HER2-Positive Circulating Tumor Cells Indicate Poor Clinical Outcome in Stage I to III Breast Cancer Patients

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Abstract

Purpose: Early metastasis in node-negative breast cancer indicates that breast cancer cells obviously can bypass the lymph nodes and disseminate directly hematogenous to distant organs. For this purpose, we evaluated the prognostic value of blood-borne, HER2-positive circulating tumor cells (CTC) in the peripheral blood from 42 breast cancer patients with a median follow-up of 95 months.

Experimental Design: Cells were isolated by the patented combined buoyant density gradient and immunomagnetic separation procedure and analyzed by immunocytochemistry.

Results: We detected one to eight CTCs in the peripheral blood of 17 of 35 patients (48.6%) presenting no overt metastasis. As a positive control, 7 of 7 (100%) patients with metastatic disease presented positive. Healthy persons and patients ($n = 32$) operated for nonmalignant diseases presented negative for CTCs. The presence and frequency of HER2-positive CTCs correlated with a significantly decreased disease-free survival ($P < 0.005$) and overall survival ($P < 0.05$). Interestingly, in 12 patients with HER2-positive CTCs, the primary tumor was negative for HER2 as assessed by immunohistochemical score and fluorescence *in situ* hybridization.

Conclusions: This study provides some evidence of a prognostic effect of HER2-positive CTCs in stage I to III breast cancer. Future studies have to determine the outcome of patients treated with HER2-targeting therapies with respect to HER2-positive CTC levels because it is not unlikely that high levels of HER2-positive CTCs reflect the activity of the tumor and may predict response to trastuzumab.

Breast cancer, even in stage I and II, obviously can bypass the lymph nodes and disseminate directly hematogenous to distant organs, consequently adjuvant therapy is given to lower the risk of metastasis (1, 2). These cells have been referred to as "micrometastases" (disseminated tumor cells; ref. 3). Clinical studies consistently report an independent negative prognostic value of disseminated tumor cells in the bone marrow of breast cancer patients at primary diagnosis (4–6). In many cancer patients, circulating tumor cells (CTC) have been identified in the peripheral blood. Because CTCs are present not only in patients with metastatic disease but also in those at stage I to III cancer, these cells may represent an undetected spread of the tumor and serve also as a prognostic marker (7). In addition, CTCs have been reported to reflect the outcome of systemic treatment (8, 9). Therefore, detection of CTCs in these patients using prognostic markers, such as HER2 expression, may help to understand their biology and clinical relevance.

The objective of the present study was to evaluate the prognostic value of HER2-positive CTCs in the peripheral blood of stage I to III breast cancer patients. The presence and frequency of HER2-positive CTCs has been correlated with clinicopathologic variables and with patients' follow-up. To the best of our knowledge, this is the first study to analyze the prognostic value of HER2-positive CTCs from the peripheral blood of breast cancer patients. Because HER2 overexpression indicates a poor prognosis in breast cancer, analysis of HER2-positive CTCs in the peripheral blood of breast cancer patients may also help to identify high-risk patients who could benefit from targeting HER2.

Patients and Methods

Patients. Forty-two patients who were diagnosed with primary breast cancer between 1995 to 1996 at the Department of Gynecology, University of Münster, Germany were included. Thirty-five patients had no sign of overt metastasis at the time of primary diagnosis and have been evaluated for further clinical follow-up. Seven patients had distant metastases at diagnosis and have been considered as a positive reference group. The follow-up group was characterized as follows: mean age at diagnosis was 51 years (range, 29-72 years). Seven (20%) patients were premenopausal, 4 (11.4%) patients were perimenopausal, and 24 (68.6%) patients were postmenopausal. None of the patients had a

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history of previous cancer. Details on clinicopathologic data (e.g., tumor stage, lymph node status, and grading) are shown in Table 1. Surgical therapy consisted of breast conserving therapy (31.4%) or modified radical mastectomy (68.6%). Breast conservation was combined with postoperative ipsilateral radiotherapy. Furthermore, information regarding adjuvant therapy was obtained: 18 patients received a cytotoxic chemotherapy (51.4%), and 8 patients were exclusively treated with endocrine therapy (22.9%). Oncologic follow-up was monitored at our Department or at an outpatient institution according to national guidelines for treatment and follow-up of breast cancer patients (*Tumorzentrum München* 1994). Median follow-up was 95 months. In general, clinical examinations were done every 3 months for the first 3 years, then every 6 months for 2 years, and once a year thereafter. Routine mammography was initially done every 6 months for the first 3 years and thereafter once a year. Other examinations were done when indicated.

Separation of HER2-positive CTCs. Analysis for hematogenously spread HER2-positive epithelial cells was done using the patented combined buoyant density gradient and immunomagnetic separation procedure as described previously (10–12). In brief, 50 mL of EDTA-treated peripheral blood was taken before surgery. For enrichment and separation of epithelial cells, the samples were processed by buoyant density gradient centrifugation (13) and an immunomagnetic separation procedure (14). For immunocytochemical staining of isolated cells, 150 μL of the positive magnetic fraction (final MACS outlet) were centrifuged onto glass slides using a cytocentrifuge (Cytospin 2, Shandon, Pittsburgh, PA). The slides were then incubated for 20 minutes with 10% human AB serum with 0.1% bovine serum albumin-C (Aurion, Leiden, the Netherlands) and for 45 minutes with a rabbit polyclonal HER2 antibody (21N, 7 μg/mL; DAKO, Hamburg, Germany). The antibody was decorated with a 5-nm gold-conjugated goat anti-rabbit antibody (Paesel and Lorei, Hanau, Germany) at a dilution of 1:50. After fixation (2% glutaraldehyde in PBS) and a blocking step (5% mouse serum, DAKO), cells already stained with a biotinylated anti-cytokeratin antibody were incubated in alkaline phosphatase–conjugated streptavidin (1.5 μg/mL; Jackson ImmunoResearch, West Grove, PA). Cytokeratin was visualized immunoenzymatically by use of Newfuchsin (DAKO). For visualization of HER2 staining, the colloidal gold particles were enhanced using a silver enhancement kit (IntenSE, Amersham, Braunschweig, Germany). A breast cancer cell line (SKBR-3) and leukocytes were used as positive and negative controls for the staining procedure, respectively. The specificity of the assay was confirmed in 15 healthy volunteers (male laboratory employees), 12 patients suffering from benign gynecologic diseases and 5 patients who underwent cardiovascular surgery, detecting no false-positive cases (10). The presence and frequency of HER2-positive CTCs have been correlated with clinicopathologic variables and follow-up data.

Table 1. Characteristics of patients examined for HER2-positive CTCs

Clinicopathologic variables	*n* (%)
Tumor stage*	
pT_1	19 (55.9)
pT_2	9 (26.5)
pT_3	3 (8.8)
pT_4	3 (8.8)
Lymph nodes*	
N_0	25 (75.8)
N_+	8 (24.2)
Histologic grade	
G1	4 (11.8)
G2	17 (50)
G3	13 (38.2)
Tumor type	
Ductal	22 (62.9)
Lobular	9 (25.7)
Tubular	3 (8.6)
Medullary	1 (2.9)

*Information on pT stage was available in 34 of 35 patients (97.1%) and information on pN stage was available in 33 of 35 (94.3%) patients.

Tumor tissue microarray. To further characterize the patients' tumors, formalin-fixed and paraffin-embedded tumor samples of these patients were obtained from the archives of the Gerhard-Domagk Institute of Pathology, serving as donor blocks for the tissue microarray. Sections were cut from each donor block and stained with H&E. Using these slides, morphologically representative regions were chosen from each of the 35 tumor samples and circled. Six cylindrical 0.6-mm cores were acquired from the circled areas of each breast carcinoma and precisely arrayed into a new recipient paraffin block (20 × 35 mm) using a manual tissue arrayer (Beecher Instruments, Silver Spring, MD). The presence of invasive breast cancer in the arrayed samples was verified on H&E-stained sections.

Immunohistochemistry. Antibodies, dilutions, and antigen retrieval methods used are as follows: estrogen receptor (ER; DAKO, 1D5; 1:200; steamer; LSAB; nuclear), progesterone receptor (PR; DAKO, 636; 1:1,000; steamer; LSAB; nuclear), HER2 (DAKO; 1:4,000; steamer, LSAB, membranous), epidermal growth factor receptor (EGFR; DAKO, H11; 1:5; Proteinase K; LSAB; membranous), Ki-67 (DAKO, Mib-1; 1:100; steamer; LSAB; nuclear), bcl-2 (DAKO, 124; 1:1,000; steamer; LSAB; cytoplasmic), p53 (DAKO, DO-7; 1:2,000; steamer; LSAB; nuclear). The tissue microarray block was cut with a microtome into 4-μm sections that were mounted on poly-L-lysine–coated glass slides and processed for immunohistochemistry. After deparaffinization and rehydration, unspecific binding was blocked. Then, antigen retrieval by different pretreatment methods was done. For immunohistochemistry, an automated immunostainer (DAKO autostainer) was used. After incubation for 25 minutes with the primary antibody and detection, sections were counterstained with hematoxylin. Appropriate negative (the first antibody was either omitted or replaced by nonimmune rabbit IgG diluted to the same concentration as the first antibody) and positive controls were used throughout. Immunohistochemical expression of ER and PR, EGFR, Ki-67, bcl-2, and p53 was graded in a binary fashion. ER, PR, and p53 were considered as positive if ≥10% of nuclei stained positive. Staining for Ki-67 was positive if >20% of cells presented with nuclear staining. In bcl-2–positive cases, >20% of neoplastic cells showed cytoplasmic staining. EGFR staining was positive in case of any membrane staining. Expression of HER2 was assessed as follows: 0, membrane staining in <10% of tumor cells; 1+, faint or incomplete membrane staining in >10% of cells; 2+, weak or moderate complete or incomplete staining in >10% of cells; 3+, strong complete membrane staining in >10% of cells. Tumors scored as 3+ were clearly HER2-positive cases; tumors scored as 0/1+ were designated as HER2-negative cases; borderline cases (2+) required further investigation by fluorescence *in situ* hybridization to assess whether they show gene amplification.

Data analysis. Semiquantitative analysis of staining results was done in blind-trial fashion without knowledge of the clinical data for the corresponding case. Statistical analysis was done with SPSS 10.0 statistical software. Correlations between presence of HER2-positive CTCs and clinicopathologic variables were tested for statistical significance by χ^2 test. For analysis of survival data related to HER2-positive CTCs, Kaplan-Meier survival estimates were generated and compared with the log-rank test. Disease-free survival was calculated as time from the date of diagnosis to the first recurrence of disease at local, regional, or distant site or death. Overall survival

Table 2. Identification and number of HER2-positive CTCs in stage I to III primary breast cancer patients ($n = 35$) and primary metastatic patients (M_+, $n = 7$) as positive controls

No. per sample	Patients (%)
Stage I-III patients	
0	18 (51.4)
1	4 (11.4)
2	2 (5.7)
3	3 (8.6)
4	3 (8.6)
7	1 (2.9)
8	4 (11.4)
0	18 (51.4)
1-4	12 (34.3)
≥5	5 (14.3)
No	18 (51.4)
Yes	17 (48.6)
M_+ patients (positive controls)	
3-50	7 (100)

ended with the date of tumor-associated death. Multivariate analysis was done using Cox's proportional hazards regression model. Level of significance was $P < 0.05$.

Results

Incidence of circulating cytokeratin/HER2 double-positive cells. Predominantly, HER2-positive CTCs were detected in the peripheral blood of 17 from 35 stage I to III patients (48.6%). In samples positive for CTCs, the mean number of cells per sample was 4 (range, 1-8). Table 2 presents the frequency of HER2-positive CTCs detected per sample. As positive controls, patients suffering from primary metastatic cancer were analyzed (stage M_+, $n = 7$). All M_+ patients presented with HER2-positive CTCs. For negative control blood from 32 persons not suffering from cancer (15 healthy employees, 5 patients from chest surgery, and 12 women with benign breast diseases) was investigated without the detection of HER2-positive CTCs.

Immunohistologic staining of primary tumors. Of the 35 primary carcinomas in follow-up, 52.9% and 58.8% were classified ER and PR positive, respectively; 11.1% tumors were classified to be HER2 positive (score, +3 or +2/fluorescence *in situ* hybridization positive) or EGFR positive. A high Ki-67 expression (MIB-1 labeling index >20%) was observed in 35.5% of the tumors; 29.2% tumors expressed mutant p53, and 52% were bcl-2 positive. Associations between expression patterns of the different immunohistochemical factors were as expected (e.g., positive correlation of ER/PR, $P < 0.001$; concordance of HER2 and EGFR expression, $P < 0.001$), supporting the representativeness of the series (data not shown).

Relationship of the detection of HER2-positive CTCs to staging, grading, hormone receptors, and immunohistochemical status. As shown in Table 3, HER2-positive CTCs were found to be associated with larger tumor size (pT stage), negative ER status, and worse histologic differentiation, significantly. Furthermore, in the blood of all patients with an inflammatory carcinoma ($n = 3$) and the patients staged primarily M_+ ($n = 7$), HER2-positive CTCs were found. There was no significant correlation with lymph node status (pN stage), PR status, or proliferation rate (Ki-67). However, we observed a significant discrepancy between the detection of HER2-positive CTCs and the HER2 status of the corresponding primary tumor: in 12 patients with HER2-positive CTCs, the primary tumor showed a negative score for HER2, indicating that the amount of HER2-positive cells in the primary tumor fall below 10%. In those cases, no fluorescence *in situ* hybridization analysis was done. We observed no correlations between HER2-positive CTCs and EGFR expression, p53 mutation, and bcl-2 expression.

Table 3. Correlation between presence of circulating HER2-positive tumor cells and clinicopathologic variables in primary breast cancer patients ($n = 35$)

Clinicopathologic variables	Patients with HER2-positive CTCs, n/total (%)	P^*
pT stage		
pT_2	11/29 (37.9)	0.006
pT_3	6/6 (100)	
Lymph node status		
Negative	10/25 (40)	NS
Positive	5/8 (62.5)	
Histologic grading		
I	0/4 (0)	0.033
II-III	17/30 (56.7)	
ER status		
Positive	6/18 (33.3)	0.039
Negative	11/16 (68.7)	
PR status		
Positive	8/20 (40)	NS
Negative	9/14 (64.3)	
HER2 status		
Negative	12/24 (50)	NS
Positive	2/3 (66.7)	
EGFR status		
Negative	2/3 (14.3)	NS
Positive	12/24 (50)	
MIB-1 labeling index		
20%	8/19 (42.1)	NS
>20%	8/12 (66.7)	
bcl-2 status		
Negative	6/6 (100)	NS
Positive	7/13 (53.8)	
p53 mutation		
No	10/17 (58.8)	NS
Yes	4/7 (71.4)	

NOTE: Staining was evaluable for HER2 and EGFR in 27 (77.1%), for Ki-67(MIB-1 labeling index) in 31 (88.6%), for bcl-2 in 25 (71.4%), and for p53 in 24 (68.6%) of 35 patients with primary breast cancer.
Abbreviation: NS, not significant.
*χ^2 test.

Table 4. Disease-free and overall survival in relationship to the detection of circulating HER2-positive tumor cells

HER2-positive CTCs	*n*	Disease-free survival, mo (95% confidence interval)	*P**	Overall survival, mo (95% confidence interval)	*P**
No	18	124 ± 7 (110-139)	0.007	125 ± 7 (110-139)	0.024
Yes	17	60 ± 11 (38-83)		89 ± 15 (60-117)	
0	18	124 ± 7 (110-139)	0.003	125 ± 7 (110-139)	0.021
1-4	12	72 ± 13 (46-97)		80 ± 12 (57-103)	
≥5	5	32 ± 16 (1-62)		61 ± 29 (4-118)	

*Log-rank test.

Association between presence of HER2-positive CTCs and survival. Median follow-up was 95 months. During that time, local recurrence occurred in two patients (5.7%). Distant metastases occurred in 8 patients (22.9%), all of whom died from metastatic disease. Median disease-free survival was 92.5 months (range, 0-132 months; mean, 74.3 months; SD, 40.1 months), and median overall survival was 93.5 months (range, 6-132 months; mean, 79.0 months; SD, 37.3 months). A significant correlation between the presence and frequency of HER2-positive CTCs and both a decreased disease-free and overall survival was observed. Results on survival analysis with respect to HER2-positive CTCs are summarized in Table 4; Fig. 1 shows the corresponding Kaplan-Meier curves. In particular, HER2-positive CTCs have been detected in the blood samples of all four patients who subsequently developed bone metastases.

Multivariate analysis. To evaluate the independent prognostic value of HER2-positive CTCs for disease-free survival and overall survival, multivariate survival analysis was done. The presence of HER2-positive CTCs (yes versus no), pT stage (pT_1-T_2 versus pT_3-T_4), lymph node status (pN_0 versus pN_+), histologic grading (G_1 versus G_2-G_3), and ER and PR status (positive versus negative) were entered as covariates. Only tumor size (pT) was an independent prognostic marker (P = 0.004). The presence of HER2-positive CTCs almost reached borderline statistical significance (P = 0.060).

Discussion

In 1998, our group first reported isolation of HER2-positive CTCs from the peripheral blood of breast cancer patients with organ-confined tumors by means of the patented combined buoyant density gradient and immunomagnetic separation technique (10, 11). Against the background that breast cancer patients with distant metastasis at diagnosis had disseminated HER2-overexpressing epithelial cells in the bone marrow (15), the hypothesis was generated that at the time of primary treatment CTCs expressing the HER2-oncogenic receptor might be intermediates for the metastasis process. This assumption is further supported by the association between bone marrow micrometastases and the occurrence of HER2-positive cells. To the best of our knowledge, this is the first study to investigate the clinical relevance of HER2-positive CTCs in the peripheral blood of nonmetastatic breast cancer patients.

In this study, HER2-positive CTCs were present in the peripheral blood of 17 of 35 (48.6%) primary breast cancer patients. We found a significantly decreased disease-free and overall survival in patients with HER2-positive CTCs. HER2-positive CTCs have been found in relationship to larger tumor size, negative ER status, poor histologic differentiation, and lymphovascular invasion. No correlation with lymph node involvement was found, which might be due to the fact that 75%

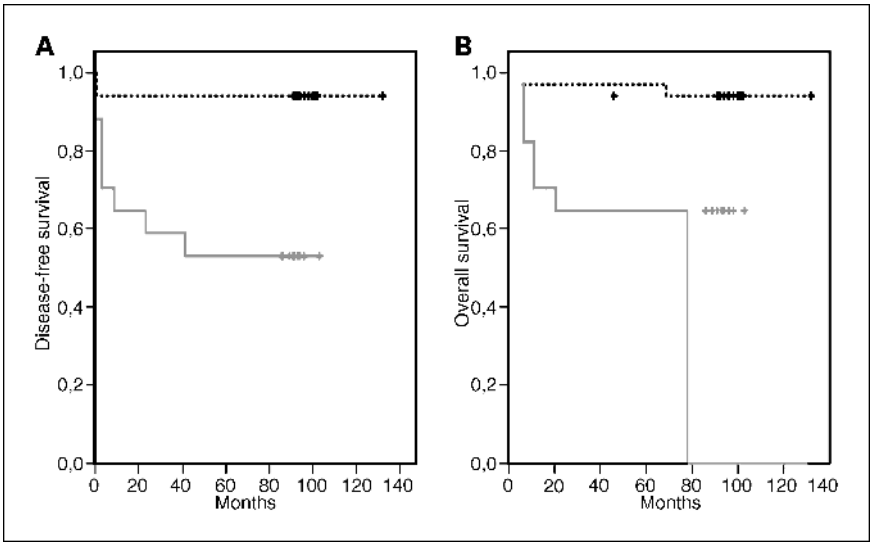

Fig. 1. Survival curves of patients with primary breast cancer. Kaplan-Meier estimates for disease-free (*A*) and overall survival (*B*) with respect to presence and frequency of circulating HER2-positive tumor cells in the peripheral blood. The black dashed line represent patients in whom no HER2-positive tumor cell was detected. The gray curve shows survival of patients with circulating HER2-positive tumor cells.

of patients in the study were node negative. Hence, the presence of HER2-positive CTCs in patients with nonmetastatic breast cancer might be a specific indicator of occult metastasis. In view of a hematogeneous metastatic tumor cell spread, prognostic markers are needed which differentiate breast cancer patients for individually based risk-adapted therapy. CTCs might represent a surrogate marker for micrometastatic disease before a metastasis becomes life threatening. In addition, the presence of disseminated tumor cells in bone marrow after adjuvant treatment may reflect an adverse outcome of the systemic treatment (16–18). However, because frequent bone marrow aspirations for the search of occult tumor cells is uncomfortable and may not easily be accepted by otherwise healthy breast cancer patients, sampling peripheral blood may be more suitable, in particular for longitudinal studies. To date, however, the clinical significance of CTCs is still unclear as it not is for disseminated tumor cells. The clinical importance of occult tumor cells in the bone marrow has been confirmed in various prospective studies showing their independent negative prognostic value (2, 4–6, 19). In addition, several studies have reported detection of CTCs in the peripheral blood of breast cancer patients (8, 9, 20). Because our technique detects HER2-positive CTCs highly sensitive not only in patients with metastatic disease but also in those with stage I and II cancer, these cells might represent an undetected spread of the tumor and serve as surrogate markers for prognosis estimates (7, 8). Furthermore, the detection of HER2-positive CTCs might influence treatment decisions that are in accordance with reports indicating CTCs to reflect the outcome of systemic treatment (9). Besides, the characterization of the CTCs using, for example, immunocytochemistry for an established prognostic marker, such as HER2 expression, underlines their conceivable prognostic and predictive importance.

The HER2 immunohistochemical score of breast carcinomas is used to guide therapy decisions for the application of humanized monoclonal antibodies, such as trastuzumab, leading to a significantly improved disease-free and overall survival (21, 22). However, to date, determination of the HER2 score by tissue testing is an one-time event. The detection of HER2-positive CTCs might serve to enable a "real-time" assessment of the HER2 status during the clinical course of disease. In addition, Braun et al. reported that HER2 overexpression on rare cytokeratin-positive cells in bone marrow predicts poor clinical outcome of stage I to III breast cancer patients (23). Thus, determination of HER2-positive CTCs might become a tool to combine the diagnostic and prognostic advantages of both the identification of CTCs and analysis of the HER2 status, respectively.

We observed a discrepancy between the detection of HER2-positive CTCs and the HER2 score of the corresponding primary tumor: in 12 patients with HER2-positive CTCs the primary tumor presented with <10% HER2-positive cells. The relatively low number of HER2-positive primary tumors in our series (11.1%) may be explained by the selection of cases with predominantly small (82% $pT_1 + T_2$), nodal-negative (76%), well or moderately differentiated (61.8%) tumors, overall implying a presumably favorable prognosis. Although careful interpretation of our data is necessary in view of the different methods used for detection of HER2 overexpression in CTCs versus breast cancer tissue, a similar discrepancy between HER2 serum markers and HER2 overexpression at the tissue level has been reported concerning the prevalence of the circulating extracellular domain of the HER2 oncoprotein, which was more frequently detected than HER2 overexpression of the primary tumor (24). In addition, in the study of Braun et al. and Zidan et al., no correlation was found between the HER2 staining score of the primary tumor and the presence of HER2-positive micrometastatic tumor cells in the bone marrow (23, 25). It has also been reported that a significant percentage of patients with HER2-negative primary tumors develop high concentrations of serum HER2 during tumor progression, supporting the possibility that HER2 gene amplification can be acquired during progression of the cancer (26). Nevertheless, our data may also reflect appearance of HER2 during tumor progression. Another explanation for these findings could be a specific preselection of HER2-positive cells during tumor cell dissemination. More likely, most of the cells will become apoptotic during their blood passage, which we described recently for blood-borne prostate cancer cell clusters (27). Moreover, it could be assumed that only a few HER2-overexpressing cancer cells may have the potential to disseminate, subsequently leading to metastases and death. This is in line with our previous results from *in vitro* extravasation experiments using disaggregated cells and cell clusters from primary breast cancer tissue (28). Most of the transendothelial invasive cells displayed HER2 expression, whereas the primary tumor had been classified as HER2 negative by immunohistochemistry.

To the best of our knowledge, this is the first study to analyze the prognostic value of HER2-positive CTCs from the peripheral blood of breast cancer patients. The data give support for the detection of circulating HER2-positive tumor cells by this new method to assess and to monitor the "real-time HER2 status" and to detect early evidence of occult metastatic disease. Due to the limited number of patients in our series and the fact that clinicopathologic differences (e.g., pT stage, pN status, and grade) and the adjuvant systemic treatment can confound interpretation of our data further studies are needed to confirm the role of monitoring HER2-positive CTCs for clinical diagnosis and treatment guidance. In particular, future studies have to determine the outcome of patients treated with HER2-targeting therapies with respect to HER2-positive CTC levels because it is not unlikely that high levels of HER2-positive CTCs reflect the activity of the cancer and may predict an improved response to trastuzumab (29).

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