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Imaging, Diagnosis, Prognosis

Clinical Cancer Research

Detection and HER2 Expression of Circulating Tumor Cells: Prospective Monitoring in Breast Cancer Patients Treated in the Neoadjuvant GeparQuattro Trial

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Abstract

Purpose: This study was aimed at detecting and characterizing circulating tumor cells (CTC) before and after neoadjuvant therapy (NT) in the peripheral blood of patients with breast cancer.

Experimental Design: The clinical trial GeparQuattro incorporated NT approaches (epirubicin/cyclophosphamide prior to randomization to docetaxel alone, docetaxel in combination with capecitabine, or docetaxel followed by capecitabine) and additional trastuzumab treatment for patients with HER2-positive tumors. We used the Food and Drug Administration–approved CellSearch system for CTC detection and evaluation of HER2 expression and developed HER2 immunoscoring for CTC.

Results: We detected ≥1 CTC/7.5 mL in 46 of 213 patients (21.6%) before NT and in 22 of 207 patients (10.6%) after NT (P = 0.002). Twenty (15.0%) initially CTC-positive cases were CTC-negative after NT, whereas 11 (8.3%) cases were CTC-positive after NT, although no CTC could be found before NT. CTC detection did not correlate with primary tumor characteristics. Furthermore, there was no association between tumor response to NT and CTC detection. HER2-overexpressing CTC were observed in 14 of 58 CTC-positive patients (24.1%), including 8 patients with HER2-negative primary tumors and 3 patients after trastuzumab treatment. CTC scored HER2-negative or weakly HER2-positive before or after NT were present in 11 of 21 patients with HER2-positive primary tumors. HER2 overexpression on CTC was restricted to ductal carcinomas and associated with high tumor stage (P = 0.002).

Conclusion: CTC number was low in patients with primary breast cancer. The decrease in CTC incidence during treatment was not correlated with standard clinical characteristics and primary tumor response. Information on the HER2 status of CTC might be helpful for stratification and monitoring of HER2-directed therapies. *Clin Cancer Res; 16(9); 2634–45. ©2010 AACR.*

Neoadjuvant treatment (NT) strategies allow the assessment of therapeutic efficacy of chemotherapy and novel targeted approaches in patients with breast cancer without the need for long follow-up periods, which are required in the adjuvant setting. The German Breast Group has conducted successful clinical trials in the neoadjuvant setting. The GeparQuattro study is a phase III trial program that incorporated different NT chemotherapy approaches with the addition of trastuzumab into current NT regimes for primary breast cancer (Fig. 1; refs. 1,2). This provides an opportunity for translational research projects to examine biomarkers that might elucidate mechanisms underlying these therapies.

Disseminated tumor cells in the bone marrow of patients with breast cancer are a significant independent predictor of poor prognosis in patients with nonmetastatic

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Translational Relevance

In patients with breast cancer, the onset of subclinical tumor cell spread is the putative precursor stage of solid metastases and can be assessed with the detection of disseminated tumor cells in bone marrow samples or circulating tumor cells (CTC) in the blood. The detection of CTC is of potential clinical relevance in the context of a growing number of therapeutic options, especially in the adjuvant setting in which no tumor is present. In the study described here, the incidence of CTC decreased during neoadjuvant treatment, but no correlation was found between the persistence of CTC and the treatment response of tumors. HER2-positive CTC could be detected in patients with HER2-negative primary tumors, and HER2-positive CTC could also survive trastuzumab treatment. Thus, determination of HER2 expression of CTC allows further insights into the effects of trastuzumab therapy in the context of neoadjuvant therapy and might also be of relevance for study designs examining new therapeutic approaches targeting HER2.

breast cancer, indicating that early tumor cell spread has biological relevance. Conclusive data on the prognostic relevance of such findings are derived from recently published studies and a pooled analysis involving 4,703 patients with early stage breast cancer (3). In addition, several studies have indicated the presence of disseminated tumor cells in bone marrow after adjuvant therapy as a predictor of poor prognosis (4–6). However, bone marrow aspirations are not widely accepted as repeated diagnostic tests in patients with breast cancer, whereas sequential peripheral blood analysis is more acceptable for therapy monitoring. Therefore, several research groups are currently assessing the clinical value of circulating tumor cells (CTC) for therapy monitoring of therapeutic efficacy (7).

Several publications have described that in patients with metastatic breast cancer, the presence of tumor cells detected with the highly standardized CellSearch assay was associated with worse prognosis and seems to allow early response evaluation (8–11). However, many aspects of the role of CTC detection in patients with early stage nonmetastatic breast cancer remain unclear, especially in treatment regimes including targeted therapy, e.g., trastuzumab directed against HER2. With the availability of improved and standardized techniques for CTC detection, it should now be possible to examine several of these important questions within a prospective multicenter study.

The development of metastatic disease is assumed to be a highly selective process. Only a small portion of tumor cells of the primary tumor probably have the ability to initiate metastatic growth in different organ sites. Therefore, the phenotype of the primary tumor may not necessarily reflect the phenotype of metastatic disease. Thus, a striking potential of CTC could be to re-evaluate therapeutic targets on these cells, which might enable a more individual and optimized antimetastatic therapy in patients with cancer. For example, it has been shown that CTC in the blood and disseminated tumor cell in the bone marrow are more frequently HER2-positive than the corresponding primary tumor (12–14).

Better insights into the biology of tumor cells surviving (neo)adjuvant treatment strategies should permit optimized treatment strategies that could increase the cure rate of patients with breast cancer. Thus far, there have only been a few reports, on a small number of patients, which have analyzed the detection of CTC in the context of NT in patients with breast cancer. These studies yielded discordant results concerning the possibility of monitoring therapeutic efficacy by detecting CTC and have not examined HER2 on CTC (15, 16).

In this study, we examined blood samples from 213 patients with nonmetastatic breast cancer before NT and 207 patients after NT prior to surgery. Our results show that CTC could be detected in patients with nonmetastatic breast cancer at primary diagnosis and also after NT; however, the incidence of CTC decreased during treatment. Our data suggests that determining the HER2 expression of CTC allows further insights into the effects of trastuzumab therapy in the context of NT and should also be of relevance for study designs examining new therapeutic approaches targeting HER2.

Materials and Methods

The GeparQuattro clinical study. Patients with either large operable or locally advanced tumors, tumors with negative hormone receptor status, or receptor-positive tumors but clinically node-positive disease were recruited to preoperatively receive four cycles of epirubicin/cyclophosphamide (90-600 mg/m^2) and to be then randomized to either four cycles of docetaxel (100 mg/m^2) or four cycles of docetaxel + capecitabine (75-1,800 mg/m^2) or four cycles of docetaxel (75 mg/m^2) followed by four cycles of capecitabine (1,800 mg/m^2; docetaxel → capecitabine). Patients with HER2-positive tumors received trastuzumab (6 mg/kg i.v. every 3 wk) concomitant to cytotoxic treatment, starting with a loading dose of 8 mg/kg i.v. on day 1 of the first epirubicin/cyclophosphamide cycle. The primary objectives were to assess the effect of capecitabine by comparing epirubicin/cyclophosphamide → docetaxel versus + epirubicin/cyclophosphamide → docetaxel + capecitabine versus epirubicin/cyclophosphamide → docetaxel → capecitabine and to assess the effect of duration (24 versus 36 wk) by comparing epirubicin/cyclophosphamide → docetaxel + epirubicin/cyclophosphamide → docetaxel + capecitabine versus epirubicin/cyclophosphamide → docetaxel → capecitabine (Fig. 1). The study was performed as a joint trial of the German Breast Group and Arbeitsgemeinschaft Gynäkologische

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Onkologie study groups and was co-chaired by Michael Untch and Gunter von Minckwitz.

Inclusion criteria for the translational subprotocol. Blood samples were collected from patients eligible for the GeparQuattro study in CellSave (Veridex) tubes before and/or after chemotherapy in the 14 participating centers. Trastuzumab was given to patients with HER2-positive tumors. HER2 positivity of the primary tumor was defined as either IHC 3+ or fluorescence *in situ* hybridization (FISH) positive. The standardized immunohistochemistry assay HercepTest by DakoCytomation was mandatory and all IHC 2+ cases had to be centrally analyzed by FISH assay in one of five German reference centers.

Ethical considerations. All patients gave informed consent to provide a prespecified amount of extra blood before entering the GeparQuattro study, although participation in the clinical trial was still possible even if a patient did not agree to provide extra blood samples. Results for CTC were linked to clinical data after irreversible anonymization of the clinical data. Patients were not informed about the laboratory results due to their experimental character. The clinical treatment study as well as the translational research project described here was approved by the central ethics committee at the University of Frankfurt as well as in all ethics committees of the participating centers.

Interventions. Full blood samples of 7.5 mL each were collected into CellSave (Veridex) tubes (*a*) before the start of treatment and (*b*) after NT but before surgery. Sample preparation and analysis were performed at the Institute of Tumor Biology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany. We have previously conducted a validation study and showed that samples could be stored and transported as well as examined in a multicenter setting (17).

The CellSearch Epithelial Cell Test (Veridex) was applied for the enrichment and enumeration of CTC. In brief, CTC were captured from the peripheral blood by anti-EpCAM antibody–bearing ferrofluid and subsequently identified by cytokeratin-positivity plus negativity for the leukocyte common antigen CD45 and 4′,6-diamidino-2-phenylindole (DAPI) staining to ensure the integrity of the nucleus.

Determination of HER2 expression on CTC. CTC were further characterized for HER2 expression within the CellSearch system by the addition of a FITC-labeled anti-HER2 antibody as described by the manufacturer (CellSearch tumor phenotyping reagent HER2/neu; Veridex). To evaluate the intensity of HER2 immunostaining, approximately 500 breast cancer cells from cell lines with known HER2 status were added into the blood from healthy donors and processed under identical conditions using the CellSearch system. The breast cancer cell lines MCF-7, BT20, T47D, SK-BR-3, and BT474 obtained from the Central Cell Service Unit of the Imperial Cancer Research Fund (London, United Kingdom) were cultured as previously described (18). The cell line MDA-MB-453 was purchased from the German Collection of Microorganisms and Cell Cultures. It was cultured in 90% Leibovitz's L-15 medium + 10% fetal bovine serum at 37°C without exposure to CO_2. In parallel, 2×10^5 cells of these cell lines cytospun onto slides were analyzed by immunocytochemistry and FISH for HER2 expression.

For immunocytochemistry, cells were fixed in 4% paraformaldehyde or 4% formaldehyde. After washing and peroxidase blocking, the polyclonal rabbit anti-human c-erbB-2 antibody (A0485, dilution 1:500; Dako Cytomation) was applied for 45 min at room temperature. Subsequently, slides were incubated with peroxidase-labeled EnVision polymer coupled with goat antibodies to rabbit/mouse immunoglobulins (Dako) for 15 min at room temperature. 3,3′-Diaminobenzidine was used as a chromogen. Slides were counterstained with

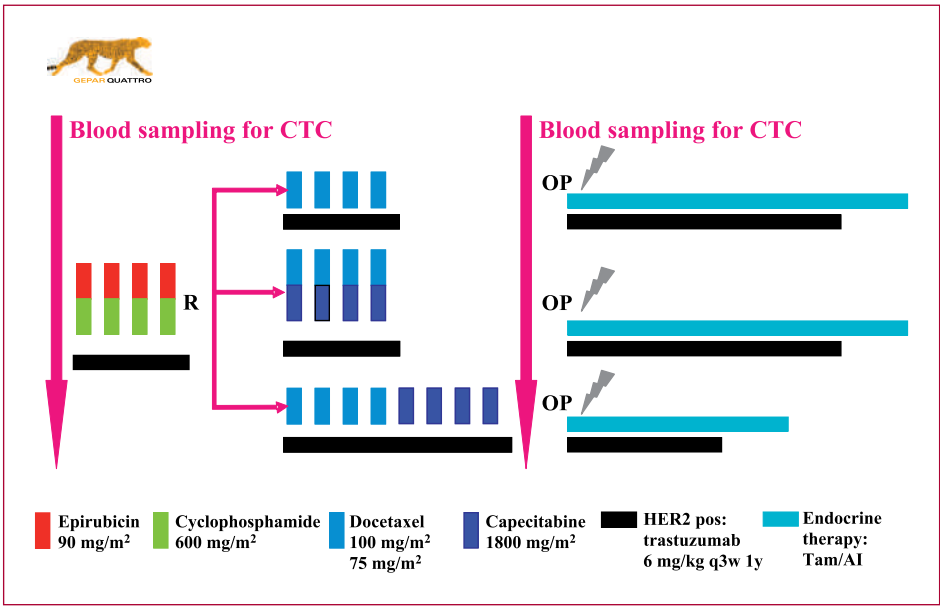

Fig. 1. Design of the GeparQuattro clinical study and time points of blood sampling. Each 7.5 mL blood sample was sampled prior to and after NT.

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Table 1. CTC detection in relation to patient characteristics

Clinical variable at baseline	Total no. of patients analyzed, *N* = 287 (%)	No. of patients with ≥1 CTC/7.5 mL (%)*	*P*	No. of patients with ≥2 CTC/7.5 mL (%)*	*P*
Age (y)					
<50	146 (50.9)	31 (21.2)	0.47	12 (8.2)	0.152
≥50	141 (49.1)	35 (24.8)		19 (13.5)	
cT					
T_1	9 (3.1)	1 (11.1)	0.849	0	0.54
T_2	187 (65.2)	44 (23.5)		21 (11.2)	
T_3	50 (17.4)	12 (24)		4 (8)	
T_4	41 (14.3)	9 (22)		6 (14.6)	
cN					
N_0	127 (44.3)	34 (26.8)	0.226	15 (11.8)	0.239
N_1	143 (49.8)	26 (18.2)		12 (8.4)	
N_2	14 (4.9)	5 (35.7)		3 (21.4)	
N_3	3 (1)	1 (33.3)		1 (33.3)	
Histology					
Ductal	233 (81.2)	56 (24)	0.462	27 (11.6)	0.404
Lobular	25 (8.7)	6 (24)		3 (12)	
Others	29 (10.1)	4 (13.8)		1 (3.4)	
Grading					
G_1	3 (1)	0	0.197	0	0.728
G_2	174 (60.6)	36 (20.7)		18 (10.3)	
G_3	97 (33.8)	28 (28.9)		12 (12.4)	
No data	13 (4.5)	2 (15.4)		1 (7.7)	
HER2					
Negative	182 (63.4)	41 (22.5)	0.804	21 (11.5)	0.596
Positive	105 (36.7)	25 (23.8)		10 (9.5)	
ER					
Negative	123 (42.9)	34 (27.6)	0.105	16 (13)	0.297
Positive	164 (57.1)	32 (19.5)		15 (9.1)	
PR					
Negative	131 (45.6)	36 (27.5)	0.098	20 (15.3)	0.026
Positive	156 (54.4)	30 (19.2)		11 (7.1)	
Triple negativity					
Yes	59 (20.6)	18 (30.5)	0.124	9 (15.3)	0.216
No	228 (79.4)	48 (21.1)		22 (9.6)	

*CTC positivity before and/or after NT.

Mayer's hemalaun solution (Merck) and permanently mounted. For negative controls, the primary antibody was omitted.

HER2-specific immunofluorescence was detected using the CB11 antibody (dilution 1:50; Novocastra) and Alexa 488 rabbit anti-mouse secondary antibody (1:200; Invitrogen) for 30 min at room temperature. MOPC-21 (at 15 μg/mL; Sigma), an unrelated mouse myeloma immunoglobulin, served as the IgG_1 isotype control. Slides were mounted with DAPI mounting medium (Vector Laboratories).

HER2 gene amplification of breast cancer cell line cells was determined by FISH using the HER2/Cen17 probes from Zytomed. After incubation at 80°C for 2 min, dehydration, and air-drying, cells were digested with a pepsin solution at 37°C for 7 min. Hybridization with the probe previously denatured for 7 min at 75°C was performed at 37°C for 16 h. After different stringent and nonstringent washing steps, cells were counterstained with DAPI. HER2 and centromer 17 signals were counted in 60 cells from each cell line and HER2 gene amplification was determined as the ratio between the mean numbers of HER2 and centromer 17 signals.

Statistical analysis. The statistical analysis was performed using SPSS 14.0 software (SPSS). Correlations between detection and HER2 expression of CTC and clinical or pathologic variables were analyzed by χ^2 or Fisher's exact tests. Two-tailed $P < 0.05$ values were considered statistically significant.

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Results

From the 287 patients enrolled in the translational subprotocol of the GeparQuattro study (Table 1), at least one blood sample was examined for CTC. The treatment outcome concerning primary tumor response for the overall patient cohort was described elsewhere (19, 20).

Detection of CTC. According to our previous investigation, samples were considered CTC-positive if ≥1 CTC per 7.5 mL blood was detected (17). In 66 out of 287 patients (23%), ≥1 CTC (mean 6, median 1) was detected in at least one sample either before or after NT. In 31 of 287 patients (10.8%), ≥2 CTC/7.5 mL blood (mean 11, median 3) were found, and a detection rate of ≥5 CTC was measured in 8 patients (2.8%).

Before NT, we detected CTC in 46 out of 213 patients (21.6%, 95% confidence interval, 16.3–27.7%). The number of CTC ranged between 1 and 200 with a mean value of 7.1. More than 1 CTC was detected in 19 of 213 (8.9%) patients and a detection rate of ≥5 CTC (i.e., prognostically relevant threshold for patients with metastatic breast cancer; ref. 8) was 3.3% (7 of 213 patients; Fig. 2A).

After NT, the incidence of CTC detection was lower than before NT ($P = 0.002$). Only 22 of 207 cases (10.6%, 95% confidence interval, 6.8–15.6%) were patients found to be CTC-positive with a mean value of 1.9 and CTC numbers ranging between 1 and 5. There was no correlation with either clinicopathologic tumor characteristics or age of the patients considering CTC positivity (≥1) at any time point (Table 1). Furthermore, the presence of CTC was not correlated with these factors either before or after NT (data not shown). This was also true for changes of CTC positivity during therapy.

In view of the fact that single cytokeratin-positive/CD45-negative cells could also be occasionally found in healthy controls (8, 21), we performed an additional analysis using a cutoff of two and more CTC/7.5 mL for CTC-positivity (Table 1). Positive findings were no longer observed in patients with small tumors (T1, $n = 9$), and the inverse correlation between CTC detection and progesterone receptor expression of the primary tumor became statistically significant ($P = 0.026$). All of the other results obtained with a cutoff of > 2 CTC remained statistically insignificant.

Matched blood samples from 133 patients were analyzed both before and after NT. We did not find statistically significant correlations of clinical and response characteristics of patients before versus after NT in this group of patients. At both time points, 100 of 133 patients (75.2%) were CTC-negative. Twenty (15.0%) initially CTC-positive cases were CTC-negative after NT, whereas 11 (8.3%) cases were CTC-positive (1 to 5 CTC) after NT, although no CTC could be found before NT. Although it did not reach statistical significance, the incidence of CTC in this smaller cohort of patients decreased during NT from 16.5% (22 of 133) to 9.8% (13 of 133). Figure 2B represents CTC values from 31 cases with changes in CTC numbers after NT. Only 2 of 133 (1.5%) patients stayed CTC-positive during NT (Fig. 2B). For the whole

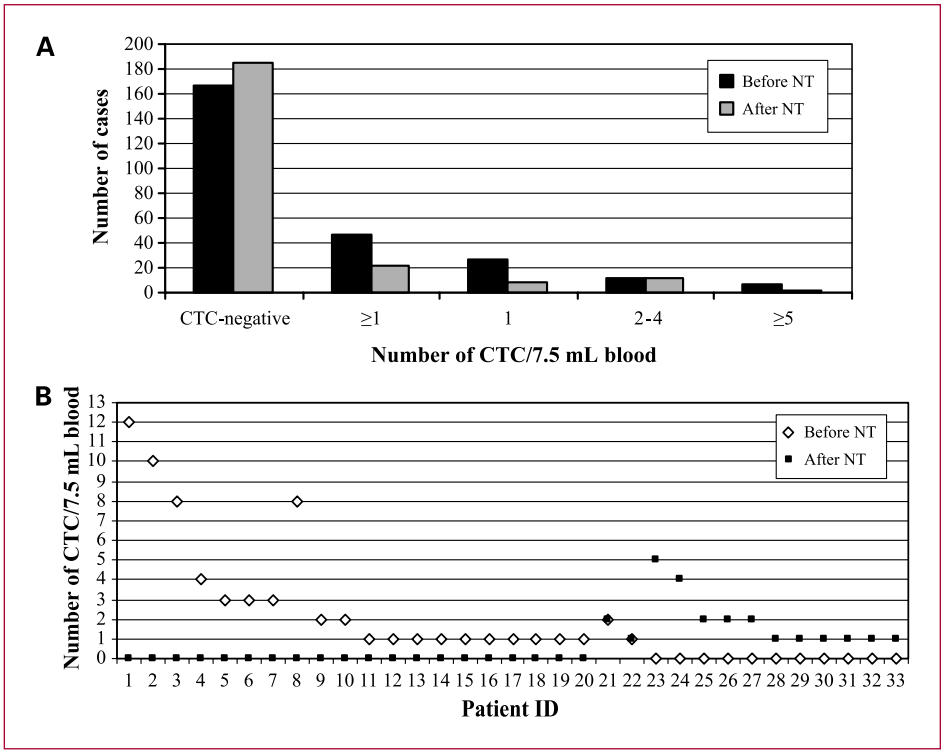

Fig. 2. A, frequency of CTC-negative versus CTC-positive patients as well as numbers of CTC detected in 7.5 mL of blood before and after NT. Black columns, number of cases with the corresponding CTC result detected before NT; gray columns, number of cases detected after NT. B, comparison of CTC numbers of individual patients detected in 7.5 mL of blood before (◇) and after (■) NT. No alterations in CTC numbers in cases 21 and 22.

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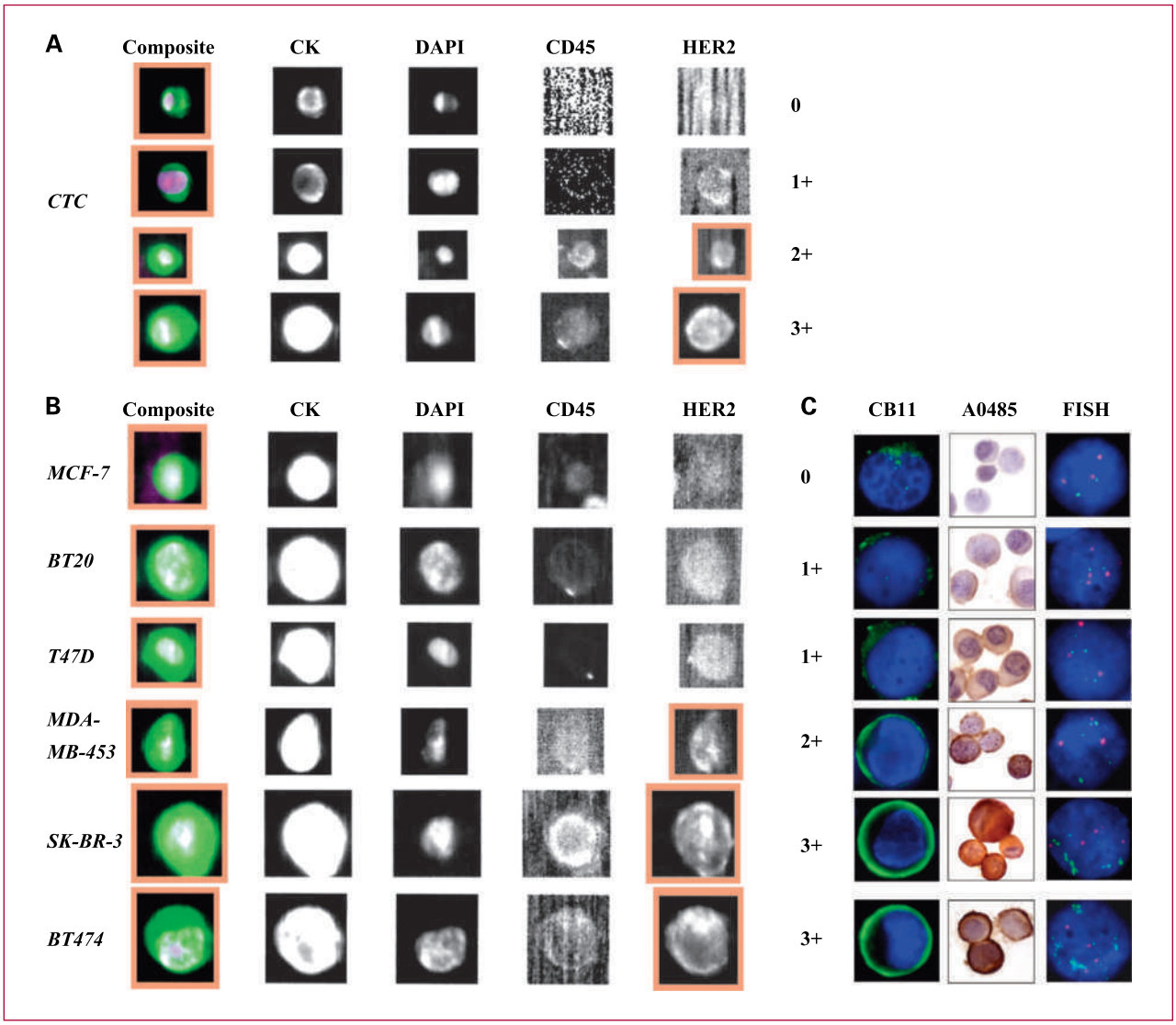

Fig. 3. Image galleries after CellSearch processing (A and B). CTC are cytokeratin (CK), DAPI-positive, and CD45-negative. HER2 expression of CTC (A) and breast cancer cell line cells added to blood (B) was determined using the FITC-labeled anti-HER2 antibody in the CellSearch system. Intensities of HER2-specific immunofluorescence of CTC (A) were categorized into negative (0), weak (1+), moderate (2+), and strong (3+) by comparing the results for CTC to those obtained with cell line cells (B). C, HER2 gene amplification of breast cancer cell line cells was determined by FISH (green signals, HER2; red signals, centromer 17). HER2 protein expression of these cell line cells was detected by immunocytochemistry using the antibodies CB11 (green immunofluorescence, HER2; blue, nuclear staining by DAPI) and A0485 (chromogenic detection: brown, HER2; blue, nuclear counterstaining).

patient cohort, the highest number of CTC measured in 7.5 mL blood before NT was 200 with no blood sample from this particular patient available after therapy.

Classification of HER2 status on CTC. MCF-7 cells with a mean HER2 gene copy number of 2 compared with a mean centromer 17 number of 2.6 did not show or exhibited only very weak immunofluorescence in the FITC channel of the CellSearch system. BT20 (mean HER2/Cen17: 3.5/3.7) and T47D (mean HER2/Cen17: 5.2/4.5) cells without HER2 gene amplification, but with an increased HER2 gene copy number presented with a weak HER2 immunofluorescence (Fig. 3). MDA-MB-453 cells with a low HER2 gene amplification (2.6-fold, mean HER2/Cen17: 8.1/3.1) were moderately to strongly HER2-positive (Fig. 3). Most SK-BR-3 (mean HER2/Cen17: clusters/5.3) and BT474 (mean HER2/Cen17: clusters/4.5) cells with strong HER2 overexpression based on HER2 gene amplification (at least 5- to 12-fold) exhibited a strong HER2 immunofluorescence (Fig. 3). The results of the CellSearch HER2 assay for these cell line cells were also consistent with results from immunostainings with the anti-HER2 antibodies A0485 (Dako) and CB11 (Novocastra). Consequently, strongly HER2-FITC–positive CTC were classified as "HER2-overexpressing or strongly positive" CTC (3+),

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whereas moderately HER2-FITC–positive CTC were termed "questionably HER2-positive" (2+), and weakly HER2-FITC–positive (1+) or FITC-negative CTC (0) were considered "HER2-negative."

In 58 out of 66 CTC-positive cases, HER2 expression of CTC was analyzed. Before NT, in 8 of 37 (21.6%) patients at least one CTC with a strong HER2-specific immunofluorescence was detected. A slightly higher percentage of HER2-positive CTC could be determined in blood samples drawn after NT (6 of 21, 28.6%; Table 2).

In 24 cases (14 cases with 2 CTC, and 10 cases with ≥3 CTC), more than 1 CTC was assessed for HER2 expression. Heterogeneity in HER2 expression of individual CTC from the same patient was observed in only three cases. One of these patients had one CTC with weak (1+) and one with strong (3+) HER2 immunofluorescence. From another patient, 9 of 11 CTC were strongly HER2-positive (3+), whereas the other 2 were moderately positive (2+). Three of five CTC from the third patient were categorized as 2+, whereas the others were only weakly HER2-positive (1+). Cases were categorized as CTC HER2 2+ or 3+ if at least one CTC showed moderate or strong HER2 immunofluorescence.

HER2 status of CTC and corresponding primary tumors. In 26 patients with HER2-negative primary tumors, CTC detected before NT were analyzed for HER2 expression (Table 2). Although in 19 (73.1%) of these cases, CTC were also classified as HER2 0 or 1+, 19.2% (5 of 26) of patients had CTC with a strong HER2 immunostaining (3+), and in 2 cases, CTC were classified as HER2 2+ (Table 2). Discordant HER2 expression was also found in patients with HER2-positive primary tumors in which 5 of 11 (45.4%) patients had CTC classified as HER2 0 or 1+. Only 3 of 11 (27.3%) patients with HER2-positive primary tumors also had HER2-positive CTC (3+).

CTC detected after NT were HER2-negative or HER2 1+ in 8 of 11 (72.7%) patients with HER2-negative primary tumors, whereas discordant results were found in 3 of 11 (27.3%) cases. Moreover, 6 of 10 patients (60.0%) with HER2-positive primary tumors had HER2-negative CTC after NT (Table 2). Moderate to strong HER2 expression of CTC was more frequently detected in patients with a high tumor stage (T_4 compared with T_1–T_3 tumors, $P = 0.002$; Supplementary Table S1). Furthermore, strong HER2 immunostaining (3+) of CTC was only found in ductal, but not in lobular, and other breast cancer types (Supplementary Table S1).

There was no correlation between HER2 expression of CTC and age, clinical lymph node stage of the patients, histologic grading, estrogen receptor/progesterone receptor (ER/PR) status, and response of the primary tumors to NT (Supplementary Table S1). Although not reaching statistical significance ($P = 0.113$), there was a higher frequency of patients with moderately and strongly HER2-expressing CTC (2+ and 3+) and HER2-positive primary tumors (10 of 21, 47.6%) compared with those with HER2-negative primary tumors (10 of 37, 27%; Supplementary Table S1).

CTC detection and tumor response to NT. Pathologic complete response of patients was significantly associated with high tumor grade, ER and PR negativity, HER2 positivity, and triple negativity (Supplementary Table S2). Neither CTC detection before nor after NT were predictive of primary tumor pathologic complete response (Fig. 4A). Changes in CTC detection during NT were also not significantly correlated to primary tumor response (Fig. 4B).

As shown in Table 2, CTC from 10 patients were still detectable after trastuzumab therapy. In six of these patients, only HER2-negative or weakly positive CTC were found after NT including trastuzumab treatment. There also were CTC with strong (3+) or moderate (2+) HER2 immunostaining from four patients after trastuzumab treatment (Table 2).

Considering patients with HER2-positive primary tumors who additionally received trastuzumab therapy, there seems to be a tendency to a higher rate of pathologic complete response in patients with HER2-positive CTC detected before NT compared with patients with HER2-negative CTC; however, patient numbers in each group were too small to reach statistical significance (data not shown).

CTC detection and preliminary clinical follow-up of patients. From 136 patients analyzed for the presence of CTC, follow-up data with a maximal observation time of 42

Table 2. Comparison of HER2 expression in CTC and corresponding primary tumors

	Primary tumors			
	HER2-negative (IHC 0, 1+ or FISH-negative)		HER2-positive (IHC 3 + or FISH-positive)	
	Before NT	After NT	Before NT	After NT including trastuzumab
No. of CTC-positive cases analyzed (before/after NT)	26	11	11	10
CTC HER2-negative (0)	15 (57.7%)	5 (45.4%)	4 (36.4%)	5 (50%)
CTC HER2-negative (1+)	4 (15.4%)	3 (27.3%)	1 (9%)	1 (10%)
CTC HER2 questionable (2+)	2 (7.7%)	0	3 (27.3%)	1 (10%)
CTC HER2-strongly positive (3+)	5 (19.2%)	3 (27.3%)	3 (27.3%)	3 (30%)

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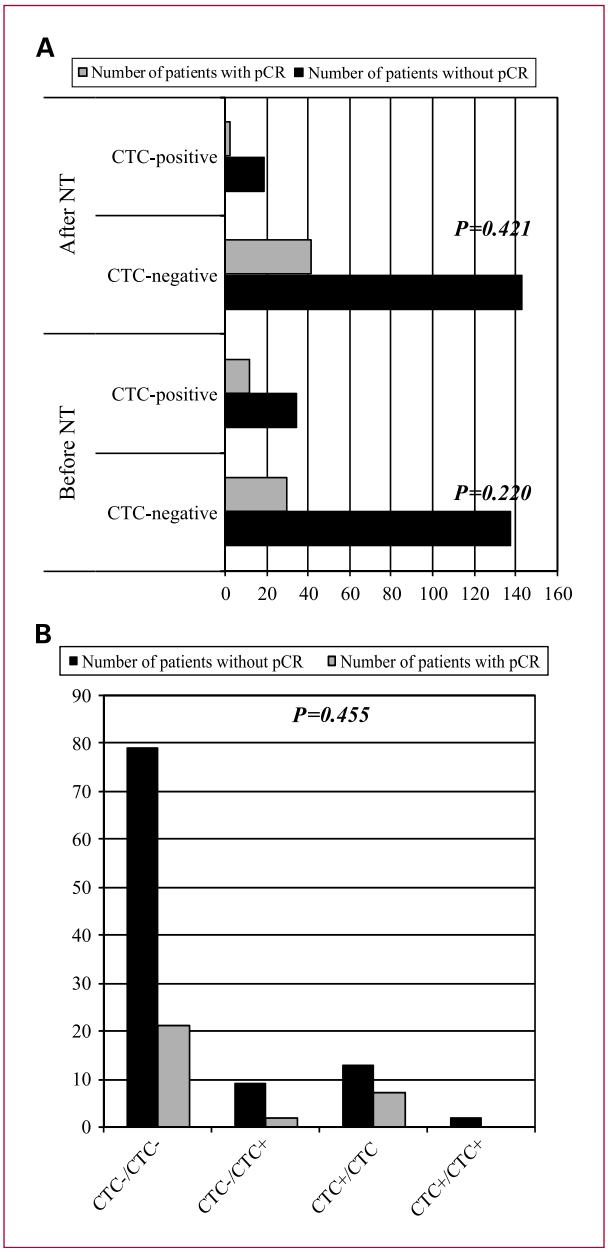

Fig. 4. A, tumor response to NT in relation to the detection of CTC before and after NT. Gray and black columns, the number of patients (*X*-axis) with and without pathologic complete response (pCR), respectively. B, tumor response to NT related to CTC detection in patients analyzed for CTC before and after NT (n = 133; CTC–/–, CTC-negative before and after NT; CTC–/+, CTC-negative before, but CTC-positive after NT; CTC+/–, CTC-positive before and CTC-negative after NT; CTC+/+, CTC-positive before and after NT). Gray and black columns, the number of patients (*Y*-axis) with and without pathologic complete response (pCR), respectively.

months were available. Mean and median observation times were 13.9 ± 8.8 months and 12 months, respectively. Observation times did not differ between patients with and without detected CTC (P = 0.45). Within the group of CTC-positive patients (n = 36), three patients had evidence of relapse of the disease and one patient died, whereas in the group of CTC-negative patients (n = 100), eight patients presented with relapse. Because of the small number of events in both groups, the effects of the Kaplan-Meier survival analysis was limited.

Discussion

The translational research project described here is of high clinical relevance because a better understanding of targeted therapies and the ability to monitor therapy in the nonmetastatic setting might help to improve the treatment outcome of patients with breast cancer. To our knowledge, the present study is the largest evaluation of CTC in the context of NT, and is the first study to examine HER2 expression on CTC in this treatment setting. Furthermore, we addressed the relevant questions, whether HER2-positive CTC still persists after trastuzumab treatment and/or whether HER2-negative CTC are being selected by trastuzumab treatment.

For metastatic breast cancer, CTC detection has proven to be of prognostic relevance (8–10, 22), whereas the role of CTC detection in patients with early breast cancer is less well described (7, 23–25), especially before surgery and during NT when the tumor is still present (15, 16). In our study, the incidence of CTC was 21.6% prior to any kind of therapy and decreased to 10.6% after NT. Similar results were obtained by Pierga et al. in a smaller cohort of patients enrolled in the phase II randomized REMAGUS 02 trial, in which HER2 status on CTC was not examined (15). We and Pierga et al. did not observe a correlation between CTC detection and primary tumor characteristics such as tumor stage, clinical lymph node stage, and HER2 or hormone receptor status. In contrast, Lang et al., also applying the CellSearch approach, reported a significantly increased incidence of CTC in patients with HER2-positive compared with HER2-negative primary breast cancer in blood samples from 92 patients (26). However, patient cohorts and percentages of patients with HER2-positive primary tumors were not comparable between the different studies (15, 26).

Similar to the study by Pierga et al. (15), we also observed no significant correlation between CTC detection and the primary tumor response to NT. However, we cannot exclude the possibility that this lack of correlation might be influenced by statistical "noise" inherent in performing analyses with small numbers of CTC, and future studies with more sensitive technologies and/or larger cohorts of patients should be helpful to draw more robust conclusions. In contrast to our findings, using the MAINTRAC method, Camara et al. correlated a decrease in the number of circulating epithelial cells with radiologic response of the primary tumor to NT (16). However, only 58 patients were enrolled and the tumor cell numbers reported with their system were several log units higher than those detected with more specific and Food and Drug Administration–approved methods. In a previous study published by these authors in a cohort of 91 patients with

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nonmetastatic breast cancer, a 10-fold or higher increase in the number of CTC at the end of systemic chemotherapy was a strong predictor of relapse (27).

Interestingly, Pierga et al. and Bidard et al. observed a prognostic effect of CTC detected before and/or after therapy for early relapse (15, 28). At the annual ASCO meeting in 2008, Rack and coworkers presented results from the adjuvant therapy trial SUCCESS A also using the CellSearch system (29). They found a prognostic relevance for CTC as well, but only for those patients with CTC detected after completion of chemotherapy. These results support the idea that CTC detected by immunocytochemistry with the same method used in our study had biological relevance. Furthermore, encouraging results on the association between CTC detection by reverse transcription-PCR approaches and metastatic relapse in patients with breast cancer at various disease stages have been recently published, indicating that the detection of CTC is of prognostic relevance (7, 30–36). In our preliminary follow-up analysis, the prognostic effect of CTC detection on the early progression and/or overall survival of the patients is still limited, underlining that longer observation times are needed.

To our knowledge, the CellSearch system used here is the only system that has validated the stability of blood samples from patients with breast cancer as a prerequisite for analyses in a multicenter setting (17, 21). Although Rack et al. analyzed a total of 22.5 mL of blood with an additional preanalytic gradient enrichment step (29), CTC detection within the neoadjuvant studies described by Pierga et al. and in our study were performed on the standard volume of 7.5 mL of blood. Another important point is the cutoff value used to consider a sample CTC-positive. For patients with metastatic breast cancer, this cutoff value was ≥5/7.5 mL, whereas there are no study results available for patients with nonmetastatic disease. We found ≥1 CTC/7.5 mL blood in ~20% of patients preoperatively, whereas 5 or more CTC were only detected in <5% of preoperative samples in our study similar to the study of Pierga et al. (15). Interestingly, Tibbe et al. developed a model describing the statistics of the different process steps that are needed for the isolation and detection of CTC (37). They concluded that elimination of the errors caused by the variability between readers of the CTC results might reduce the current cutoff value of 5 to 1 per 7.5 mL of blood at least for patients with metastatic breast cancer (37). As previously described by us and others, CTC with a threshold of 1 CTC were not detected in healthy individuals (15, 17, 38). Thus, we decided to consider patients with ≥1 CTC per 7.5 mL blood as CTC-positive.

Moreover, it is under debate whether the CellSearch system could detect CTC from all breast cancer subtypes (39–42). Very recently, Sieuwerts et al. published that the CellSearch system, using EpCAM and cytokeratin expression for tumor cell enrichment and detection, respectively, was not able to recognize breast cancer cell line cells of normal-like type (42). This tumor type is very aggressive and markers to specifically identify these cells are very difficult to establish. In our study, we did not observe a correlation between CTC detection and triple negativity for HER2, ER, and PR, a frequent characteristic of normal-like breast cancers as described by Sieuwerts et al. (42). Sieuwerts et al. also reported that normal-like breast cancer cells express the CD44+/CD24− stem cell phenotype, and vimentin, a possible indication of an epithelial-mesenchymal transition (43). However, because antibodies against CD44+/CD24 and vimentin detect hematopoietic cells, they are applicable for CTC detection only when used in combination with epithelium-specific markers that are not completely downregulated in the course of epithelial-mesenchymal transition such as cytokeratin 5 (44).

HER2 is a prominent therapeutic target in breast cancer, and trastuzumab, a monoclonal antibody directed against this epidermal growth factor receptor, prolongs survival in the adjuvant and metastatic setting (45, 46). The expression of HER2 in primary tumors is a prerequisite for trastuzumab treatment of patients with breast cancer (45). However, the optimal use of trastuzumab and various other drugs targeting HER2 currently in different phases of development is not yet clear. Therefore, there is an urgent need for identifying factors that enable the monitoring of therapy especially in the adjuvant setting. Apostolaki and coworkers (47) have observed a prognostic effect from CTC after adjuvant treatment using HER2 mRNA as marker. In this study, the detection of HER2 mRNA-positive CTC after chemotherapy was associated with a reduced disease-free survival. Considering the frequency of HER2-positive CTC in their study (21%), one has to bear in mind that HER2 is usually not overexpressed or amplified in all CTC, as indicated by our present findings. Furthermore, RNA-based methods are not able to detect heterogeneity among CTC populations. Thus, methods that detect HER2 expression of individual CTC might circumvent these drawbacks. As indicated by Wülfing and coworkers in a cohort of 42 patients, the presence and frequency of HER2-positive CTC detected by immunocytochemistry correlated significantly with both decreased disease-free and overall survival (13).

Currently, there is no standardized and widely accepted method available for the determination of HER2 status on CTC. Therefore, in the studies reported here, we have performed extensive validation experiments of the immunocytochemical method for HER2 determination on CTC using breast cancer cell line cells with known HER2 gene amplification status.

We observed a strong HER2 expression of CTC in ~20% of patients with primary tumors classified as HER2-negative (19.2% before and 27.3% after NT). This frequency was not significantly different from that observed in patients with HER2-positive primary tumors (27.3% before and 33% after NT). Nevertheless, there was an association between HER2 expression in primary tumors and CTC in certain breast cancer subgroups. The incidence of HER2-overexpressing CTC was higher in patients with inflammatory breast cancer

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(stage T_4 tumors) and ductal breast carcinomas known to also exhibit a higher frequency of HER2 overexpression in their respective primary tumors (48–51). Wülfing and coworkers also found HER2-positive CTC to be associated with larger tumor size, but additionally with negative ER status and worse histologic differentiation (13). Our results suggest that patients with either HER2-positive or HER2-negative primary tumors might benefit from anti-HER2 treatment strategies. This corroborates published data by Paik et al. in 1,787 patients with follow-up data, in which some patients with HER2 negative primary tumors also seemed to benefit from trastuzumab treatment (52). Whether trastuzumab in the context of the NT regimen has an additional response effect on the tumors of patients with HER2-positive CTC remains to be elucidated in larger patient cohorts. In a study by Gajda et al. on only 26 patients with HER2-positive tumors, that had an excellent tumor response to NT (including trastuzumab), CTC were still detected (53). In their study, a decrease in the number of CTC (without determining the HER2 status) was only observed in patients who received trastuzumab after surgery and this was correlated to a decreased relapse rate (53). Interestingly, in our study, CTC from 10 patients were still detected after trastuzumab treatment (including HER2-negative or weakly HER2-positive CTC from six patients with HER2-positive primary tumors), which suggests a therapy-induced selection of HER2-negative tumor cells. Additionally, HER2-overexpressing CTC were present in three patients with HER2-positive primary tumors after trastuzumab treatment, probably representing CTC resistant against this therapy. These results underline the urgent need for the identification of additional therapeutic targets. However, the lack of correlation in HER2 expression between the primary tumors and CTC observed in the current study could be biased by the fact that only one CTC was available in most patients for HER2 analysis. Thus, the development of more sensitive CTC assays is also of high relevance for the characterization of CTC.

Several small studies reported the use of CTC for re-evaluating the HER2 status in metastatic breast cancer. Meng et al. (14) reassessed the HER2 status in 31 metastatic patients with CTC. Nine of 24 patients (37.5%) with initially HER2-negative tumors had HER2-positive CTC. Four of these nine patients were treated with trastuzumab, three of which showed partial or complete remission. HER2 status was reassessed by Fehm et al. (54) on CTC at the time of metastatic disease in 21 patients with initially HER2-negative breast cancer that indicated HER2 overexpression in 8 of these cases (38.1%). These data support the conclusion that measurement of HER2 expression of CTC from patients with metastatic breast cancer will also help identify patients who might benefit from trastuzumab treatment.

One of our goals for future research will be to understand the biology of cells surviving trastuzumab treatment that are probably responsible for metastatic spread. Based on these results, it will be of great interest to design clinical trials to correlate clinical responses to HER2-targeted therapy based on HER2-positive CTC in adjuvant and metastatic breast cancer. Determining the HER2 expression of CTC will also be of relevance for study designs including new therapeutic approaches targeting HER2. The ongoing study GeparQuinto randomizes patients between trastuzumab and lapatinib, also targeting HER2, and additionally, epidermal growth factor receptor as NT; we are currently examining HER2 expression on CTC in these patients (http://www.germanbreastgroup.de/geparquinto). In the context of this trial, it should be possible to examine if trastuzumab and lapatinib treatment affect the kinetics and HER2 expression on CTC.

In summary, our findings support the hypothesis that detection and characterization of CTC could help to better understand the effect of NT on disseminating tumor cells, which may eventually lead to an improvement of current treatment strategies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Clinical Cancer Research

Detection and HER2 Expression of Circulating Tumor Cells: Prospective Monitoring in Breast Cancer Patients Treated in the Neoadjuvant GeparQuattro Trial

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