HER2 Status on Persistent Disseminated Tumor Cells after Adjuvant Therapy May Differ from Initial HER2 Status on Primary Tumor

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Abstract. *Background: Persistence of disseminated tumor cells (DTCs) is observed in 10 to 15% of breast cancer patients and is associated with poor prognosis. These patients might benefit from secondary adjuvant targeted therapy. The aim of this study was to assess HER2 status of persistent DTCs to determine whether the use of HER2-targeted agents might be a therapeutic option in patients with tumor cell persistence. Patients and Methods: Bone marrow was obtained from 85 primary breast cancer patients intraoperatively and after completion of systemic treatment (median follow-up of 13 months; range: 6-30 months). Immunofluorescence double staining was used for identification of cytokeratin-positive, HER2-positive cells. Results: A total of 31 out of 85 (36%) patients had DTCs preoperatively. Out of 85 (16%) patients, 14 were DTC positive after completion of surgery and adjuvant cytotoxic therapy. Five of these patients had HER2-positive DTCs, however, the corresponding tumor was HER2 positive in only one case. The remaining nine patients with HER2-negative DTCs had HER2-negative primary tumors. Conclusion: HER2-positive DTCs can be detected in patients with HER2-negative tumors, even after adjuvant therapy. Such patients may benefit from (secondary) HER2-targeted therapy in an adjuvant setting.*

Persistence of disseminated tumor cells (DTCs) in bone marrow after completion of surgery and adjuvant chemotherapy can be observed in 10 to 15% of breast cancer patients. As demonstrated by the European pooled analysis, tumor cell persistence is associated with poor clinical outcome (1). Therefore, the targeted elimination of these cells might be a highly promising therapeutic strategy to improve prognosis in these patients. While the accurate nature of DTCs is still under research, attempts have been made over the last decade to characterize these cells with regard to both pheno- and genotype.

Human epidermal growth factor receptor 2 (HER2), one of the tyrosine kinase erb-B receptors, belongs to the most relevant predictive factors in breast cancer (2). HER2-positive tumors tend to be of a more aggressive biological behavior (2). The clinical role of HER2 gained in importance after the introduction of the anti-HER2 monoclonal antibody trastuzumab and other novel anticancer agents such as pertuzumab and lapatinib (3, 4). HER2 is overexpressed in 20 to 30% of primary breast cancer patients and this group may benefit from targeted therapy (5, 6). The indication for molecular antibody therapy is based on HER2 overexpression or gene amplification in the primary tumor. However, several studies suggested that disseminated and circulating breast cancer cells may acquire positive HER2 status independently of the primary tumor and may become a potential target for a molecular antibody therapy in an adjuvant or metastatic setting (7-10). Abandonment of a targeted therapy in this patient collective could thus result in unintentional undertreatment.

The aims of our study were (a) to assess how many patients have persistent DTCs after completion of adjuvant therapy and (b) to evaluate the HER2 status of DTCs themselves at the time of diagnosis and after the therapy to determine whether trastuzumab and other molecular targeted agents might be a therapeutic option for the elimination of persistent DTCs.

Patients and Methods

Patients. After written informed consent, bone marrow samples were obtained intraoperatively from 85 primary breast cancer patients who were treated at the Department of Gynecology and Obstetrics (University Hospital Tuebingen, Germany) from 2001 until 2006. The patients were then treated with adjuvant

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Key Words: Breast cancer, persistent disseminated tumor cells, trastuzumab, targeted therapy, HER2 status.

chemotherapy, hormone therapy, or both based on current St. Gallen recommendations and national treatment guidelines (www.ago-online.de). After a median follow-up of 13 months (range: 6-30 months), a second bone marrow aspiration was performed. Clinical data of patients are shown in Table I. Only two patients received trastuzumab as part of their adjuvant therapy, since the majority of patients in this study were diagnosed with breast cancer before trastuzumab was considered as a standard therapy in HER2-positive breast cancer.

Detection and characterization of DTCs. Ten to twenty ml of bone marrow were prepared by centrifugation on a Ficoll-Hypaque density gradient (1.077 g/ml; Biochrom, Germany) followed by lysis of red blood cells with lysis solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA pH 7.2). Mononuclear cells (10^6 MNC/spot) were then cytospun onto a glass slide (Hettich cytocentrifuge, Germany) and air-dried overnight at room temperature. A double immunofluorescence staining procedure was performed for the detection of HER2-positive tumor cells. Slides were fixed with 0.5% neutral-buffered formalin for 10 minutes and washed twice in phosphate-buffered saline (PBS). Nonspecific antibody binding was blocked using 10% Goat Serum Normal (DAKO, Denmark) in PBS for 30 minutes. Primary rabbit HER2-antibody CB11 (1:100; Biogenex, CA, USA) was applied for 30 minutes, followed by incubation with secondary goat anti-rabbit antibody, labeled with Texas Red (1:100; Vector Laboratories, CA, USA) for 30 minutes. Subsequently, directly labeled FITC-C11 mouse monoclonal antibody against pan-cytokeratin (CK) (1:100; Sigma, MI, USA) was added and slides were incubated for 30 minutes. Nuclei were counterstained with 4'6-diamidino-2-phenylindole in mounting media (Vector Laboratories). The breast cancer cell line SKBR3 (ATCC®-Nr. HTB-30, American type culture collection, Manassas, VA, USA) was used as a positive control for cytokeratin and HER2 staining and the cell line MCF7 (ATCC®-Nr. HTB-22D, American type culture collection) as negative control for HER2 staining. Leukocytes of a healthy volunteer served as a negative control for both. The microscopic analysis of slides was performed by two independent investigators (NK and TF). Evaluation for the presence of tumor cells was carried out using a computerized fluorescence microscope (Axiophot; Zeiss, Germany). A single-pass filter for individual fluorochromes (FITC, Texas Red or DAPI) and a dual-pass filter for FITC/Texas Red were used to screen for HER2-positive tumor cells. Only cells with moderately or strongly stained membrane were considered HER2-positive. Criteria for the identification of single HER2-positive DTCs by immunofluorescence are described in more detail by Meng *et al*. (11) and Solomayer *et al*. (8).

Staining of the primary tumor. Core cut biopsies or surgically resected specimens were analyzed immunohistochemically for expression of HER2 protein. Sections 3-5 μm-thick of formalin-fixed, paraffin-embedded tissue were stained using commercially available ABC kit (Vectastain; Vector Laboratories). Sections were incubated with primary polyclonal HER2 antibody (clone A 0485) diluted 1:200 in Tris-HCl according to the manufacturer's instruction (HERCEPTM test; Dako, Glostrup, Denmark). Color development was achieved with 3,3'-diaminobenzidine (DAB). Slides were counterstained with hematoxylin and mounted for examination. HER2 expression was evaluated using HercepTest criteria. The HER2 score was based on a 0 to +3 scale. Tumors with a score of +2/+3 were considered HER2 positive. In case of a score of +2, fluorescence *in situ* hybridization was performed to determine HER2 amplification using the Pathvysion™ kit (HER2/NEU) (Vysis, Downers Grove, IL, USA). The scoring conditions followed the recommendations given by the manufacturer.

Statistical analysis. Chi-squared test was used to examine the association between clinicopathological factors and detection of CK and/or HER2-positive tumor cells. Statistical analysis was performed using SPSS (Version 16, SPSS GmbH Software, Germany) considering *p*-values less than 0.05 to be statistically significant.

Results

Bone marrow status before adjuvant treatment. Bone marrow aspirates from 85 patients were analyzed for the presence of persistent DTCs. The first bone marrow aspiration was performed at the time of surgery and the second after a median follow-up of 13 months (range: 6-30 months). The identification of DTCs was based on cytokeratin positivity and morphological criteria according to the Consensus Recommendations for Standardized Tumor Cell Detection (12). Typical morphology of a representative cytokeratin-positive tumor cell is shown in Figure 1. In 31 (36%) patients, DTCs were detected in bone marrow. The number of DTCs ranged from 1 to 5 cells per patient (2×10^6 mononuclear cells). A statistical correlation was found between intraoperative DTC-positive bone marrow status and negative estrogen receptor status of primary tumor but not with any other of the established prognostic markers, including the HER2 status of the primary tumor (Table I).

In 8 out of 31 (26%) cases with DTC-positive bone marrow status, HER2 positivity of DTCs was observed. Nevertheless, only one of these 8 patients demonstrated an HER2 overexpressing primary tumor. Four out of 23 (17%) patients with HER2-negative DTCs showed HER2 positivity of their primary lesion. The comparison of HER2 status between primary tumor and DTCs is shown in Table II.

Heterogeneity of HER2 expression in DTCs. In 23 out of 31 (74%) patients with detectable DTCs in bone marrow at the time of primary diagnosis, only HER2-negative DTCs were found. In the remaining 8 (26%) patients, HER2-overexpressing cells were observed. In 3 out of 7 (43%) patients with more than one tumor cell in bone marrow there was heterogeneity of HER2 expression (Figure 2).

HER2 status of DTCs after completion of adjuvant therapy. Persistent DTCs after therapy were found in 14 out of 85 (16%) patients. No statistical correlation between DTC-positive bone marrow status after treatment and any of the established prognostic markers including the HER2 status of the primary tumor was observed (Table I).

Five out of 14 (36%) patients with tumor cell persistence had HER2-positive DTCs. However, the corresponding tumor was HER2 positive in only one case. All nine patients with HER2-negative DTCs also had HER2-negative primary tumors (Table II). Nevertheless, the percentage of patients with HER2-positive DTCs was higher during follow-up (5 out of 14; 36%) compared to the intraoperative time point (8 out of 31 patients; 26%).

Five patients with HER2-positive tumors showed DTCs at the time of first diagnosis. Since trastuzumab was not part of the standard treatment before 2005, these patients did not receive HER2-targeted therapy. After completion of adjuvant treatment, only one of these patients had persistent tumor cells which were HER2 positive, although initially only HER2-negative DTCs had been detected.

Discussion

Tumor cell persistence. Recent studies suggest that a selected subgroup of patients may benefit from extended adjuvant treatment. Of all validated prognostic factors, monitoring of minimal residual disease is the only one available after the primary tumor has been removed. A large pooled analysis demonstrated a strong negative impact of persistent DTCs on both disease-free and overall survival (1). Thus, follow-up bone marrow screening might help to identify patients who are most likely to develop disease recurrence and would potentially benefit from a secondary adjuvant therapy. While the exact biological nature of DTCs is still to be further investigated, various study groups examined their phenotype with regard to novel therapeutic agents. Since biological factors of DTCs differ from those of the primary tumor, their correct assessment may improve our understanding of the natural history of breast cancer and enable us to optimize therapy regimens. HER2 status has proven to be one of the most important predictive factors in breast cancer and is routinely determined in primary tumor. Targeted therapy drugs, such as trastuzumab, pertuzumab and lapatinib, were introduced into breast cancer treatment in both metastatic and adjuvant settings.

HER2 status of DTCs does not reflect the HER2 status of the primary tumor. Several aspects of HER2 status on DTCs must be considered. Firstly, DTCs reflect only a subpopulation of cancer cells from primary tumor. This selected group of cells seem to feature factors commonly associated with poorer clinical outcome, such as negative hormonal status and up-regulation of urokinase-type plasminogen activator receptor (13, 14). Additionally, HER2-positive tumor cells have an enhanced extravasative potential, and thus a growth and survival advantage, and can therefore be encountered more frequently in bone marrow or blood (15). As a result, the HER2 status of DTCs or other metastatic sites does not necessarily reflect the HER2 status of the primary tumor. In our patient group, HER2-positive tumor cells were detected in the bone marrow of seven patients despite their having HER2-negative primary tumors. This finding is consistent with previous publications (7, 8, 16-19) (Table III). As the indication for trastuzumab-targeted therapy is based on HER2 overexpression or gene amplification of the primary tumor, a subgroup of patients with HER2-positive DTCs but HER2-negative tumors is not eligible for this treatment. However, several studies have demonstrated that trastuzumab-based therapy is able to eliminate HER2-positive circulating tumor cells (11, 20, 21). Whether the indication for trastuzumab treatment in an adjuvant setting should be extended to patients with HER2-positive DTCs regardless of primary tumor status must be further evaluated.

Table I. *Clinical data of patients*.

		Before treatment		After treatment	
	N	BM positive (%)	*p*-value	BM positive (%)	*p*-value
Total	85	31 (36)		14 (16)	
Menopausal status					
Premenopausal	28	13 (46)	n.s.	10 (17)	n.s.
Postmenopausal	57	18 (32)		4 (14)	
Tumor size					
pT1	53	21 (37)	n.s.	8 (15)	n.s.
pT2-4	32	10 (31)		6 (19)	
Nodal status					
Node negative	58	24 (41)	n.s.	8 (16)	n.s.
Node positive	26	7 (27)		6 (23)	
Histology					
Ductal	59	22 (37)	n.s.	8 (14)	n.s.
Lobular	19	7 (37)		3 (16)	
Other	7	2 (29)		3 (43)	
Grading					
I	3	1 (33)	n.s.	1 (33)	n.s.
II	63	22 (35)		11 (17)	
III	17	7 (41)		0 (0)	
ER status					
Negative	20	11 (55)	0.05	5 (25)	n.s.
Positive	65	20 (31)		9 (14)	
PR status					
Negative	29	13 (45)	n.s.	7 (24)	n.s.
Positive	56	18 (32)		7 (12)	
HER2					
Negative (0/+1)	71	27 (38)	n.s.	13 (19)	n.s.
Positive (+2/+3)	14	4 (29)		1 (7)	
Systemic adjuvant therapy					
Chemotherapy	19	11 (58)	n.s.	4 (21)	n.s.
Endocrine therapy	25	8 (32)		3 (12)	
Both	41	12 (29)		7 (17)	

n.s.: Not significant; BM positive: presence of disseminated tumor cells in bone marrow; ER: estrogen receptor; PR: progesterone receptor.

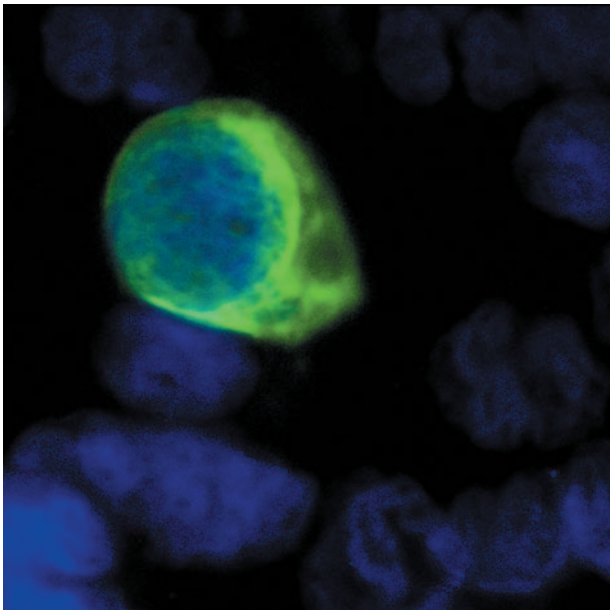

Figure 1. *Typical cytomorphology (nuclear size clearly enlarged, high nuclear to cytoplasmic ratio) and immunophenotype (irregular cytoplasmic staining for cytokeratin, cytokeratin filaments can be seen) of a representative DTC from a breast cancer patient. Tumor cell is stained with an anti-CK-fluorescein isothiocyanate (green) antibody (×40 oil immersion objective).*

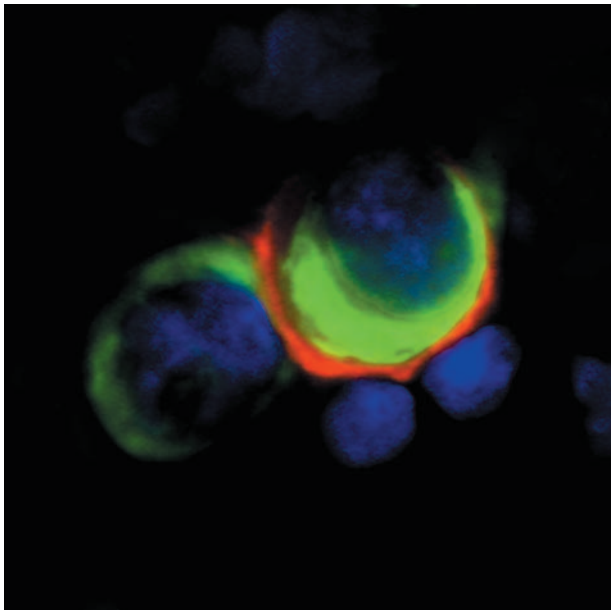

Figure 2. *Heterogeneity of HER2 expression on DTCs from a primary breast cancer patient. Tumor cells were stained with an anti-CK-fluorescein isothiocyanate (green) and anti-HER2 detected by a secondary Texas Red labeled goat anti-rabbit (red) antibodies. Nuclei are stained blue with DAPI (×40 oil immersion objective). Cluster of HER2-negative and HER2-positive DTCs from a breast cancer patient.*

HER2 overexpression can be acquired during dissemination and progression. Furthermore, persistent DTCs may acquire a more aggressive phenotype in the course of the disease. We observed an increase of patients with HER2-positive DTCs (26% at the time of surgery, 33% after follow-up). As shown before, conventional adjuvant chemotherapy fails to eliminate DTCs from bone marrow (22). One major reason for this inefficiency is the dormant state of DTCs with a small proliferation index (23). However, at some point, single tumor cells might increase their metabolism, leaving the dormant state, and thus cause subsequent metastasis.

Recently, Jückstock *et al*. presented preliminary results of an interventional post-adjuvant trastuzumab-based pilot trial (24). Twelve asymptomatic breast cancer patients with persistent HER2-positive DTCs received trastuzumab. All patients completed chemotherapy at least 6 months prior to entering the study. Trastuzumab treatment was able to eradicate DTCs in seven of these patients. Another interesting approach was proposed by Bernhard *et al*. Autologous HER2-specific T-lymphocytes were transferred to a patient with metastatic HER2-positive breast cancer. This experimental treatment was able to eliminate HER2-overexpressing tumor cells from the bone marrow, but did not penetrate into solid metastases (25). However, elimination of minimal residual disease may not have a direct impact on survival outcome. Whether patients with persistent DTCs actually benefit clinically from additional targeted therapy strategies will have to be evaluated in further prospective randomized studies.

Disease monitoring and response to therapy. The acquisition of more aggressive genomic aberrations, such as HER2 amplification, may indicate tumor progression and play a role in the metastatic cascade (11). This patient group might benefit from additional targeted therapy. This underlines the need for re-evaluation and monitoring of DTC status in the course of the disease (10). In contrast to tissue evaluation with regard to HER2 overexpression – a single event – monitoring minimal residual disease gives an opportunity for real-time insight into disease progression. The persistence of HER2-positive circulating tumor cells after completion of adjuvant chemotherapy was shown to be linked to poor clinical outcome (16).

Conclusion

Concluding, in the present report, we were able to show that the HER2 status on persistent DTCs differs not only from that of the primary tumor, but also from the intraoperative

Table II. *Course of DTCs in primary breast cancer during treatment and follow-up.*

HER2 status		N	DTC-positive after treatment	DTC HER2 status after treatment	
Primary tumor	DTCs before treatment			Positive	Negative
Negative	Negative	19	3	0	3
	Positive	7	2	1	1
	No DTCs	44	8	3	5
Positive	Negative	4	1	1	0
	Positive	1	0	-	-
	No DTCs	10	0	-	-
Total		85	14	5	9

Table III. *Correlation of HER2 status between primary tumor and DTCs.*

Study group	N	HER2 positivity (%)		Concordance
		Primary tumor	DTCs	
Present[a]	31	5 (16)	8 (26)	64%
Present[b]	14	1 (7)	5 (36)	71%
Jueckstock et al. (24)[b]	129	34 (26)	49 (38)	68%
Apostolaki et al. (16)[a]	212	24 (11)	52 (25)[c]	69%
Solomayer et al. (8)[a]	45	14 (29)	20 (44)	63%
Becker et al. (9)[a]	105	26 (25)	22 (21)	77%
Braun et al. (19)[a]	24	7 (29)	15 (63)	58%
Meng et al. (11)[d]	33	15 (46)	11 (33)	88%

[a]DTC at the time of diagnosis; [b]persistent DTC after completion of therapy; [c]CTC in peripheral blood, not DTC in bone marrow; [d]breast cancer patients at recurrence.

DTC status. HER2 positivity may be acquired during dissemination and tumor progression. Whether the indication for targeted trastuzumab treatment should be based on both primary tumor and DTC status must be further evaluated.

Acknowledgements

We thank Professor Ludwig Spätling, MD, Ph.D. (Department of Gynecology and Obstetrics, Klinikum Fulda) for his support.

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Received May 29, 2009
Revised August 16, 2009
Accepted September 3, 2009