Circulating Tumor Cells in Breast Cancer: Correlation to Bone Marrow Micrometastases, Heterogeneous Response to Systemic Therapy and Low Proliferative Activity

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Abstract

Purpose: The incidence and biological characteristics of circulating tumor cells in the blood of patients with breast cancer were examined and subgroups were evaluated in the context of systemic treatment and the presence of disseminated tumor cells in bone marrow.

Experimental Design: Circulating tumor cells were isolated from the peripheral blood of patients with breast cancer using a gradient system designed for the enrichment of circulating tumor cells (OncoQuick). Circulating tumor cells were identified with the anti-cytokeratin antibody, A45-B/B3. In subsets of patients, expression of the proliferation-associated Ki-67 antigen in circulating tumor cells and the concomitant presence of micrometastases in bone marrow were examined.

Results: In patients with primary breast cancer (stage M_0), circulating tumor cells were detected in 5 of 60 patients (8.3%) after surgery and before initiation of adjuvant chemotherapy; a positive correlation to the presence of disseminated tumor cells in bone marrow was observed ($P = 0.030$, $n = 53$). During the course of adjuvant chemotherapy, repeated analysis of 20 M_0 patients revealed the occurrence of circulating tumor cells in 7 of 16 patients that were initially negative. Patients with metastatic disease (stage M_1) showed circulating tumor cells in 25 of 63 cases (39.7%, $P < 0.0001$ as compared with M_0 patients), and a positive finding was correlated with elevated concentrations of the serum tumor marker CA15.3 ($P = 0.0093$). Performing repeated analysis in a subgroup of 25 M_1 patients, circulating tumor cells were found more frequently in patients with progressive disease than in patients with stable disease or remission (87.5% versus 43.8% of patients with circulating tumor cells, respectively; $P = 0.047$). Independent of the disease-stage, none of the 47 patients examined for the proliferative status of their circulating tumor cells showed coexpression of Ki-67.

Conclusions: Circulating tumor cells seem to be nonproliferating cells that persist during chemotherapy. Circulating tumor cell detection is linked to disease progression and elevated tumor marker concentrations in patients with metastatic breast cancer.

Peripheral blood could be an ideal source for the detection of disseminated tumor cells due to the simple sampling procedure. However, the clinical relevance of circulating tumor cells in the blood of breast cancer patients is less well-established than the prognostic relevance of disseminated tumor cells in bone marrow (1). The recent study by Cristofanilli et al. (2) has indicated the potential of circulating tumor cell detection as a tool to monitor therapy in patients with metastatic breast cancer. However, only limited information is available about the frequency of circulating tumor cells in patients with primary breast cancer, in particular in the context of adjuvant chemotherapy, and the biological characteristics of circulating tumor cells are largely unknown.

Studies examining circulating tumor cells have reported varying detection rates (1). One of the reasons for these discrepancies might be the differences in methods used for the enrichment and detection of circulating tumor cells. Cell enrichment by conventional density gradients such as Ficoll results in a large number of cells in the enriched fraction consisting mainly of blood mononuclear cells. A promising new approach is the enhanced density gradient system used in our study (OncoQuick) where, in contrast to Ficoll gradients, less slides have to be screened for positive events as the number of co-enriched mononuclear cells is lower (3, 4).

Besides their detection, further characterization of circulating tumor cells is a major future goal to understand the biology of circulating tumor cells (5). In this context, analysis of the

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proliferative activity of circulating tumor cells might be important to understand why cytotoxic chemotherapy regimens fail to eliminate disseminated cells in a substantial number of patients with breast cancer. A simple estimate to assess proliferative activity *in situ* is immunostaining for expression of the Ki-67 antigen, which is absent in the G_0 and early G_1 phases of the cell cycle (6).

Here, we have used the OncoQuick device for the enrichment of circulating tumor cells from blood and observed a correlation between the presence of disseminated tumor cells in bone marrow and circulating tumor cells in the peripheral blood of patients with primary breast cancer. Moreover, in patients with primary and metastatic breast cancer, circulating tumor cells seemed to be rather resistant to chemotherapy, and these cells were in a dormant, nonproliferating state. The presence of circulating tumor cells in patients with metastatic disease was correlated with disease progression and elevated levels of the tumor marker, CA15.3, suggesting that circulating tumor cell measurements are indeed a reflection of the metastatic tumor burden. Thus, the present work presents novel insights into the biology and dynamics of circulating tumor cells in breast cancer.

Materials and Methods

Patients. For this study, samples from 60 patients with primary breast cancer (stage M_0) were taken after surgery and before initiation of adjuvant therapy. In 20 of these patients randomly chosen from the entire cohort, repeated blood samples were collected during and at the end of their adjuvant chemotherapy. There was no evident difference between the patient characteristics of the entire patient cohort and the subgroup with repeated blood examinations concerning primary tumor stage. No clinical consequences were drawn from circulating tumor cell test results.

As a second cohort, blood samples from 63 patients with metastatic disease (stage M_1) were drawn before and during the course of treatment, and in 25 of these patients, who were randomly chosen, repeated blood examinations were done during their treatment.

Patients treated for primary breast cancer underwent surgery including lumpectomy and dissection of axillary lymph nodes by axillary clearance or modified radical mastectomy. All patients had epithelial breast cancer and metastatic spread was excluded by chest radiology, liver ultrasound scan, and bone scan. Paraffin-embedded sections of the primary tumors were stained for the expression of HER-2/neu (antibody CB 11, Novocastra, Newcastle upon Tyne, United Kingdom), estrogen receptor (antibody NCL-6F11, Novocastra), progesterone receptor (antibody NCL-PGR-312, Novocastra) and the proliferation antigen Ki-67 (clone MIB-1 Dako, Hamburg, Germany). Staining for HER-2/neu was expressed as "DAKO-Score" with values between 0 and 3+; staining for estrogen and progesterone receptors was evaluated according to the system of Remmele and Stegner (7) and a score >1 was regarded as positive. Proliferation was evaluated as the percentage of cells staining positive with the antibody MIB-1. Blood samples from patients with primary breast cancer were taken after surgery and before initiation of adjuvant therapy (n = 60). These patients received adjuvant treatment (endocrine treatment and/or anthracycline-containing chemotherapy as well as radiotherapy) according to national guidelines, without knowledge of the circulating tumor cell status. The median age of these patients was 59 years (range 29-82). Patients with metastatic disease (stage M_1) received chemotherapy, endocrine treatment, or treatment with the humanized anti HER-2/neu antibody trastuzumab (Herceptin) alone or in combination with chemotherapy. Tumor response to treatment was evaluated usually every 3 months.

All patients and healthy volunteers gave their informed consent and the examination of blood samples and bone marrow was approved by the local Ethics Review Board.

Enrichment of circulating tumor cells from the blood by gradient centrifugation. Approximately 20 mL EDTA-blood were drawn by vein puncture from 123 patients with breast cancer treated at our institution and 25 healthy volunteers. In total, 255 blood samples were evaluated for this study. To avoid contamination with skin cells, 10 mL blood were discarded before the study samples were taken. Blood was cooled to 4°C and processed within 24 hours with the OncoQuick density gradient system (Greiner Bio-One GmbH, Frickenhausen, Germany). OncoQuick is a separation device composed of a centrifugation tube with a liquid density separation medium and a porous barrier membrane optimized for the enrichment of circulating tumor cells from blood. The blood was layered on top of the gradient, and centrifuged for 20 minutes with 1,600 × g at 4°C. The complete supernatant above the porous barrier was transferred into a new tube pretreated with the washing buffer delivered with the OncoQuick device and cells were washed twice with 50 mL of washing buffer using a centrifugation step of 200 × g at 4°C. After the second washing step, the cells were resuspended in 1 mL washing buffer, counted in a Neubauer chamber, and centrifuged at 110 × g for 3 minutes using a cytocentrifuge (Hettich model 16 A, Tuttlingen, Germany) on adhesive slides (Superfrost Plus, Menzel Glassware, Braunschweig, Germany) at a concentration of 5×10^5 cells per area of 240 mm^2 or an equal cellular density of a smaller area when less cells were retrieved from the gradient. Cytospins were air-dried overnight and stored at −80°C until staining.

For the evaluation of the enrichment procedure (tumor cell recovery), MCF7 breast cancer cells were used. Cells were counted after trypsinization using a Neubauer chamber and approximately 250 cells were spiked into 18 mL of blood from a healthy control person. The blood was processed as described for patient samples. To accurately estimate the number of cells spiked into the blood, control slides were prepared from the same volume of the cell suspension and cells were counted. The average number of cytokeratin-positive cells from these control slides was used to calculate the cell recovery.

Immunocytochemical staining for the identification of circulating tumor cells in the blood. Slides were fixed according to the manufacturer's instructions with Solution B of the Epimet Kit (Micromet, Martinsried, Germany) containing formaldehyde. After blocking with a serum-free blocking reagent (Dako) for 20 minutes, slides were incubated with the anti-cytokeratin monoclonal antibody, A45-B/B3, directly labeled with the fluorochrome Cy3 at a concentration of 2 μg/mL (Micromet), and simultaneously counterstained with a monoclonal antibody directed against the CD45 antigen directly labeled with FITC at a dilution of 1:50 (Clone HI 30, BD Biosciences, Heidelberg, Germany) for 45 minutes. The monoclonal antibody A45-B/B3 is directed against a common epitope of cytokeratin polypeptides, including the cytokeratin heterodimers 8 to 18 and 8 to 19 (8). This antibody is well evaluated (9) and was applied to obtain clinical relevant information in breast cancer (10, 11) and other solid tumors (12–14). The CD45 antigen is also known as "common leukocyte antigen" and was used as an exclusion marker for CD45-stained cytokeratin-positive leukocytes in addition to the morphologic criteria that were intact nuclei and increased nuclear-cytoplasmatic ratio (15). Finally, the slides were incubated for 1 minute with 4′,6-diamidino-2-phenylindole (Sigma, Deisenhofen, Germany), mounted with 0.9% (w/v) NaCl and covered with coverslips. Cells were classified as circulating tumor cells when staining was positive for cytokeratin, was negative for CD45, and when morphologic criteria were fulfilled.

A subset of 47 patients was randomly chosen from the entire patient cohort, and in addition to the cytokeratin and CD45 expression, cells were analyzed for the expression of the Ki-67 antigen. Twenty-three of these patients had primary breast cancer and 24 patients had metastatic disease. For Ki-67 staining, the Dako ChemMate detection kit (Dako) was used. Briefly, slides were fixed in acetone, then incubated with a

Ki-67 monoclonal antibody (clone MIB-1 Dako) at a concentration of 1 μg/mL for 45 minutes, washed and incubated for 10 minutes with the biotinylated secondary antibody, again washed and incubated for 5 minutes with the peroxidase blocking solution. After washing, the slides were incubated with streptavidin peroxidase for 10 minutes, washed, and diaminobenzidine solution was added as substrate. A slight counterstain was applied with Haemalaun (Merck, Darmstadt, Germany) and blocking with mouse serum and staining for cytokeratin and CD45 were done as described above. The antibody MOPC-21 (Sigma, Deisenhofen, Germany) served as IgG_1 isotype control (1 μg/mL).

The breast cancer cell lines MCF7 (ATCC code HTB 22) and BT474 (ATCC code HTB 20) were harvested from tissue culture flasks before reaching confluency, and these cells were spiked into the blood of healthy individuals serving as positive controls and were processed in the same manner as the patient samples. The cytospins were screened by fluorescence microscopy for the presence of cytokeratin-positive cells and the expression of the Ki-67 antigen was determined in the bright field. A Leica DM LB microscope (Leica, Wetzlar, Germany) equipped with a Kappa color CCD camera and a computer with the Kappa Image Base software (Kappa Image, Gleichen, Germany) were used for all microscopic evaluations. The cytospins were evaluated by independent reviewers blinded to the clinical data.

Enrichment and detection of disseminated tumor cells in bone marrow. In 53 patients with primary breast cancer, bone marrow was aspirated directly after surgery under general anesthesia from both iliac crests, and screened for the presence of cytokeratin-positive cells. In brief, 2×10^6 mononuclear cells of each bone marrow specimen were analyzed. The monoclonal antibody A45-B/B3 (IgG_1, Micromet), was used at a concentration of 2 μg/mL to detect tumor cells in the cytospin preparation. A negative staining control was obtained by using an unrelated mouse-myeloma IgG_1 antibody (MOPC 21, Sigma, 2 μg/mL). The breast carcinoma cell line BT-20 (ATCC code HTB 19) served as a positive control for cytokeratin immunostaining in each staining batch. The specific reaction of the primary antibody was developed with the alkaline phosphatase anti-alkaline phosphatase technique (Dako), combined with the fuchsin stain, to indicate antibody binding as described before (10). Cytospins were analyzed with the automated cellular imaging system (ChromaVision Medical Systems, Inc., San Juan Capistrano, CA), as described before (16).

Determination of the tumor marker CA15.3. The concentration of the tumor marker CA15.3 was determined in serum from patient samples at the time of blood evaluation for circulating tumor cells using a standard ELISA (IMx CA15.3 second generation by Abbott Laboratories, Chicago, IL). Values >25 kU/L are considered to be increased according to the laboratory standards.

Statistics. All calculations were done with the SPSS software for Windows, version 11.0. Statistical two-sided P values <0.05 were considered significant.

Results

Tumor cell recovery and assay specificity. In order to evaluate the recovery of the enrichment and staining procedure in a model system, MCF7 breast cancer cells were spiked into the blood of healthy control subjects. Tumor cell enrichment and staining were done under the same conditions as the preparation of patient samples. Cytokeratin-positive/CD45-negative cells were designated as "tumor cells." In four separate experiments, the observed mean recovery rate was 70.6% (range 62.7-75.7%), which is in line with results published for other enrichment and staining systems (3, 17, 18).

To establish the specificity of the enrichment, staining, and evaluation procedure, blood from healthy individuals was examined. In one of the blood samples collected from 25 healthy individuals, two cytokeratin-positive cells were detected; one of these cells was negative for the leukocyte marker CD45. Blood sampling was repeated 2 weeks later and cytokeratin-positive cells were no longer found (data not shown).

Incidence of circulating tumor cells in blood samples drawn before initiation of adjuvant therapy for primary (stage M_0) breast cancer. One of the aims of our study was to evaluate the presence of circulating tumor cells as a marker for the detection of minimal residual disease at the time of primary diagnosis and surgical intervention. Samples from 60 patients without overt metastases [stage M_0] were taken after surgical removal of the primary tumor and axillary lymph nodes and before initiation of systemic therapy. Circulating tumor cells were detected in only five patients (8.3%). There was no significant correlation between the presence of circulating tumor cells and axillary lymph node involvement (pN stage), tumor size (pT stage), the differentiation grade, estrogen receptor status, progesterone receptor status, HER-2/neu expression, or the expression of Ki-67 in the primary tumor (Table 1). In samples positive for circulating tumor cells from patients with stage M_0 disease drawn before initiation of adjuvant therapy, a mean number of 1.6 circulating tumor cells (range 1-3 cells) per sample was detected. An example of a cytokeratin-positive circulating tumor cell is shown in Fig. 1.

Table 1. Characteristics of patients with primary breast cancer (stage M_0) examined for circulating tumor cells

Tumor/patient characteristics	No. of patients (%)	No. of patients with tumor cells (%)	*P*
Tumor size*			
T_1	38 (64.4)	4 (10.5)	0.75
T_2	19 (32.2)	1 (5.3)	
T_3/T_4	2 (3.4)	0	
Nuclear grading			
G_1	5 (8.5)	1 (20)	0.16
G_2	30 (50.8)	3 (10)	
G_3	24 (40.7)	1 (4.2)	
Axillary lymph node status			
Positive	22 (36.7)	0	0.147
Negative	38 (63.3)	5 (13.2)	
Estrogen receptor status			
Positive	46 (78)	4 (8.7)	1
Negative	13 (22)	1 (7.7)	
Progesterone receptor status			
Positive	40 (67.8)	3 (7.5)	0.41
Negative	19 (32.2)	2 (10.5)	
Ki-67 expression			
Low (≤30%)	41 (71.9)	4 (10.8)	1
High (>30%)	16 (28.1)	1 (6.2)	
HER-2/neu expression			
Positive (2+/3+)	13 (22)	2 (15.4)	0.3
Negative (0/1+)	46 (78)	3 (6.5)	

NOTE: Data were not available from all patients. Percentage refers to the number of patients with data available.
*Total of 60 patients.

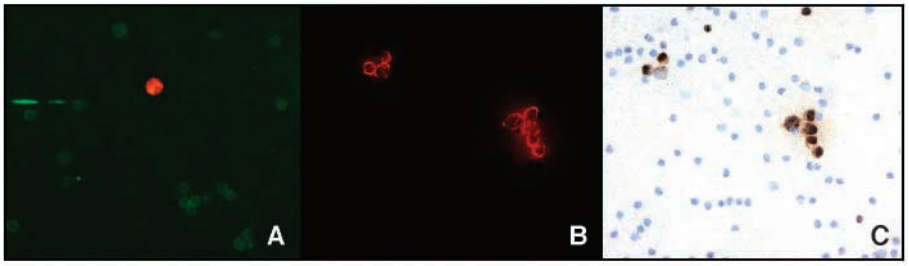

Fig. 1. *A,* cytokeratin-positive cell from a patient with breast cancer (*red*) with surrounding leukocytes (*green*); *B* and *C,* breast cancer cells BT 474 spiked into the blood of healthy individuals as positive staining controls. BT 474 cells in fluorescence microscopy (*red*, *B*) and staining for expression of the Ki-67 antigen in bright field (*dark brown*, *C*) with a nuclear counterstain (Haemalaun, *light blue*). Magnification, x400.

Changes of circulating tumor cell detection in repeated blood sampling during the course of adjuvant chemotherapy for primary breast cancer. To obtain information about the frequency of circulating tumor cells during the course of adjuvant chemotherapy, serial examinations were done before, during, and at the end of adjuvant chemotherapy in 20 of the patients with primary breast cancer; these patients were randomly chosen from the entire patient cohort (i.e., the selection was not based on baseline circulating tumor cells). All patients received anthracycline-containing therapy. Eleven patients (55%) showed circulating tumor cells either before or during the course of treatment. Among the 11 patients with circulating tumor cells, on average, 3.6 samples were examined (range 3-6). During chemotherapy, circulating tumor cells disappeared in four patients who had initially detectable circulating tumor cell levels, whereas circulating tumor cells were detected in seven initially negative patients (Fig. 2). Nine patients remained consistently negative before and during the course of treatment, and an average of 3.7 samples was examined (range 3-5). Also, no significant differences in patient characteristics (e.g., primary tumor size and lymph node status) or treatment were observed between patients with or without circulating tumor cells during the course of treatment. Therefore, a sampling bias due to different numbers of samples examined can be excluded and the changes in circulating tumor cells during the course of adjuvant chemotherapy seem to reflect tumor cell biology.

Correlation between the presence of circulating tumor cells in the blood and disseminated tumor cells in bone marrow of patients with primary breast cancer. We also examined if circulating tumor cells could serve as a surrogate marker for the presence of disseminated tumor cells in the bone marrow. The presence of disseminated tumor cells in the bone marrow was previously shown to represent a clinically relevant prognostic marker (1); however, blood sampling is easier to perform. Therefore, in 53 of the patients with stage M_0 breast cancer, the presence of disseminated tumor cells in bone marrow was also examined. Bone marrow was aspirated after surgery from both iliac crests and tumor cells were enriched over a Ficoll gradient. Cells were stained with the monoclonal antibody A45-B/B3 according to our procedure which was validated in a large prospective study (10).

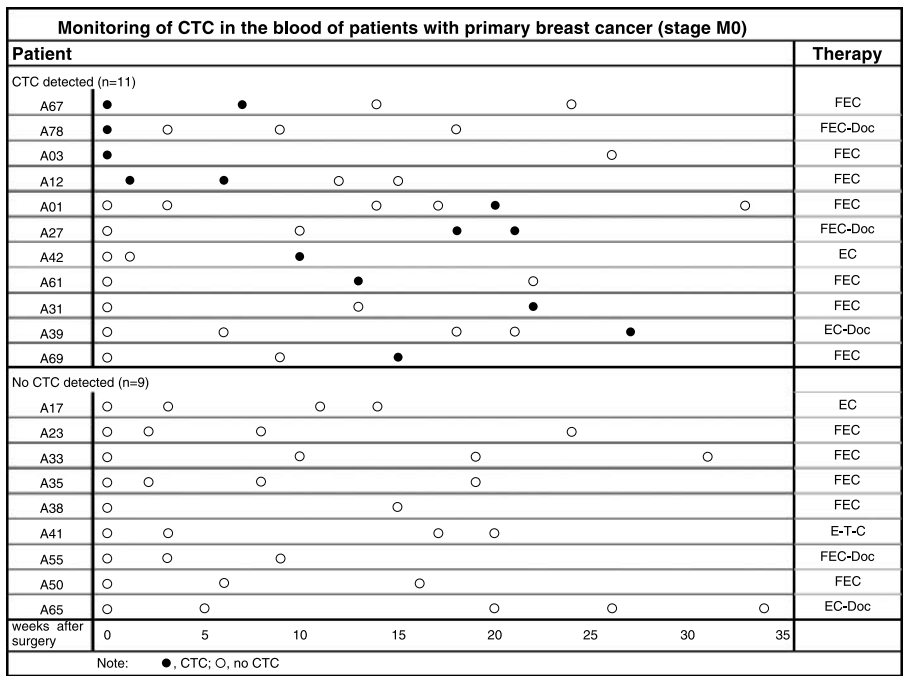

Fig. 2. Presence of circulating tumor cells in patients with primary breast cancer (stage M_0) in repeated blood examinations before initiation and during the course of adjuvant chemotherapy. Abbreviations: F, 5-fluoruracil; E, epirubicin; C, cyclophosphamide; Doc, docetaxel; T, paclitaxel.

At the time of first blood examination, 3 of the 9 patients (33.3%) with positive bone marrow status showed circulating tumor cells, whereas only 2 of 44 patients (2.3%) with negative bone marrow status showed circulating tumor cells at the time of first blood examination. Hence, an increased rate of circulating tumor cells in the blood of patients with disseminated tumor cells in bone marrow was observed compared with those without disseminated cells in bone marrow. This difference was statistically significant ($P = 0.030$, Fisher's exact test).

Incidence of circulating tumor cells in patients with metastatic breast cancer. One possible application of circulating tumor cell detection might be the monitoring of therapy in patients with advanced disease. We therefore also evaluated the frequency of circulating tumor cells in 63 patients with metastatic disease (TNM stage M_1) in the context of systemic treatment and examined a potential correlation between the presence of circulating tumor cells and the clinical outcome. Sixty-five percent of the patients had visceral disease and 35% of the patients had nonvisceral disease (e.g., locoregional or lymph nodes). In 25 (39.7%) patients, we found circulating tumor cells in the peripheral blood at the time of first examination. This incidence is statistically different to the rate of patients with circulating tumor cells after surgery for primary breast cancer ($P < 0.0001$, Fisher's exact test). In patients with stage M_1 disease, positive blood samples showed a mean number of 3.3 circulating tumor cells (range 1-19 cells). Eleven of the patients were examined before the initiation of therapy and six of them (54.5%) showed circulating tumor cells. Patients that were treated received either chemotherapy or endocrine treatment as well as therapy with the antibody trastuzumab (Herceptin) alone or in combination with chemotherapy. No correlation of disease variables (number of metastatic sites, visceral versus no visceral metastasis) with the presence of circulating tumor cells was observed (data not shown).

In order to obtain information about the presence of circulating tumor cells during the course of treatment, serial examinations were done on a randomly chosen subset of 25 patients with metastatic breast cancer without apparent differences in patient characteristics as compared with the entire patient cohort. In particular, circulating tumor cell status played no role in selection of the subjects. From 24 patients with evaluable tumor response, 6 patients achieved a complete or partial remission and 10 patients showed a stable disease status. The remaining 8 patients showed a disease progression.

Of the six patients with remission, only one patient (M35, Fig. 3) initially showed circulating tumor cells and remained positive, and another patient (M29) showed circulating tumor cells in only one of three examinations. The remaining four patients (M31, M54, M57, and M65) remained consistently negative (Fig. 3). Five of ten patients with stable disease showed circulating tumor cells at first examination but all except one sample from patient M06 became negative in later examinations. Moreover, the five patients initially negative also remained negative for circulating tumor cells during treatment. In five of eight patients with progressive disease, circulating tumor cells were detected at first examination and two patients went positive for circulating tumor cells during the course of their treatment, so seven of eight patients were positive for circulating tumor cells. Hence, 7 of 16 (43.8%) patients with remission or stable disease showed circulating tumor cells, whereas 7 of 8 (87.5%) with progression showed positive cells. Regarding all samples examined during the course of treatment from patients with either remission or stable disease, 9 of 38 (23.7%) samples were positive for circulating tumor cells. In

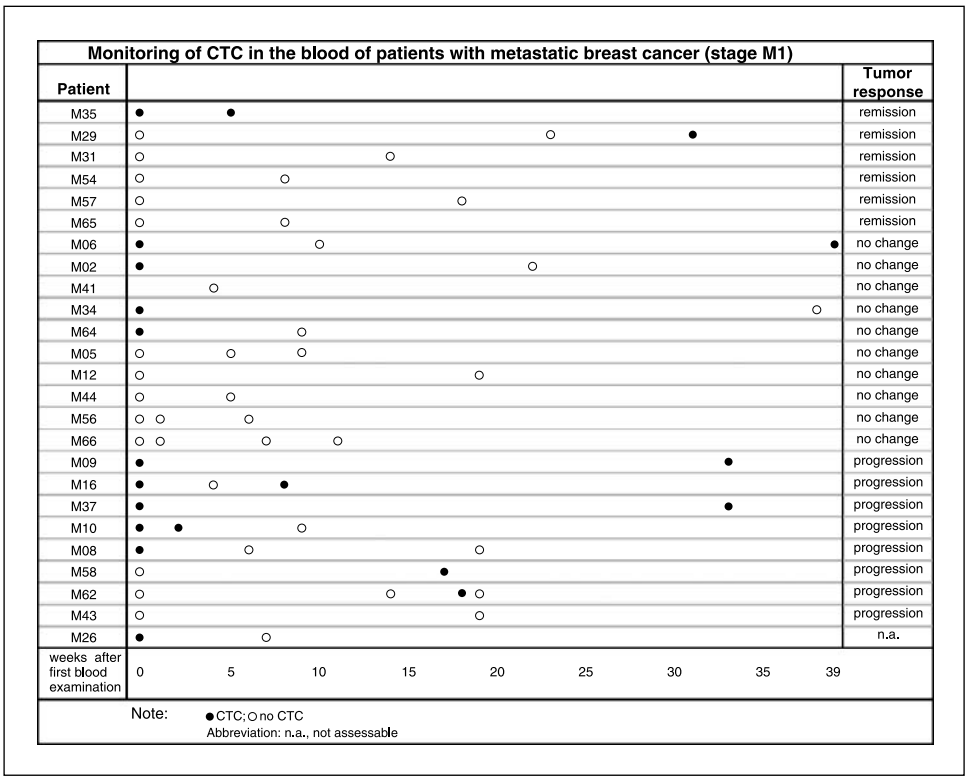

Fig. 3. Presence of circulating tumor cells in patients with metastatic breast cancer (stage M_1) in repeated blood examinations before initiation and during the course of treatment for metastatic disease.

contrast, patients with disease progression showed circulating tumor cells during the course of treatment in 11 of 19 samples (57.8%). Hence, patients with progressive disease showed an increased frequency of circulating tumor cells compared with those achieving remission or stable disease. Taken together, the frequency of circulating tumor cell – positive blood samples was significantly higher in patients with progressive disease than in those with remission or stable disease (P = 0.0473, Mann-Whitney U). This difference was not simply due to a sampling bias caused by a different number of blood samples examined because the total number of samples drawn was not different between the two groups (P = 0.37, Mann-Whitney U test).

Correlation of serum levels of the tumor marker CA15.3 with the detection of circulating tumor cells in patients with metastatic breast cancer. The tumor marker CA15.3 is known to be associated with disease burden in patients with metastatic breast cancer and used as a variable for the monitoring of therapy (19). Blood was examined simultaneously for the presence of circulating tumor cells and the serum levels of CA15.3 at the first examination (n = 52). A positive correlation of the presence of circulating tumor cells with increased serum levels of the tumor marker was observed (P = 0.009, Mann-Whitney U test). The median CA15.3 level was 236 kU/L in patients with circulating tumor cells in the blood and 50 kU/L in those with no circulating tumor cells (Fig. 4). No such correlation was observed for patients with stage M_0 breast cancer (data not shown).

Expression of the proliferation-associated antigen Ki-67 in circulating tumor cells. In order to examine the proliferative state of circulating tumor cells, we developed a triple labeling approach, using an antibody directed against the proliferation-associated antigen Ki-67 known to be present in all phases of the cell cycle except G_0 and early G_1 (6). As control for positive staining, we used proliferating tumor cells (BT 474) spiked into the blood of healthy volunteers; the spiked samples were processed under the same conditions as the patient samples. On the control slides, we were able to observe spiked BT 474 tumor cells positive for Ki-67 expression as well as Ki-67-negative tumor cells (Fig. 1), demonstrating that the triple staining technique worked.

Blood samples taken before and during systemic therapy from 47 randomly chosen patients (i.e., 23 M_0 and 24 M_1 patients) were examined for the expression of the proliferation-associated Ki-67 antigen in circulating tumor cells. In nine of these patients (1 M_0, 8 M_1 patients), a total of 22 circulating tumor cells were detected, but none of these cells were Ki-67-positive. Of the nine patients with circulating tumor cells, three had received no systemic treatment and six had received either chemotherapy and/or trastuzumab (Herceptin). Three patients with metastatic disease showed a progression, four patients had no change of disease status, and one patient was in remission. This indicates that proliferation of circulating tumor cells, as it would be reflected by Ki-67 expression, seems to be a rare event independent of treatment or disease status.

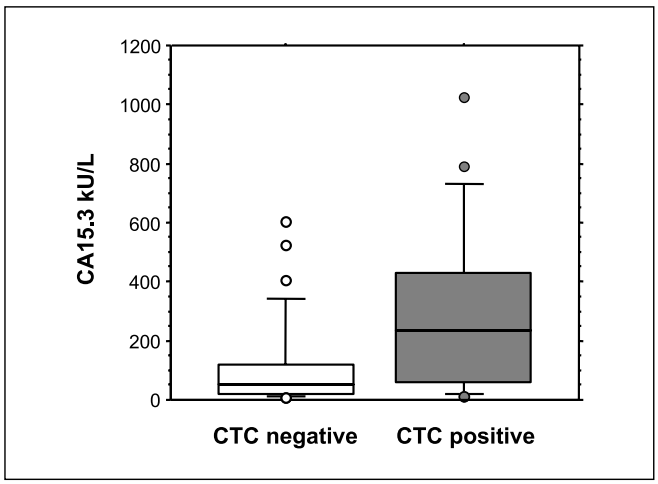

Fig. 4. Concentration of the tumor marker CA15.3 determined in serum samples drawn at the time of first blood examination for circulating tumor cells in patients with metastatic breast cancer. Box plots show 25 and 75 percentiles with lines indicating the median value. In the group with no circulating tumor cells, the median CA15.3 level was 50 kU/L; in the group with circulating tumor cells detected, the median level was 236 kU/L. The difference between the two groups is statistically significant (P = 0.009, Mann-Whitney U test).

Discussion

In this study, we were able to show that circulating tumor cells are present in a substantial fraction of patients with breast cancer undergoing systemic therapy. We showed that circulating tumor cells are usually nonproliferative and a fraction of these cells seems to be resistant to chemotherapy. Our data suggest that the detection of circulating tumor cells in patients with metastatic cancer might allow monitoring of response. Up to now, only a limited number of reports examined the occurrence of circulating tumor cells in the context of systemic therapy for primary or metastatic breast cancer. In addition, the biological characteristics of circulating tumor cells are still not well understood.

The specificity of cytokeratin-based immunoassays for detection of epithelial tumor cells in blood and bone marrow has been established in many previous studies (20). In the healthy individuals analyzed in this study, circulating cytokeratin-positive cells were very rare (if at all existent). The malignant nature of the vast majority of cytokeratin-positive cells in patients with breast cancer has also previously been shown by the detection of genomic alterations (21 – 24). Thus, the immunocytochemical approach indeed seems to detect tumor cells.

Using this approach, we took blood samples a few days after surgery (i.e., before initiation of systemic adjuvant chemotherapy) to evaluate the potential of circulating tumor cells as a marker for the detection of minimal residual disease in patients with primary breast cancer (stage M_0). We detected circulating tumor cells in 5 of 60 (8.3%) samples from these patients, despite removal of the tumor and in the absence of manifest metastases, which is in line with other results (18, 25). The fact that no significant correlation of circulating tumor cell detection with the lymph node status (pN_0 versus pN_1) was observed in our study, as well as in other publications on the detection of disseminated tumor cells in blood or bone marrow (18, 25), could support the hypothesis that hematogeneous tumor cell dissemination is not closely linked to the tumor cell load or the lymphatic route of dissemination in patients with early stage breast cancer (20, 26, 27). However, considering the small number of patients with positive cells in this group, the statistical power to detect possible correlations with prognostic factors like lymph node status is low (Table 1).

Interestingly, when we examined repeated blood samples during the course of adjuvant therapy, we also observed the occurrence of circulating tumor cells in patients being initially negative for circulating tumor cells (Fig. 1), suggesting that circulating tumor cells might be released from distant organs into the circulation (28). However, this change might also be due to false-negative findings in the initial analyses or a short transit time of circulating tumor cells in the circulation. On the other hand, patients with initially detectable circulating tumor cells rendered to a negative status, which may suggest a cytotoxic effect of chemotherapy on circulating tumor cells in a subset of patients. Taken together, these findings could reflect the heterogeneous response of different breast cancer cell populations and might correspond to the observation that adjuvant chemotherapy has a curative effect only in a subset of breast cancer patients (29). However, we cannot exclude the possibility that the assay itself has imperfect sensitivity and specificity, which can account for some of these apparent transitions in the circulating tumor cell status of patients analyzed repeatedly. At present, there is no "gold standard" for circulating tumor cell measurements that could be used to exclude false-negative findings, and multiple examinations at one time point to examine the interassay reproducibility require rather large blood volumes, which can pose a problem for the acceptances of research studies on cancer patients by Ethics Review Boards. However, in an earlier study on a limited number of patients with epithelial tumors, we observed a good concordance between duplicate measurements of circulating tumor cells in blood (30).

In patients with primary breast cancer, a significantly increased rate of circulating tumor cells in the blood of patients with disseminated tumor cells was observed in bone marrow, although the number of events in both compartments was relatively low. We compared the incidence of a positive finding in the two compartments in order to evaluate the potential of circulating tumor cells to act as a surrogate marker for minimal residual disease in bone marrow. To date, most experience with screening for occult metastatic breast cancer cells exists for immunocytochemical analyses of disseminated tumor cells in bone marrow and numerous studies report the strong prognostic impact of the presence of such cells. Thus, the clinical value of disseminated tumor cells in bone marrow is clearer than that of circulating tumor cells in blood because larger studies and longer clinical follow-up data exist for bone marrow screening (1). Our results are in line with the observations of Pierga et al. (25), who recently described a correlation between the occurrence of disseminated tumor cells in bone marrow and circulating tumor cells in patients with primary and metastatic breast cancer. However, we as well as Pierga et al., observed no complete concordance between the presence of tumor cells in blood and bone marrow. This might reflect the fact that blood is only a temporary compartment for disseminated cells. The half-life of circulating tumor cells may be short (31) and not all circulating tumor cells may settle in distant organs such as the bone marrow. Taken together, we recommend the measurement of both circulating tumor cells and disseminated cancer cells in bone marrow in the context of clinical studies in M_0 patients in order to obtain more information on the added clinical value of circulating tumor cell measurements.

It should be noted that the observed incidence of bone marrow–positive M_0 patients in our present study was lower than previously reported by Braun et al. (10), although the same antibody was used for the detection of cells in both studies. This difference can be explained by a migration to lower tumor stages due to mammography screening and a more rigid interpretation of a positive bone marrow finding due to our international standardization efforts (15).

The follow-up time in our patient cohort with primary breast cancer was too short to draw conclusions regarding the prognostic impact of circulating tumor cell detection in these patients. We therefore examined blood of patients with metastatic breast cancer to see if the presence of circulating tumor cells is correlated with response to treatment. In addition, we wanted to evaluate if circulating tumor cells can be detected during systemic treatment. In total, circulating tumor cells were detected in 39.7% of patients with metastatic breast cancer at first blood examination. This incidence is statistically different from that observed in patients with stage M_0 disease ($P = 0.0001$) and indicates a correlation between circulating tumor cell detection and disease progression. The majority of blood samples in our cohort of patients with stage M_1 disease was taken during systemic therapy for metastatic disease including chemotherapy as well as endocrine therapy and antibody treatment with the HER-2/neu-directed humanized monoclonal antibody trastuzumab (Herceptin). The observation that not all patients with metastatic disease showed circulating tumor cells could be explained by the assumptions that the half-life of circulating tumor cells in the blood might be short, circulating tumor cells might not be released permanently into the circulation, and a fraction of circulating tumor cells might be eliminated by systemic therapy. The correlation between circulating tumor cell detection in metastatic patients and clinical outcome argues against the assumption that most circulating tumor cells are apoptotic as it was suggested by Mehes et al. (32).

In a subset of 25 patients, repeated blood examinations were done during the course of treatment for metastatic breast cancer. We found a lower incidence of circulating tumor cells in patients with remission or with stable disease than in patients with progressive disease (Fig. 2). This finding suggests a link between the presence of circulating tumor cells and the response to treatment. Hence, circulating tumor cell detection might enable a "real-time evaluation" of therapy response (2, 33, 34). However, patients received different forms of treatment, and therefore this claim has to be reevaluated in a larger prospective cohort of patients with well-defined treatment.

The tumor marker CA15.3 is a glycoprotein product of the *MUC-1* gene. It is assumed to be correlated with disease burden in patients with metastatic breast cancer. In the present study, the concentrations of the tumor marker CA15.3 were higher in those patients with metastatic cancer with circulating tumor cells compared with those without tumor cells in the blood (Fig. 4), indicating a higher likelihood of detecting circulating tumor cells in those patients with an increased metastatic load. This is the first report demonstrating such a correlation in breast cancer, which strengthens the concept that circulating tumor cell measurements reflect metastatic tumor load. In future trials on metastatic breast cancer, the clinical value of circulating tumor cell detection should be directly compared with tumor marker determinations (35).

A considerable advantage of the immunocytochemical approach described here is the possibility to characterize

tumor cells by multiple staining. We have not observed circulating tumor cells expressing the proliferation antigen Ki-67, irrespective of the disease or treatment status of the patients. With our study, we obviously cannot exclude that proliferation of circulating tumor cells occurs at all. However, it seems to be at least a rare event. In line with these observations, our previous analysis showed that disseminated tumor cells in bone marrow of breast cancer patients rarely express Ki-67 at the time of primary diagnosis (36). These findings may explain in part why cytotoxic chemotherapy regimens can fail to eliminate disseminated cells in a substantial number of breast cancer patients. However, cell culture experiments with disseminated tumor cells in bone marrow indicate that these cells can escape their proliferative dormancy when transferred to a host bed consisting of the appropriate growth factors (22).

In conclusion, our results indicate that the detection of circulating tumor cells could add additional information about tumor cell persistence in patients without measurable clinical disease and about tumor response in patients with metastatic breast cancer. Therefore, the established blood test could be implemented into clinical trials to evaluate its potential as a surrogate marker for the efficacy of systemic treatment. This could support the development of new anticancer agents and treatment protocols as well as it could help to optimize the individualized treatment of cancer patients.

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