Filtration based assessment of CTCs and CellSearch® based assessment are both powerful predictors of prognosis for metastatic breast cancer patients

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Abstract

Background: The assessment of circulating tumor cells (CTCs) has been shown to enable monitoring of treatment response and early detection of metastatic breast cancer (MBC) recurrence. The aim of this study was to compare a well-established CTC detection method based on immunomagnetic isolation with a new, filtration-based platform.

Methods: In this prospective study, two 7.5 ml blood draws were obtained from 60 MBC patients and CTC enumeration was assessed using both the CellSearch® and the newly developed filtration-based platform. We analyzed the correlation of CTC-positivity between both methods and their ability to predict prognosis. Overall survival (OS) was calculated and Kaplan-Meier curves were estimated with thresholds of ≥1 and ≥5 detected CTCs.

Results: The CTC positivity rate of the CellSearch® system was 56.7% and of the filtration-based platform 66.7%. There was a high correlation of CTC enumeration obtained with both methods. The OS for patients without detected CTCs, regardless of the method used, was significantly higher compared to patients with one or more CTCs (p < 0.001). The median OS of patients with no CTCs vs. ≥1 CTC assessed by CellSearch® was 1.83 years (95% CI: 1.63–2.02) vs. 0.74 years (95% CI: 0.51–1.52). If CTCs were detected by the filtration-based method the median OS times were 1.88 years (95% CI: 1.74–2.03) vs. 0.59 years (95% CI: 0.38–0.80).

Conclusions: The newly established EpCAM independently filtration-based system is a suitable method to determine CTC counts for MBC patients. Our study confirms CTCs as being strong predictors of prognosis in our population of MBC patients.

Keywords: CTC, CellSearch, Breast cancer, Overall survival, Filtration

Background

Breast cancer is the most common cancer in women, with one out of eight women developing this type of cancer during life [1]. Even though the therapeutic management has significantly improved during the last decades, especially metastatic breast cancer (MBC) is still an incurable disease with a 5-year survival rate of less than 25%. This long term outcome for MBC is influenced by various biological factors. Tumor characteristics, which are associated with breast-cancer related deaths, like blood-derived metastatic potential and the presence of micrometastases are difficult to assess by classical morphological imaging techniques. Within the last years, liquid biopsy procedures for gaining prognostic information associated with the possibility of metastasis formation were developed [2–4]. Circulating tumor cells (CTCs) are potential founder cells for metastasis...
and can be collected and enriched from patients’ blood samples. Their numeration has been proven to be of highly prognostic impact [5] and furthermore allows physicians to recommend a personalized therapy and to monitor treatment response.

Different methods for the assessment of CTCs have been described so far. Most of them rely on the identification of CTCs by targeting antigens specific for epithelial cells (e.g. epithelial cell adhesion molecule, EpCAM) [6], by physical characteristics [7, 8] or expression patterns [9, 10]. The gold standard for CTC counting is the FDA approved semi-automated CellSearch® system (Veridex, LLC, Warren, NJ, USA). This technique uses an immunomagnetic selection of EpCAM-positive CTCs followed by immunostaining of cytokeratins (CKs) and CD45 [11]. So far, many studies presented a significant correlation of the CTC count assessed by CellSearch® (CTC\textsubscript{CS}) and the progression-free as well as the overall survival of MBC patients [12–15]. Thus, CellSearch\textsuperscript{®} represents a platform of high impact to analyze the prognosis and treatment response of breast cancer patients. However, limitations of this method are the missing detection of EpCam-negative CTCs and the difficulties in adding downstream applications like RNA, DNA or protein analysis of captured CTCs.

In this study we aimed to compare the established CellSearch\textsuperscript{®} system with a new, filtration-based method on an integrated CTC platform for automated cellular protein and nucleic acid analysis. Overall, we focused on the comparability of both units and the prognostic value for MBC patients.

**Methods**

**Study design and patient characteristics**

CTC analysis was performed for a total of 60 MBC patients enrolled in the iMode-B (imaging and molecular detection breast) study. Patients were included between 2010 and 2012 at the University Breast Center Franconia, Erlangen. Inclusion criteria were radiologically measurable or clinically assessable MBC and a written informed consent given by the patients for the use of their blood samples. The study was approved by the ethics committee of the Medical Faculty, Friedrich-Alexander University Erlangen-Nuremberg. There were no exclusion criteria based on tumor subtype, age or other patient characteristics. Physicians were blinded to CTC test results and investigators performing CTC analysis were blinded to the clinical data.

**Data capturing**

Data was documented in an electronic case report form specialized on the documentation of MBC by trained and dedicated staff. The database had the same structure like the PRAEGNANT study [16, 17] and data are monitored using automated plausibility checks. The documented data comprised information about primary diagnosis, surgery, treatment as well as progression and information about death. Histopathological data from the primary tumor were documented from pathology reports. Patients were considered estrogen receptor (ER) or progesterone receptor (PR) positive if by immunohistological (IHC) staining at least 1% of cells were stained positive. HER2 positivity was defined as either having an IHC score of 3+ or a gene amplification as shown by chromogenic in situ hybridization.

**Circulating tumor cell detection with the CellSearch\textsuperscript{®} system (CTC\textsubscript{CS})**

Blood samples were drawn into CellSave Tubes (Veridex, LLC) and shipped overnight to an experienced and dedicated laboratory (T.N.F.). The CellSearch\textsuperscript{®} Epithelial Cell Test (Veridex, LLC) was applied for CTC enrichment and enumeration as described before [6, 10, 18, 19]. In brief, CTCs were captured with the automated CELLTRACKS\textsuperscript{®} AUTOPREP\textsuperscript{®} System by using anti-EpCAM-antibody bearing ferrofluid followed by their detection with immunostaining of CKs 8, 18 and 19 and the leukocyte common antigen CD45 as well as 4’,6-diamidino-2-phenylindole (DAPI) to ensure integrity of the cell nucleus. CTCs were identified and enumerated by automated fluorescence microscopy using the CELLTRACKS ANALYZER II\textsuperscript{®} System.

**Circulating tumor cell detection with the filtration based system (CTC\textsubscript{FB})**

For the filtration based method blood samples (7.5 ml EDTA-blood) were processed with the modified pipetting robot of the VERSANT\textsuperscript{®} kPCR Sample Prep system (SIEMENS Healthcare GmbH, Erlangen). Up to 8 samples could be processed in parallel. For that purpose, 50 ml Falcon tubes, each filled with 22.5 ml red blood cell- (RBC-) lysis buffer (1.5 M NH\textsubscript{4}Cl, 100 mM NaHCO\textsubscript{3}, 10 mM disodium EDTA in Millipore water) were placed into a rack of the pipetting robot. The 7.5 ml EDTA blood samples were transferred into individual falcon-tubes by the robot and incubated at RT for 15 min by back and forth aspiration of the pipettes. Subsequently the RBC-lysed diluted blood samples were pipetted into individual vacuum-based filtration units (Siemens Healthcare). CTCs were selected by cell size using Whatman nuclepore track etched membranes (GE) with a defined pore size of 8 \(\mu\)m and 1 inch diameter. This filter system, in combination with dedicated filtration-pressure control (10–30 mbar negative pressure) enables the retention of 85–100% of tumor cells with a background of approx. 0.1% remaining white blood cells. After cell capture and fixation by 3.6% Formaldehyde (Sigma Aldrich) in PBS, the cells were washed and the membrane was permeabilized using Triton X100
In order to perform automated immunostaining, non-specific binding sites were blocked using Blocking Solution (Candor) and cells were stained for cytokeratin 8, 18 (5 μg/ml mouse anti-CK8/18-Dy550, clone UCD/PR 10.11, Siemens Healthcare) and cytokeratin 19 (5 μg/ml mouse anti-CK19-Dy550, clone A53-B/A2, Siemens Healthcare), CD45 (20 μg/ml mouse anti-CD45-Dy650, clone 9.4, Siemens Healthcare) and DAPI (1.1 μg/ml, 4′,6-Diamidino-2-phenylindole dihydrochloride, Sigma Aldrich) by pipetting corresponding antibody-fluorophore-conjugate solutions together with DAPI for cell nucleus staining. Cover medium (1,4-Diazabicyclo [2.2.2] octane, DABCO, Sigma Aldrich) was added to preserve the fluorescence intensity. Finally, the filtration membranes were removed from the processing robot for optical investigation. Cytokeratin positive/CD45 negative/DAPI positive cells (CTCs) were counted by fluorescence scanning microscopy using a dedicated software solution (SIEMENS Healthcare GmbH).

Statistical analysis
CTC assessments were described with cross tables using two different cut-offs (0 vs. ≥1) [20] and (≤5 vs. ≥5) [10]. CTC positivity with regard to prognostic value was defined as detecting at least one CTC in the blood samples with the respective method for CTC detection. A Wilcoxon signed-rank test was performed to compare CTC counts assessed by the different detection methods. A significant test result indicates that there are systematic differences between both detection methods. Furthermore, Spearman's rank correlation coefficient was calculated.

Overall survival was defined as the elapse time between the blood draw and the time of death or last follow-up, if no death event occurred during observation time. The maximal observation time of a patient was approximately 5 years. Survival rates were estimated using the Kaplan-Meier product limit method. Confidence interval of median survival time was estimated as described in [21]. Survival rates of patients with or without CTCs were compared using the log-rank test. Cox proportional hazards models were used to investigate the prognostic value of each CTCs assessment (one model for CTC_{CS} and one model for CTC_{FB}) in addition to other known prognostic factors [22]. Those prognostic factors were age at diagnosis (continuous), hormone receptor and HER2 status (positive vs. negative), grading (ordinal), therapy line (ordinal).

All tests were two-sided, and a p-value of ≤0.05 was regarded as statistically significant. Calculations were carried out using the software package SPSS (Version 21, IBM).

Results
Patient and tumor characteristics
The patient population consisted of 60 patients with a mean age of 60.9 years (SD, 11.2). A total of 16 patients were treated with first-line therapy, 12 with second-line therapy, 12 with third-line therapy and 18 with higher therapy-lines than third line (Table 1). A total of 27 patients received a chemotherapy at time of blood draw, 18 were treated with an antihormon treatment (AH) at time of blood draw and 42 patients were treated with a therapy other than the standard AH or chemotherapy. Of all 60 patients 70.0% had an ER, 63.3% PR and 20.0%
HER2 positive tumor (Table 1). Further detailed patient characteristics are shown in Table 1.

**CTC results**
At least one CTC was found in 66.7% (n = 40) of the patients with the filtration method and in 56.7% (n = 34) with the CellSearch® method. There were 4 cases which were CTC positive according to the CellSearch® method, but CTC negative using the filtration method. Vice versa, in 10 cases the filtration method detected CTCs and the CellSearch® method did not. Overall accuracy rates comparing positive vs. negative test results was 76.7% (n = 46). Considering a classification with CTC negative vs. 1–4 CTCs vs. ≥5 CTCs, the overall accuracy rate was 60% (n = 36) (Table 2).

Comparing the CTC counts assessed by CellSearch® method and the filtration based system of each patient, we found a high correlation (Spearman’s correlation 0.733) of the CTC enumeration (Fig. 1). The CellSearch® system detected a range of 1 to 2000 CTCs while the filtration based method counted CTCs from 1 to 1900. Overall the CTC enumeration by CellSearch® (median: 2.5 cells) was slightly higher compared to the one assessed with the filtration method (median: 1.5 cells). The cell count was lower with the filtration method in 33 cases and higher in 9 cases, a tie was seen in 18 cases of which 16 were a pair of 0 and 0 counts (p < 0.001, Wilcoxon test).

CTC significantly influenced overall survival in addition to the considered predictors. The adjusted hazard ratio (HR) for CTC_CS was 5.2 (95% CI: 2.2–12.4) and for CTC_FB the HR was 4.2 (95% CI: 1.9–9.4). The results of both Cox models are shown in Table 3 and Table 4. Kaplan-Meier curves for overall survival grouped into positive or negative CTC count assessed by CellSearch® or the filtration based method are shown in Fig. 2a and Fig. 2b. Kaplan-Meier curves with a threshold of ≥5 CTCs are displayed in Fig. 2c and Fig. 2d.

The median overall survival of 1.83 years (95% CI: 1.63–2.02) for patients with < 1 CTC_CS (Fig. 2a) was similar to the median overall survival of 1.88 years (95% CI: 1.74–2.03) for patients with no CTC_FB count (Fig. 2b). In contrast the median overall survival of 0.59 years (95% CI: 0.38–0.80) for patients with 1 or more CTCs assessed by the filtration based method (Fig. 2b) was slightly shorter compared to the overall survival of 0.74 years (95% CI: 0.51–1.52) for patients with ≥1 CTC_CS (Fig. 2a). Similarly, significant differences regarding the overall survival were detected for both CTC_CS and CTC_FB counts with a threshold of ≥5 CTCs (Fig. 2c and Fig. 2d). The median overall survival of 1.68 years (95% CI: 1.10–2.26) for patients with < 5 CTC_CS (Fig. 2c) was slightly longer than the median overall survival of 1.29 years (95% CI: 0.89–1.69) for patients with < 5 CTC_FB counts (Fig. 2d). In comparison, the median overall survival of 0.33 years (95% CI: 0.00–0.66) for patients with ≥5 CTC_CS (Fig. 2c) was similar to the median overall survival of 0.47 years (95% CI: 0.00–1.24) for patients with 5 or more CTCs assessed by the filtration based method (Fig. 2d).

**Discussion**
In this study we used CellSearch®, a commonly used method for CTC detection, and a new established automated filtration-based method to assess the prognostic Table 2 Comparison of CTC enumeration by CellSearch® and filtration based method

| CTC_FB n (%) | Negative | 1–4 CTCs | ≥5 CTCs | Total |
|--------------|----------|----------|---------|-------|
| CTC_CS n (%) | Negative | 16 (26.7%) | 8 (13.3%) | 2 (3.3%) | 26 (43.3%) |
| 1–4 CTCs | 3 (5.0%) | 5 (8.3%) | 10 (16.7%) | 18 (30.0%) |
| ≥5 CTCs | 1 (1.7%) | 0 (0%) | 15 (25.0%) | 16 (26.7%) |
| Total | 20 (33.3%) | 13 (21.7%) | 27 (45.0%) | 60 (100%) |

| Table 3 Cox Regression model for the prediction of OS using CTC count by CellSearch® and covariates |
|-----------------------------------------------------|
| Characteristic | HR | 95% CI | p-value |
|----------------|----|--------|---------|
| Age            | Per year | 1.00 | 0.97–1.03 | 0.91 |
| Hormone receptor status | Negative | 1 (reference) | |
| Positive | 0.44 | 0.17–0.85 | 0.08 |
| HER2 Status | Negative | 1 (reference) | |
| Positive | 0.32 | 0.13–0.85 | 0.02 |
| Grading | Per grade | 1.08 | 0.58–2.02 | 0.82 |
| Therapy line | Per line | 1.01 | 0.78–1.32 | 0.93 |
| CTC count | 0 | 1 (reference) | |
| ≥1 | 5.20 | 2.18–12.43 | 0.0002 |
value of CTC count in peripheral blood in a cohort of MBC patients. The CTC count within 7.5 ml of blood draw was determined in a study cohort of 60 radiologically measurable or clinically assessable MBC patients. We calculated the overall survival to determine and compare the prognostic impact of both methods. Even though the most commonly used cutoff for CTC positivity is five or more, it is still unclear whether a presence of one or more CTCs might be an even more accurate predictor depending on the tumor subgroup analyzed [20, 23]. Several prospective, multicenter studies showed a significant prognostic value for progression-free and overall survival of MBC patients with CTC levels < 5 or ≥1 [12, 14, 24]. Additionally, CTC assessment was proven to be a good setting for valuation of treatment response and as an individual predictive test for metastatic relapse [14, 24, 25]. Here, we set out to compare both thresholds (≥1 and ≥5 CTCs) for both methods. A significant prognostic value of CTC count could be achieved using the CellSearch® as well as the filtration-based system. There were no differences between a threshold of one CTC or five CTCs, indicating that the new filtration-based method is also suitable for sensitive detection of less than five CTCs.

Table 4 Cox Regression model for the prediction of OS using CTC count by the filtration based method and covariates

| Characteristic       | HR   | 95% CI          | p-value |
|----------------------|------|-----------------|---------|
| Age                  |      |                 |         |
| Per year             | 0.99 | 0.96–1.02       | 0.47    |
| Hormone receptor status |    |                 |         |
| Negative             | 1 (reference) |                |         |
| Positive             | 1.50 | 0.56–4.06       | 0.41    |
| HER2 Status          |      |                 |         |
| Negative             | 1 (reference) |                |         |
| Positive             | 0.89 | 0.35–2.25       | 0.80    |
| Grading              |      |                 |         |
| Per grade            | 1.45 | 0.70–3.00       | 0.32    |
| Therapy line         |      |                 |         |
| Per line             | 0.98 | 0.75–1.26       | 0.85    |
| CTC count            |      |                 |         |
| 0                    | 1 (reference) |                |         |
| ≥1                   | 4.20 | 1.86–9.46       | 0.001   |

and invasive phenotype [29, 30]. This emphasizes that a method only relying on EpCAM positivity may not be suitable for detection of all CTCs and thus might give inadequate results concerning the prognostic value or the biological classification the CTCs. In contrast to the CellSearch® system, the filtration-based system does not select CTCs based on EpCAM positivity and thus we hypothesize this system might be suitable for detection of CTCs with a wider range of different phenotypes. We assume the detection of patients with a positive CTC_FB count but negative CTC_CS enumeration might be due to the missing EpCAM positivity of these cells.

Overall, the assessment of the filtration based method was feasible. The CTC positivity was within the expected rate and similar to results from different studies [6, 10]. Interestingly, even though the filtration based method does not only select EpCam positive CTCs but in contrast to the CellSearch® system also EpCAM negative ones, we observed an overall smaller CTC count with the filtration based system compared to CellSearch®. Nevertheless, we could show a significant prognostic value for overall survival using both methods. We hypothesize that the smaller number of detected CTCs might be due to the defined pore size of the filtration based system. It was shown earlier that CTCs from prostate cancer patients, which were isolated using the CellSearch® system, had a significant smaller average diameter (7.97 μm) compared to cultured prostate cancer cells [31]. Even though, to our knowledge, there are no studies regarding the tumor cell size of CTCs from breast cancer patients collected by the CellSearch® system, we assume similar findings would occur. Our filtration based system only collects CTCs with a diameter of 8.0 μm or larger and thus, this might be causative for the overall smaller cell numbers and the CTC_CS positive, but CTC_FB negative enumerations.

The assessment of tumor characteristics on CTCs is an attractive opportunity to avoid repeated tissue biopsies. CTC counts from peripheral blood samples are defined as liquid biopsies. In contrast to tissue biopsies, this is a non-invasive, quick and feasible real-time method to gain tumor cells for further analysis. Tumor characteristics can help to stratify therapy decisions. Even if primary tumor biopsies are negative for certain tumor markers (e.g. HER2), CTCs often show a different expression pattern (HER2 positive) [23]. These characteristics of CTCs are important hallmarks to define the treatment strategy and can help to avoid overtreatment. The ongoing DETECT III trial (NCT01619111) is currently investigating the therapeutic relevance of HER2-targeted therapy for MBC patients with HER2-negative tissue biopsies but HER2-positive CTCs [32]. Additionally, protein or gene expression profiles and analysis of epigenetic or genetic alterations of the DNA could help to characterize CTCs and thus the tumor even further.
It even might help to stratify the therapeutic strategy for MBC patients [35]. As the filtration-based setting for CTC isolation is based on an automated nucleic acid preparation system (VERSANT® kPCR sample Prep system), it might not only help to determine the CTC count but also to purify DNA, RNA or proteins from CTCs for further analysis [36].

Nevertheless, this study has several limitations. First, the small sample size only allows to coarsely compare the two methods with regard to their prognostic value. Second, the lack of standardized treatment is a potential bias as it might influence the prognostic value regardless of the CTC count. Additionally, the time of blood draw was not defined precisely. But overall this setting represents the common clinical practice and was sufficient enough to compare two different CTC detection techniques in regard of overall survival.

Conclusions
In summary, our data indicates that the newly established EpCAM independently filtration-based method might be equivalent to the CellSearch® method in regard to sensitivity of detecting CTCs from MBC patients and predicting prognosis. The filtration-based method might be easier to be used for automated RNA, DNA or Protein extraction from isolated CTCs allowing an in-depth characterization of the CTCs and the related biological background of the metastatic disease.

| Group | Median | 95% C.I. | Log-rank P |
|-------|--------|----------|------------|
| CTC <1 | 1.68   | 1.10-2.26 | <0.0001    |
| CTC ≥1 | 0.33   | 0.00-0.66 | <0.0001    |
| CTC <5 | 1.29   | 0.89-1.69 | <0.05      |
| CTC ≥5 | 0.47   | 0.00-1.24 | <0.05      |
Abbreviation
CTC: Circulating tumor cells; CTCs: CTCs assessed using CellSearch® method; CTCeng: CTCs assessed using the filtration-based method; ER: Estrogen receptor; IHC: Immunohistological; MBC: Metastatic breast cancer; PR: Progesterone receptor

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The described filtration method for the detection of CTCs has been done under research conditions and is no commercial platform for the detection of CTCs.

Authors’ contributions
HH, MR and PAF performed the analysis and interpretation of data and drafted the manuscript. WG, MA, PP and KF developed the filtration-based method and acquisition of CTC count and revised the manuscript critically. SI, CR, MPL, BV, MWB and PG were responsible for the collection of blood draws, the acquisition of clinical data and revised the manuscript critically. LH performed the statistical analysis and was involved in drafting the manuscript. FMS and TNF performed the analysis of CTC counts using the CellSearch method and revised the manuscript critically. AH, HH and KA contributed to conception and design of the study and revised the manuscript critically. All authors gave final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate
The study was approved by the ethics committee of the Medical Faculty, Friedrich-Alexander University Erlangen-Nuremberg and complies with the current laws of the country in which it was performed. A written informed consent was obtained from all patients. This consent included the approval of biomaterial collection and analysis as well as the access of patient/clinical data and storage in a database.

Consent for publication
Not applicable

Competing interests
PAF received honoraria from Novartis, Pfizer, Roche, Celgene and his institution conducts research with research grant from Novartis. MPL received honoraria from Novartis, Pfizer, AstraZeneca, Roche, Celgene and his institution conducts research with research grant from Novartis. CR received honoraria from Novartis and Roche and her institution conducts research with research grant from Novartis. PG has received honoraria from Novartis. WG, MM and PP are employees of Siemens Healthcare GmbH. All other authors declare that they do not have any conflicts of interest.

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References
1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin. 2016;66:7–30.
2. Alix-Panabieres C, Pantel K. Circulating tumor cells: liquid biopsy of cancer. Clin Chem. 2013;59:1110–8.
3. Crowley E, Di Nicolantonio F, Loquats F, Bardelli A. Liquid biopsy: monitoring cancer- genetics in the blood. Nat Rev Clin Oncol. 2013;10:472–84.
4. Heitzer E, Ute P, Geogl JB. Circulating tumor DNA as a liquid biopsy for cancer. Clin Chem. 2015;61:112–23.
5. Cristofanilli M, Hayes DF, Budd GT, Ellis MJ, Stopeck A, Reuben JM, Doyle GV, Materia J, Allard JW, Miller MC, et al. Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. J Clin Oncol. 2005;23:1420–30.
6. Riethdorf S, Fritsche H, Muller V, Rau T, Schindlbueck C, Rack B, Janni W, Coith C, Beck K, Janicke F, et al. Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the cell search system. Clin Cancer Res. 2007;13:920–8.
7. Huang T, Jia CP, Jun Y, Sun WJ, Wang WT, Zhang HL, Cong H, Jing FX, Mao HJ, Jin QH, et al. Highly sensitive enumeration of circulating tumor cells in lung cancer patients using a size-based filtration microfluidic chip. Biosens Bioelectron. 2014;51:213–4.
8. Kahn HJ, Presta A, Yang LY, Bloniald J, Trudeau M, Lickley L, Holloway C, McCready DR, Maclean D, Marks A. Enumeration of circulating tumor cells in the blood of breast cancer patients after filtration enrichment: correlation with disease stage. Breast Cancer Res Treat. 2004;86:237–47.
9. Larioudo ES, Markou A. Circulating tumor cells in breast cancer: detection systems, molecular characterization, and future challenges. Clin Chem. 2011;57:1242–55.
10. Muller V, Riethdorf S, Rack B, Janni W, Faeching PA, Solomander E, Attaus B, Kasmir-Bauer S, Pantel K, Fehm T. Prognostic impact of circulating tumor cells assessed with the CellSearch System and AdnaTest Breast in metastatic breast cancer patients: the DETECT study. Breast Cancer Res Treat. 2012;141R118.
11. Miller MC, Doyle GV, Terstappen LW. Significance of circulating tumor cells detected by the CellSearch system in patients with metastatic breast colorectal and prostate cancer. J Oncol. 2010;2010:6517421.
12. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Materia J, Miller MC, Reuben JM, Doyle GV, Allard JW, Terstappen LW, Hayes DF. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med. 2004;351:781–91.
13. Hayes DF, Cristofanilli M, Budd GT, Ellis MI, Stopeck A, Miller MC, Materia J, Allard JW, Doyle GV, Terstappen LW. Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. Clin Cancer Res. 2006;12:2418–24.
14. Liu MC, Shields PG, Warren RD, Cohen P, Wilkinson M, Ottaviano YL, Rao SB, Eng Wong J, Sellier-Moisewitsch F, Noone AM, Isaac C. Circulating tumor cells: a useful predictor of treatment efficacy in metastatic breast cancer. J Clin Oncol. 2009;27:513–9.
15. Nakamura S, Sayata H, Ohino S, Yamauchi H, Iwata H, Tsunoda I, Ito Y, Tokudome N, Toi M, Kuroi K, Suzuki E. Multi-center study evaluating circulating tumor cells as a surrogate for response to treatment and overall survival in metastatic breast cancer. Breast Cancer. 2010;17:199–204.
16. Faeching PA, Brucker SY, Fehm TH, Overkamp F, Janni W, Wallwiener M, Hadji P, Belleville E, Habeke L, Taran FA, et al. Biomarkers in patients with metastatic breast cancer and the PRAEGNANT study network. Geburtshilfe Frauenheilkd. 2015;75:41–50.
17. Hein A, Gass P, Walter CB, Taran FA, Hartkopf A, Overkamp F, Kolberg HC, Hadji P, Tesch H, Ettl J, et al. Computerized patient identification for the EMBRACA clinical trial using real-time data from the PRAEGNANT network for metastatic breast cancer patients. Breast Cancer Res Treat. 2016;158:55–65.
18. Hauch S, Zimmermann S, Lankiewicz S, Zieglschmid V, Bocher O, Albert WH. The clinical significance of circulating tumour cells in breast cancer and colorectal cancer patients. Anticancer Res. 2007;27:1337–41.
19. Riethdorf S, Muller V, Zhang L, Rau T, Loibl S, Komor M, Roller M, Hueber J, Fehm T, Schneider I, et al. Detection and HER2 expression of circulating tumor
cells: prospective monitoring in breast cancer patients treated in the neoadjuvant GeparQuattro trial. Clin Cancer Res. 2010;16:2634–45.
20. Gazzaniga P, Raimondi C, Gradilone A, Biondi Zoccai G, Nicolazzo C, Gandini O, Longo F, Tomaso S, Lo Russo G, Seminara P, et al. Circulating tumor cells in metastatic colorectal cancer: do we need an alternative cutoff? J Cancer Res Clin Oncol. 2013;139:1411–6.
21. Barker C. The mean, median, and confidence intervals of the Kaplan-Meier survival estimate—computations and applications. Am Stat. 2009;63:78–80.
22. Loehberg CR, Almstedt K, Jud SM, Haeberle L, Fasching PA, Hack CC, Lux MP, Thiel FC, Schrauder MG, Brunner M, et al. Prognostic relevance of Ki-67 in the primary tumor for survival after a diagnosis of distant metastasis. Breast Cancer Res Treat. 2013;138:899–908.
23. Liu Y, Liu Q, Wang T, Bian L, Zhang S, Hu H, Li S, Hu Z, Wu S, Liu B, Jiang Z. Circulating tumor cells in HER2-positive metastatic breast cancer patients: a valuable prognostic and predictive biomarker. BMC Cancer. 2013;13:202.
24. Bidard FC, Mathiot C, Delaloge S, Brain E, Giachetti S, de Cremoux P, Marty M, Pierga JY. Single circulating tumor cell detection and overall survival in nonmetastatic breast cancer. Ann Oncol. 2010;21:729–33.
25. Böhnke J, Wang T, Liu Y, Zhang HQ, Song JJ, Zhang SH, Wu SK, Song ST, Jiang ZF. Evaluation of treatment response for breast cancer: are we entering the era of “biological complete remission”. Chin J Cancer Res. 2012;24:403–7.
26. Raimondi C, Nicolazzo C, Gradilone A. Circulating tumor cells isolation: the “post-EpCAM era”. Chin J Cancer Res. 2015;27:461–70.
27. Martelotto LG, Ng CK, Piscuoglio S, Weigelt B, Reis-Filho JS. Breast cancer intra-tumor heterogeneity. Breast Cancer Res. 2014;16:210.
28. Gorges TM, Tinhofer I, Drosch M, Rose L, Zollner TM, Krahn T, von Ahsen O. Circulating tumor cells escape from EpCAM-based detection due to epithelial-to-mesenchymal transition. BMC Cancer. 2012;12:178.
29. Gires O, Stoecklein NH. Dynamic EpCAM expression on circulating and disseminating tumor cells: causes and consequences. Cell Mol Life Sci. 2014;71:4393–402.
30. Spizzo G, Gast G, Obrist P, Fong D, Haun M, Grunewald K, Parson W, Eichmann C, Millinger S, Fiegl H, et al. Methylation status of the ep-CAM promoter region in human breast cancer cell lines and breast cancer tissue. Cancer Lett. 2007;246:253–61.
31. Park S, Ang RR, Duffy SP, Baoz J, Chi KN, Black PC, Ma H. Morphological differences between circulating tumor cells from prostate cancer patients and cultured prostate cancer cells. PLoS One. 2014;9:e85264.
32. Hagenbeck C, Melcher CA, Janni JW, Schneeweiss A, Fasching PA, Aktas B, Pantel K, Solomayer EF, Oetmann U, Jaeger BAS, et al. DETECT III: A multicenter, randomized, phase III study to compare standard therapy alone versus standard therapy plus lapatinib in patients (pts) with initially HER2-negative metastatic breast cancer but with HER2-positive circulating tumor cells (CTC). J Clin Oncol. 2013;31:20 Meeting Abstract: TPS1146-TPS1146.
33. Pixberg CF, Raba K, Muller F, Behrens B, Honisch E, Niederacher D, Neubauer H, Fehm T, Goering W, Schulz WA, et al. Analysis of DNA methylation in single circulating tumor cells. Oncogene. 2017;36(23):3223–31.
34. Schneck H, Blassl C, Meier-Stiegen F, Neves RP, Janni W, Fehm T, Neubauer H. Analysing the mutational status of PIK3CA in circulating tumor cells from metastatic breast cancer patients. Mol Oncol. 2013;7:976–86.
35. Lee JS, Magbanua MJ, Park JW. Circulating tumor cells in breast cancer: applications in personalized medicine. Breast Cancer Res Treat. 2016;160:411–24.
36. Poizot B, Medoro G, Pasch S, Fontana F, Zorzino L, Pestka A, Andergassen U, Meier-Stiegen F, Cyzy ZT, Alberter B, et al. Molecular profiling of single circulating tumor cells with diagnostic intention. EMBO Mol Med. 2014; 6:1371–86.