Archives of Gynecology and Obstetrics (2018) 297:785–790
https://doi.org/10.1007/s00404-018-4669-9

GYNECOLOGIC ONCOLOGY

Simultaneous detection of circulating and disseminated tumor cells in primary breast cancer patients following neoadjuvant chemotherapy

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Received: 3 August 2017 / Accepted: 11 January 2018 / Published online: 27 January 2018

Abstract

Purpose Pathological complete response (pCR) is a common endpoint in neoadjuvant chemotherapy (NACT) of primary breast cancer patients (PBC), but does not address the systemic prevalence of minimal residual disease. In this study, we compared pCR with the detection of circulating (CTC) and disseminated tumor cells (DTC) following NACT, as well as their impact on survival.

Methods Patients with PBC receiving NACT and consecutive surgery were eligible for this study. CTCs were detected using the CellSearch® system and DTCs were determined using immunocytochemistry (cytokeratin staining with the A45-B/B3 antibody). pCR was defined as ypT0/ypTis and ypN0.

Results 58 patients were included in the analysis with a median follow-up of 30 months. Of these, 5 (9%) presented with CTCs and 36 (62%) with DTCs. 16 patients (28%) achieved a pCR. No significant correlation between CTCs, DTCs and pCR and no statistically significant impact on disease free (DFS) or overall survival (OS) was apparent.

Conclusions Both CTCs and DTCs are detectable after NACT. As we could not show a significant relationship between CTC detection, DTC detection and pCR, all three methods may provide independent information regarding treatment response. Since we were unable to show a significant impact on survival, larger prospective studies that include CTCs and DTCs are needed. These trials should include the molecular characterization of primary tumor tissue, CTCs and DTCs to determine whether these cells are independent subpopulations of malignant cell clones.

Keywords Breast cancer · Neoadjuvant chemotherapy · Minimal residual disease · Circulating tumor cells · Disseminated tumor cells · Pathological complete response

Abbreviations

NACT	Neoadjuvant chemotherapy
PBC	Primary breast cancer
DFS	Disease free survival
OS	Overall survival
pCR	Pathological complete response
MRD	Minimal residual disease
DTC	Disseminated tumor cell
BM	Bone marrow
CTC	Circulating tumor cell
HR	Hormone receptor

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Introduction

Neoadjuvant chemotherapy (NACT) in primary breast cancer (PBC) aims to minimize the extent of surgery by reducing tumor size. Moreover, it enables in situ chemosensitivity testing [1, 2]. No differences with respect to disease free (DFS) and overall survival (OS) between neoadjuvant and adjuvant chemotherapy are evident [3, 4].

Pathological complete response (pCR) is a common endpoint in neoadjuvant clinical trials. The prognostic value of pCR, which ideally should be defined as no evidence of invasive tumor in breast or lymph nodes (ypT0/ypTis, ypN0), has been demonstrated in various trials including

a large meta-analysis and is mostly pronounced in patients with aggressive tumor subtypes [5, 6]. However, even after achieving pCR, patients can still relapse and tumor cells can survive at secondary sites, a phenomenon which is termed minimal residual disease (MRD). As current definitions of pCR do not include a potentially persistence of MRD, surrogate markers for its detection during NACT are being searched.

In recent years, two surrogate markers of MRD have been extensively described in primary breast cancer: disseminated tumor cells (DTCs), that are found in the bone marrow (BM) and circulating tumor cells (CTCs) from the peripheral blood [7–10]. DTCs are predictive of locoregional relapse, distant relapse and overall survival [7, 11]. Because of the invasiveness of BM sampling and the resulting difficulty of acquiring several probes at different time points to monitor disease progression, detection of CTCs is a promising alternative that is easy to perform in the clinical routine. The most common method for CTC detection is the FDA-approved CellSearch® system (Menarini Silicon Biosystems, Huntingdon Valley, PA, USA). Their detection is predictive of an impaired prognosis [10, 12]. Simultaneous detection of CTCs and DTCs in patients with PBC shows concordance rates of 66–94% [13], however, the prognostic impact of DTCs seems more pronounced [9].

Both, CTCs [14, 15] and DTCs [16–18], were shown to be independent prognostic factors following NACT and their detection does not correlate with the primary tumor's response to NACT. Therefore, it is reasonable that CTC and DTC determination after NACT may provide additional clinical information with respect to the patient's prognosis, the persistence of MRD after NACT and the potential need for additional (post-neoadjuvant) treatment. Hence, the aim of this study was to compare the prognostic impact of DTCs and CTCs with that of pCR in patients with PBC that have completed NACT.

Methods

Study population

Patients that received NACT and subsequent surgery to treat PBC between January 2009 January 2015 at the Department of Obstetrics and Gynecology at Tuebingen University Hospital, Germany, were eligible for this retrospective study. The CTC- and DTC-statuses were determined after the completion of NACT i.e., at the time of surgery. Patients with recurrent or metastatic disease, bilateral breast cancer, R1-resection, or another secondary malignancy were excluded. All patients provided written informed consent and the study was approved by the Ethics Committee of Tuebingen University (reference number: 560/2012R).

CTC detection

CTCs were detected using the CellSearch® system (CellSearch® Epithelial Cell Kit/CellSpotter Analyzer, Menarini Silicon Biosystems). In brief, 7.5 ml of venous blood are collected in a CellSafe tube (Menarini Silicon Biosystems), kept at room temperature and processed within 72 h. Cells expressing the epithelial cell adhesion molecule (EpCAM) are immunomagnetically enriched using anti-EpCAM-coated magnetic beads and thereafter labelled with 4′,6-diamidino-2-phenylindole (DAPI), staining nucleic acid. Monoclonal antibodies directed against the leukocyte common antigen CD45 and against epithelial markers (cytokeratin 8,18,19-phycoerythrin) were used to differentiate between epithelial cells and leukocytes. Cells with intact nuclei expressing cytokeratin, but not CD45 were counted as CTCs. A sample was defined as CTC-positive if at least one CTC was evident in 7.5 ml blood.

DTC detection

10–20 ml of BM aspirates was collected during primary surgery and processed within 24 h. Mononuclear cells were separated through density centrifugation (1.077 g/mL; Ficoll, Biochrom, Germany), spun down onto a glass slide (Hettich cytocentrifuge, Hettich, Tuttlingen, Germany) and thereafter fixed in 4% formalin. Using the DAKO Autostainer (Dako, Glostrup, Denmark), the monoclonal mouse A45-B/B3 antibody directed against pancytokeratin (Micromet, Munich, Germany), and the DAKO-APAA detection kit (Dako) the DTC-status was determined. Following consensus recommendations for standardized tumor cell detection [19], two slides (2×10^6 cells) were evaluated for each patient. An unspecific isotype-matched antibody was used as isotype control on an additional slide. With every batch of samples, leukocytes from healthy volunteers were analyzed and served as negative control, whereas the human breast cancer cell lines MCF-7 and SKBR-3 served as positive control.

Evaluation of pCR

Mammary tissue and axillary lymph nodes were removed during primary surgery according to national guidelines [20] and examined by an expert pathologist. pCR was defined as ypT0 or ypTis and ypN0.

Statistical analysis

The association between categorical variables was analyzed using Chi square test, or, in case of an expected frequency lower than five, Fisher's exact test. DFS was defined as time

from primary surgery to local recurrence or detection of distant metastasis and OS as the respective time to death. If none of these events occurred until the last inquiry, data were censored. Kaplan–Meier curves of survival were plotted and compared using the log-rank test. Median follow-up duration was determined by the Kaplan–Meier estimate of potential follow-up. The significance level was set to $p < .05$. Statistical tests were performed using JMP®, Version 12. SAS Institute Inc., Cary, NC, USA, 1989–2015.

Results

Patient characteristics

58 patients were included in the analysis. Detailed characteristics are shown in Table 1. The median age was 48 years. In 40 (69%) of the cases axillary lymph nodes were involved and the median baseline tumor size was 29 mm. 32 (55%) were ER positive, 26 (45%) PR positive and 16 (28%) HER2 positive. 28 (48%) of the patients had a grade 3 tumor, whilst 30 (52%) were graded 1 or 2. The mean duration of the NACT was 150 days (SD 22 days, range 60–200 days). All patients received taxanes, in 57/58 (98%) anthracyclines were administered and 13/58 (22%) received HER2-targeted treatment (trastuzumab, trastuzumab + pertuzumab, trastuzumab + lapatinib).

Table 1 Patient characteristics and CTC-/DTC-status

	CTC positive	*p* value	DTC positive	*p* value
All	5 (9%)		36 (62%)	
Menopausal status				
Premenopausal	5/36 (14%)	.146[a]	26/36 (72%)	.042
Postmenopausal	0/22		10/22 (46%)	
Tumor grade				
1–2	1/30 (3%)	.187[a]	20/30 (67%)	.455
3	4/28 (14%)		16/28 (57%)	
Axillary lymph node status				
Positive	5/40 (13%)	.311[a]	27/40 (68%)	.204
Negative	0/18		9/18 (50%)	
ER status				
Positive	3/32 (9%)	1[a]	22/32 (69%)	.245
Negative	2/26 (8%)		14/26 (54%)	
PR status				
Positive	3/26 (12%)	.648[a]	19/26 (73%)	.119
Negative	2/32 (6%)		17/32 (53%)	
HER2 status				
Positive	2/16 (13%)	.609[a]	8/16 (50%)	.242
Negative	3/42 (7%)		28/42 (67%)	

[a]Two-sided Fisher's exact test

CTC-status, DTC-status and pCR

One or more CTCs per 7.5 ml peripheral blood could be detected in 5 (9%) of the patients. DTCs were found in 36 (62%) patients. The concordance between CTC and DTC detection was 40%. 16 of 58 patients (28%) achieved pCR. No significant correlation between pCR and DTC- or CTC-status was apparent (Table 2), but pCR was significantly less frequently achieved in hormone receptor (HR) positive HER2 negative tumors than in other subtypes (Table 3). The median follow-up time was 30 months (95% CI 16–34 months). Concerning DFS and OS, there was no significant association with DTC-status, CTC-status and pCR, respectively (Fig. 1).

Discussion

This study analyzed the association of pCR-, CTC- and DTC-status, as well as their prognostic impact in PBC patients, who received NACT. To the best of our knowledge, this study is the first to compare the impact of a cytokeratin-based detection of the CTC-status, obtained using the CellSearch® system, with a consensus based DTC detection

Table 2 Comparison of DTC- and CTC-status

	DTC		*p* value
	−	+	
CTC			
−	20/58 (34%)	33/58 (57%)	1[a]
+	2/58 (3%)	3/58 (5%)	

[a]Two-sided Fisher's exact test

Table 3 Comparison of CTC-status, DTC-status and tumor subtypes with pCR

	pCR		*p* value
	−	+	
All patients	42/58 (72%)	16/58 (28%)	
CTC			
−	39/58 (67%)	14/58 (24%)	.609[a]
+	3/58 (5%)	2/58 (3%)	
DTC			
−	14/58 (24%)	8/58 (14%)	.242
+	28/58 (48%)	8/58 (14%)	
Tumor subtype			
HR+/HER2−	22/24 (92%)	2/24 (8%)	.021
HER2+	9/16 (56%)	7/16 (44%)	
Triple negative	11/18 (61%)	7/18 (39%)	

[a]Two-sided Fisher's exact test

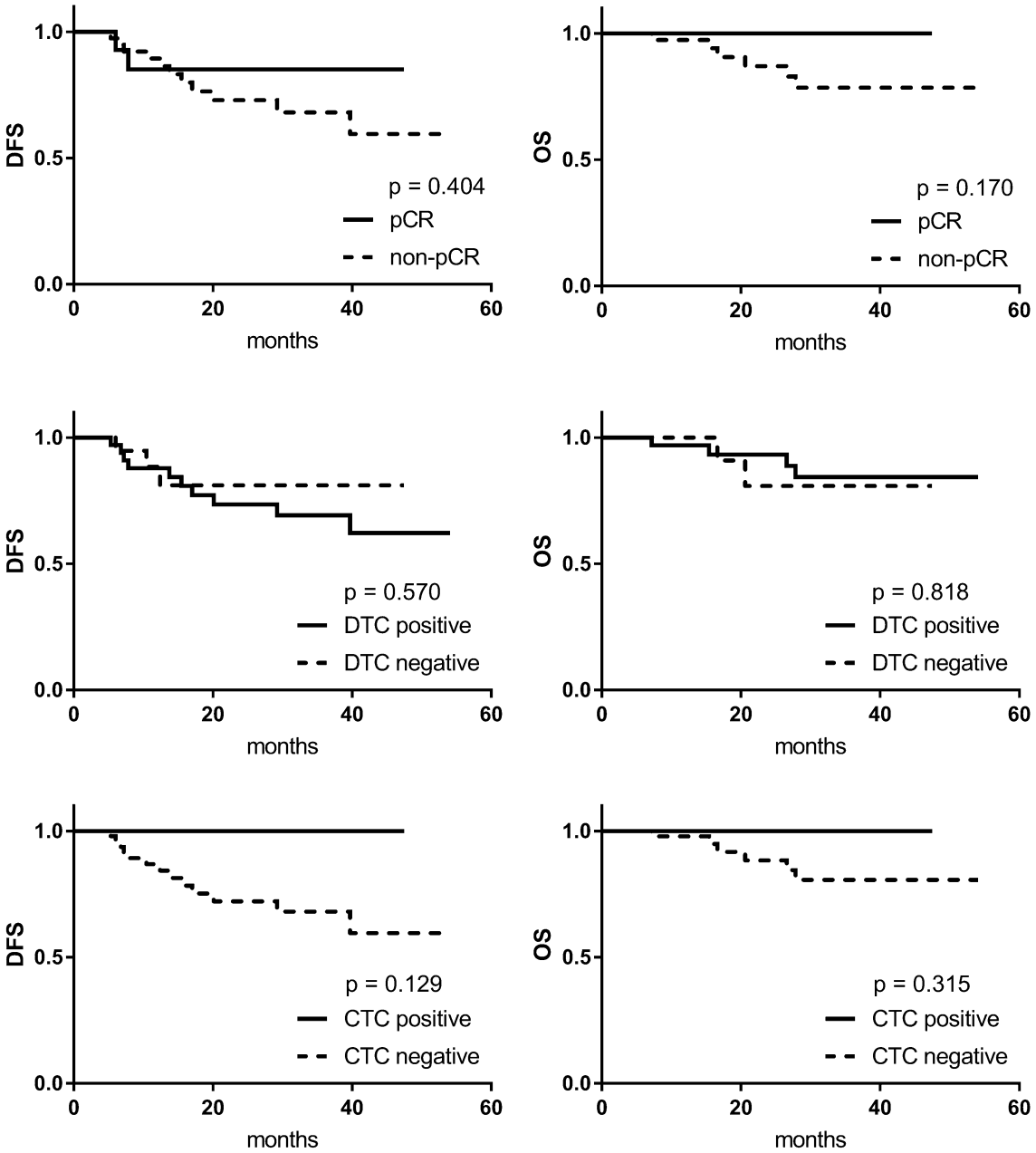

Fig. 1 Kaplan–Meier curves of DFS (left) and OS (right) comparing pCR and non-pCR (top), DTC-status (middle), and CTC-status (bottom). *p* values were calculated using the log-rank test

method on survival in PBC patients that have received NACT. We found no statistically significant correlation between any of the three markers. Moreover, in the analyzed cohort, which was comparatively small, neither pCR nor CTC- or DTC-status significantly impacted DFS and OS.

In a similar setting, Kasimir-Bauer et al. [21] recently reported in a study of 190 patients with PBC that, neither the CTC-status as determined by RT-PCR based AdnaTest, nor the DTC-status following NACT had a significant impact on either progression free survival or OS. In another study Mathiesen et al. [17] assessed the DTC-/CTC-status of 236 PBC patients at three different time points (pretreatment, at surgery following NACT, at 1-year follow-up) and found the DTC-status determined 1 year after starting NACT to be a negative prognostic factor. Regarding CTCs, however, only positivity before the beginning of NACT was associated with reduced survival. In a recently conducted international meta-analysis of the prognostic impact of CTCs in 2156 PBC patients receiving NACT (IMENO trial), Bidard et al. [22] were able to show a CTC-count of two or higher both

at pretreatment and presurgery time points to be significant negative prognostic factors for OS and distant disease-free survival. It was also shown that patients whose CTCs persisted during NACT had a shorter OS than patients, who continually presented without CTCs. Whereas in the meta-analysis presented by Bidard et al. the CellSearch® System was used for CTC detection, Mathiesen et al. prepared cytospins and used immunocytochemistry not only for DTC-, but also for CTC detection. Although reported previously, most studies do not show a significant correlation between DTCs and CTCs; presumably due to a lower sensitivity of CTC-detection [9, 13, 23, 24].

PCR has been shown to be a prognostic factor for improved survival [5, 6] in all subtypes of breast cancer. As expected and previously reported in larger study populations [6], pCR was achieved less frequently in HR+/HER2− patients than in HER2+ or triple negative patients in our cohort. The fact that we were unable to show a prognostic value of pCR is most likely due to the small size of our study population and the relatively short follow up. In line with our data, several other studies have also shown that neither the CTC- [14, 15, 25] nor the DTC-status [16–18] after NACT are significantly associated with pCR and therefore seem to provide independent prognostic information. Through this, the different biological concepts of pCR and detection of tumor cell dissemination become apparent. While pCR describes the primary tumor's response to NACT, DTCs and CTCs are representative of the systemic character of breast cancer disease and have the ability to seed later metastasis [26]. Therefore, even patients achieving a pCR may relapse due to the persistence of MRD.

With 9% our CTC detection rate was lower than expected when compared to earlier findings. CTCs were detected in only five of the patients, which can certainly be the cause of insignificant results regarding our survival analysis. This comparably small number of CTC-positive patients is conceivably due to the small number of patients in our cohort. Moreover, only 7.5 ml blood was analyzed, while other studies used larger volumes of blood samples for CTC detection [8]. As no blood sampling was performed before the start of NACT, we cannot rule out that CTCs were eliminated through the administration of cytotoxic and targeted therapy, as recently shown by Bidard et al. [22]. In contrast, our DTC detection rate was comparably high, being almost twofold of what would usually be expected in PBS patients. This observation is similar to previous findings [11, 16, 27] and might be due to the following reasons: most DTCs are in a non-proliferative stage [28] and may therefore escape from NACT. Also, our cohort contained a large number of patients with high-grade, HER2-positive, and ER/PR-negative tumors, which are factors that have been described to be associated with increased tumor cell dissemination into the bone marrow [11]. Moreover, chemotherapy may result in increased shedding of apoptotic cells from the primary tumor [27], which can then be found in patients' bone marrow. In earlier studies, we found that the detection of apoptotic DTCs, which were detectable in 29–48% of the patients in those cohorts, is associated with response to NACT [16, 29]. There, it was also found that patients with apoptotic DTC were less likely to relapse, although the impact on DFS was not statistically significant.

Our inability to show a significant impact of the DTC-status on DFS/OS is most likely due to the small sample size but may also be explained by apoptotic tumor cell shedding and the earlier observation that the prognostic impact of DTC detection is mostly pronounced in luminal tumors [30], which are less often treated with NACT.

Conclusion

Both CTCs and DTCs are detectable after NACT, irrespective of the primary tumors response to treatment. MRD detection using single tumor cells from blood and/or bone marrow after NACT might therefore be useful to monitor systemic treatment response. It is, however, not yet clear whether DTCs and CTCs represent independent compartments of MRD. Larger translational analyses within the context of prospective clinical trials are needed to evaluate the clinical value of CTC/DTC detection after NACT. Ideally, these analyses should include molecular characterization of DTCs, CTCs, and tumor tissue from the same patient to investigate whether these compartments are independent subpopulation of malignant tumor cells and which compartment is most likely to seed a later metastatic relapse.

Author contributions VPW: data collection, data analysis, manuscript writing. FAT: project development. MW: data collection. MH: data collection. SYB: Project development. ADH: data collection, data analysis, manuscript editing.

Compliance with ethical standards

Ethical standards The experiments conducted comply with current German laws.

Conflict of interest The authors declare that they have no conflict of interest.

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