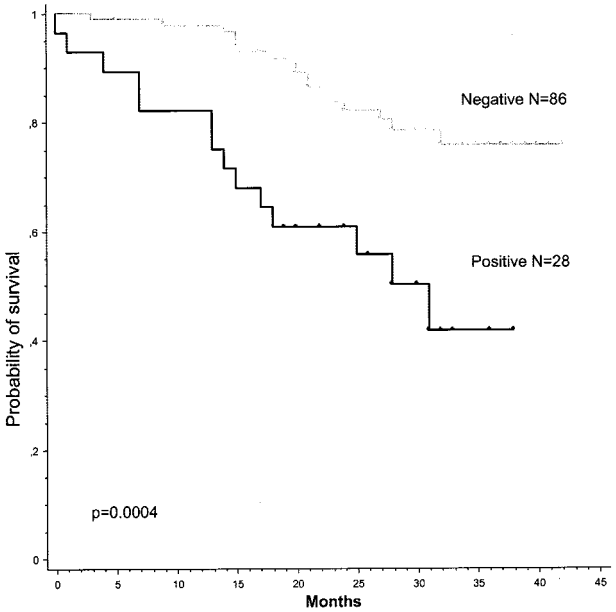

Fig. 4 Overall survival in the 114 patients according to the presence of cytokeratin-positive cells in peripheral blood.

status ($P = 0.05$) and histological nodal involvement ($P = 0.04$; Table 1). The presence of CK+ cells in the BM in nonmetastatic patients was correlated with premenopausal status ($P = 0.024$), high tumor size (T4; $P = 0.025$), ER-negative tumors ($P = 0.026$), and tumor emboli ($P = 0.043$). Age was not significantly different in the two groups: 50 years for CK+ in BM *versus* 53 in CK− ($P = 0.18$, *t* test). For the whole population ($n = 114$), the presence of CK+ in blood and/or BM was strongly correlated with clinical metastatic disease (Table 4).

Survival. Median follow-up was 28 months, and 31 deaths have occurred in these 114 patients. Four deaths have occurred on nonmetastatic patients ($n = 75$). The overall survival prognosis cannot be assessed due to this small number of events and short follow-up in nonmetastatic patients. Thirteen patients developed distant metastases and 6 developed local relapses. Prognostic factors were evaluated for disease-free survival. The same prognostic factors were observed for metastasis-free survival (data not shown).

In the 114 patients, on univariate analysis, poor prognostic factors for overall survival were clinical metastatic stage (stage IV; $P < 0.0001$), presence of CK+ cells in BM ($P = 0.0004$; Fig. 3), presence of CK+ cells in peripheral blood ($P = 0.0004$; Fig. 4), and premenopausal status ($P = 0.047$; Table 5). On multivariate analysis, metastatic clinical stage ($P < 0.0001$) and presence of CK+ cells in BM ($P = 0.051$) were independent factors of poor prognosis (Table 6).

In the subgroup of nonmetastatic patients ($n = 75$), prognostic factors for poor disease-free survival were absence of ER ($P = 0.004$), presence of CK+ cells in BM ($P = 0.012$; Fig. 5), clinical nodal involvement (0.016), large tumor size (T4; $P = 0.029$), and presence of tumor emboli ($P = 0.048$; Table 7). Presence of circulating CK+ cells in peripheral blood was not statistically significant (Fig. 6). On multivariate analysis, the independent indicator for disease-free survival was absence of ER ($P = 0.043$). The second factor in the model was presence of CK+ cells in BM ($P = 0.076$; Table 8).

DISCUSSION

In the present study, we have demonstrated the feasibility of detection of CK+ cells in the peripheral blood of breast cancer patients at various stages of the disease by ICC performed on a small volume of blood. The incidence of CK+ cells detected was significantly lower in blood than in BM (24 *versus* 59%). The number of cells was also lower in blood (median of 3 *versus* 6 CK+ cells in BM). The presence of CK+ cells in blood is strongly correlated with the presence of CK+ cells in BM. Only 2 patients had positive blood without BM involvement (93% concordance). In contrast to our results, the concordance was very low in Schoenfeld's study (30), which compared tumor cell detection in blood and BM by two techniques: 6% concordance by immunohistochemistry and 27% by RT-PCR. In the recent article published by Stathopoulou *et al.* (15), using RT-PCR for CK 19 gene, all positive cases in blood were positive in BM (100% concordance).

In the present study, most of the cases positive for blood were observed in metastatic patients or in patients with locally advanced breast cancer. The incidence of CK+ cells in patients

Table 5 Prognostic factors for overall survival on univariate analysis (log-rank test) in all patients ($n = 114$)

	Patients $n = 114$	%	χ^2	*P*
Clinical stage IV	39	34	61.52	<0.0001
Cytokeratin-positive cells in bone marrow	67	59	12.67	0.0004
Cytokeratin-positive cells in peripheral blood	28	25	12.49	0.0004
Pre- *versus* postmenopausal	53	46	3.91	0.047
Ductal versus other histology	99	87	1.99	0.157
Estrogen receptor negative *versus* estrogen receptor positive	40	35	1.79	0.181

Table 6 Multivariate analysis (Cox model) for disease-free survival in 114 patients

$n = 114$	Risk Ratio	χ^2	*P*	95% confidence interval
Stage IV	16.39	21.27	<0.0001	5.37–50
Cytokeratin-positive cells in bone marrow	3.12	3.80	0.051	1–9.80
Cytokeratin-positive cells in peripheral blood	1.63	1.44	0.228	0.73–3.66

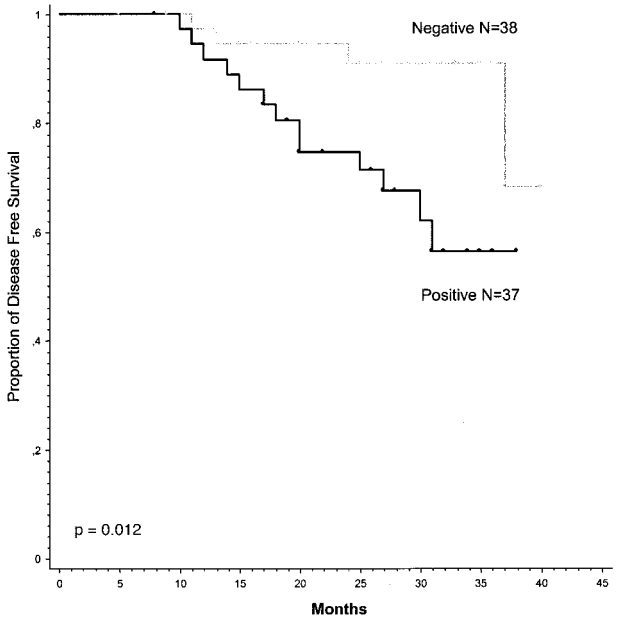

Fig. 5 Disease-free survival in nonmetastatic patients ($n = 75$) according to the presence of cytokeratin-positive cells in bone marrow.

at an operable stage was low (7.5%). In a large study on 155 patients before high-dose chemotherapy with autologous stem cell support, tumor cells were detected by ICC staining of peripheral blood cytospins in 17% of cases, mostly in stage IV patients (31). The incidence of CK-19 mRNA-positive cells was 30% in peripheral blood in early breast cancer and 52% in metastatic breast cancer in Stathopoulou's study (15). The overall sensitivity of detection is, however, lower in blood than in BM. CK+ cells may indeed not circulate continuously, and the small volume of blood may result in sampling errors.

In our study with ICC and automated detection, we obtained a level of detection comparable with that observed with PCR techniques. Slade *et al.* (32) compared a semiquantitative RT-PCR assay to ICC for the detection of CK 19-positive cells in the peripheral blood of breast cancer patients. In this study, PCR was not significantly superior to ICC, 49.6% of these samples were positive by RT-PCR, and 42% were positive by ICC. Automated-assisted screening appears to increase the sensitivity of epithelial cell detection by allowing the routine examination of a larger number of BM or PB cells (26, 33). This sensitivity can be potentially increased by other techniques such as immunomagnetic bead enrichment of larger sample volumes. The tumor-rich magnetic fraction can be processed by RT-PCR, ICC, or *in situ* hybridization. This should lead to more accurate quantification and molecular characterization of these tumor cells (34).

The use of density centrifugation in the present study is likely to result in the loss of many circulating tumor cell (CTC; particularly CTC clumps). Immunomagnetic separation, followed by specific RT-PCR (35), allows semiquantitative detection of circulating mammary cells without density centrifugation. A significant correlation was found in the latter study between the frequency of CTC and the clinical stage of breast tumors: 24% in the group with localized disease (*versus* 16% in the present study) and 45% in the group with metastatic disease (*versus* 41% in the present study). In Witzig's series (33), using immunomagnetic separation (immunomagnetic separation) after Ficoll on peripheral blood of breast cancer patients, the percent-

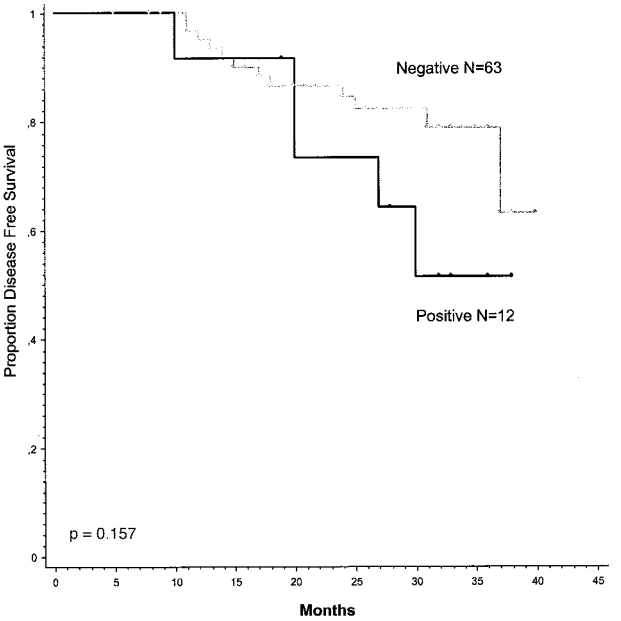

Fig. 6 Disease-free survival in nonmetastatic patients ($n = 75$) according to the presence of cytokeratin-positive cells in peripheral blood.

Table 7 Prognostic factors for disease-free survival on univariate analysis (log-rank test) in nonmetastatic patients

$n = 75$	Patients	%	χ^2	P
Estrogen receptor negative *versus* estrogen receptor positive	31	41	8.13	0.004
Cytokeratin-positive cells in bone marrow	37	49	6.24	0.012
Clinical N1N2 *versus* N0	39	52	5.79	0.016
Clinical T4 *versus* T3/T2/T1	16	21	4.75	0.029
Tumor emboli	33	44	3.90	0.048
Cytokeratin-positive cells in peripheral blood	12	16	1.99	0.157
Tumor grade II/III *versus* I	61	81	1.44	0.230
Pre- *versus* postmenopausal	43	57	0.92	0.337
Progesterone receptor negative *versus* progesterone receptor positive	45	76	0.88	0.347
Histological pN > 3N+	33	44	0.85	0.354
Ductal *versus* other histology	65	87	0.01	0.957

Table 8 Multivariate analysis (Cox model) for disease-free survival in 75 nonmetastatic patients

$n = 75$	Risk ratio	χ^2	P	95% confidence interval
Estrogen receptor negative *versus* estrogen receptor positive	3.02	4.08	0.043	1.03–8.83
Cytokeratin-positive cells in bone marrow	2.82	3.14	0.076	0.90–8.85
Clinical N1/N2 *versus* N0	2.57	2.58	0.108	0.81–8.13

age of detection of CK+ cells by ICC in blood was 76% in metastatic stages *versus* 40% in our series without enrichment. In a series of operable breast cancers, reported by Krag *et al.* (36), tumoral cells were detected using high gradient magnetic cell sorting in blood samples preceding surgical removal of the primary tumor in 18 of 19 patients. Prognostic factors for disease-free survival in our series were classical: tumor size; clinical nodal status; tumor emboli; and BM micrometastatic disease. Pathological nodal status was not a prognostic factor in this series but was assessed after neoadjuvant chemotherapy in the majority of nonmetastatic patients. ERs are crucial factors because all receptor-positive patients received tamoxifen. Hormonal receptors have been described as an early prognostic factor, which loses its significance with long follow-up. This could explain the high value of this parameter in this series with short follow-up (37).

The clinical value of circulating epithelial cells has yet to be established. BM cells appear to be more predictive of relapse than peripheral blood cell detection. BM may indeed act as a filter to concentrate breast cancer cells, as reflected by the propensity of breast cancer cells to form metastases in bone. A similar observation has been reported in Ewing's sarcoma, where RT-PCR was performed to look for EWS-FLI-1 fusion transcripts, resulting from the specific t(11;22) translocations. Among patients with localized tumors, BM micrometastasis predicted significantly poorer disease-free survival rates, but the presence of CTCs was not predictive of relapse (38). With a larger number of patients and longer follow-up, BM micrometastasis remained a major prognostic factor, and CTC became associated with a poor outcome among patients with clinically localized disease (39).

Because of the simplicity of repeated sampling, CTCs would constitute a possible alternative or complement to BM surveillance. It could be used to monitor the efficacy of treatment in advanced or metastatic breast cancer. According to Smith *et al.* (40), the number of cancer cells evaluated by RT-PCR or ICC reflects the outcome of systemic treatment in the majority of patients. This assay could screen for high-risk patients, determine the need for and monitor response to adjuvant or neoadjuvant therapy, and detect early recurrence of breast cancer.

In conclusion, the presence of tumor cells in blood is strongly correlated with the presence of these cells in BM. The number of cells detectable in blood by ICC is lower than in BM. Using an automated detection device, ICC is feasible for CTC detection in blood, but without any immunoselection technique, the level of detection is low even in metastatic patients with a high tumor burden. The prognostic value of CK+ cell detection in blood is lower than that in BM, but this may because of a lower detection sensitivity of the present method (low sampling volume). The prognostic value of BM involvement is clearly demonstrated by a number of large studies, which is not the case for blood. Blood could be more effectively used for sequential monitoring of treatment in adjuvant or metastatic situations.

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