Circulating Tumor-Derived Endothelial Cells: An Effective Biomarker for Breast Cancer Screening and Prognosis Prediction

Tuo Han, Juanjuan Zhang, Dong Xiao, Binhui Yang, Liang Chen, Chao Zhai, Feifei Ding, Yue Xu, Xiaoyu Zhao, and Jiangman Zhao

1Department of Oncological Surgery, 3201 Hospital Affiliated to Xi’an Jiaotong University School of Medicine, Hanzhong 723000, Shaanxi, China
2Department of Surgical Oncology, Quanzhou First Hospital Affiliated to Fujian Medical University, Quanzhou 362000, China
3Shanghai Biotecan Pharmaceuticals Co. Ltd, Shanghai Zhangjiang Institute of Medical Innovation, Shanghai 201204, China

Correspondence should be addressed to Xiaoyu Zhao; xyzh07@126.com and Jiangman Zhao; zhaojiangman86@163.com

Received 8 May 2022; Accepted 28 June 2022; Published 28 August 2022

Academic Editor: Jie Mei

Copyright © 2022 Tuo Han et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Background. Circulating tumor-derived endothelial cell (CTEC) is a new potential tumor biomarker to be associated with cancer development and treatment efficacy. However, few evidences are available for breast cancer.

Methods. Eighty-nine breast cancer patients were recruited, and preoperative and postoperative blood samples were collected. Besides, 20 noncancer persons were enrolled as controls. An improved subtraction enrichment and immunostaining-fluorescence in situ hybridization (SE-iFISH) method was adopted to codetect CD31+ aneuploid CTEC and CD31− aneuploid circulating tumor cell (CTC). Then, the clinical significance of CTCs and CTECs on breast cancer screening and prognosis prediction was evaluated and compared.

Results. The positive rate of CTCs and CTECs in newly diagnosed breast cancer patients was 68.75% and 71.88%. Among detected aneuploid circulating rare cells, CTEC accounts for a greater proportion than CTC in breast cancer patients. CTEC-positive rate and level were significantly high in breast cancer patients with lymph node metastasis (LNM) than those without LNM (P = 0.043), while there was no significant difference in CTC. CTEC (area under the curve, AUC = 0.859) had better performance than CTC (AUC = 0.795) to distinguish breast cancer patients from controls by receiver operator characteristic curve analysis. Preoperative CTEC count ≥ 2 was a significant risk factor for reducing PFS of breast cancer patients.

Conclusions. CTECs may function as a reliable supplementary biomarker in breast cancer screening and prognosis prediction.

1. Introduction

Breast cancer is the most common cancer in women worldwide [1]. About 20–30% of breast cancers have developed locally advanced or metastasis, which results in poor prognosis [2]. Postoperative recurrence and metastasis are the major causes of mortality in breast cancer patients [3]. Because of its complexity, the traditional methods of cancer detection are limited to comprehensively capturing the characteristics of breast tumors. Hence, there are still several limitations of breast cancer management including early diagnosis, prediction of relapse and prognosis, and monitoring response to treatment.

As a liquid biopsy method, circulating tumor cell (CTC) is a commonly used biomarker for breast cancer diagnosis and prognosis prediction. Previous studies have shown that CTCs numbers in peripheral blood were directly associated with the prognosis of breast cancer patients [4–8]. Persistently high CTCs numbers in patients always indicate significantly increased risks for relapse and postoperative micrometastasis [9, 10]. The presence of CTCs after neoadjuvant chemotherapy was also found to be relevant to early metastatic relapse and worse disease-free survival [11]. CTCs have also been used for therapeutic evaluation, in which patients with persistent CTCs after completion of (neo) adjuvant chemotherapy have an increased risk of relapse [12–14]. For patients receiving palliative chemotherapy, CTCs numbers after the first cycle of treatment showed strong relevance to the therapeutic response [15].
The technical approaches of enrichment and identification were multifarious, mainly based on polyploidy [16, 17], CK positivity, and EpCAM positive [18–21], which always confused circulating tumor-derived endothelial cells (CTECs) with CTCs. CTECs are tumor-derived endothelial cells shed into the peripheral circulation of patients [22, 23]. Peter Ping Lin et al. developed an improved subtraction enrichment and immunostaining-fluorescence in situ hybridization (SE-iFISH) method, which could codetect aneuploid CTC and CTEC [22]. Increased CTECs counts were found to be a high-risk factor for poor outcomes in nonsmall cell lung cancer (NSCLC) patients [24]. CTECs quantification is also a promising tool for treatment monitoring for neoadjuvant letrozole plus low-dose cyclophosphamide therapy in estrogen receptor-positive breast cancer [25]. However, there are few studies comprehensively expounding the characteristics of CTECs and CTCs and their clinical significance during breast cancer diagnosis and treatment.

In this study, we applied the SE-iFISH method developed by Cytelligen (San Diego, CA, USA) to codetect aneuploid CTC and CTEC [22]. A total of 89 breast cancer patients and 20 noncancer controls were recruited, and peripheral blood was collected to detect CTCs and CTECs. Cytogenetic characteristics of CTCs and CTECs during the process of diagnosis and treatment and their correlation with clinical and pathological factors were comprehensively analyzed. The clinical significance of CTCs and CTECs on breast cancer screening and prognosis prediction was also evaluated and compared.

### 2. Materials and Methods

#### 2.1. Participants and Sample Collection

A total of 89 female breast cancer patients and 20 noncancer controls were recruited from 3201 Hospital Affiliated to Xi’an Jiaotong University School of Medicine and Quanzhou First Hospital Affiliated to Fujian Medical University between April 2018 and April 2020. The clinical characteristics of 89 breast cancer patients are given in Table 1. Seventy-five patients received surgery following adjuvant therapy, and 14 patients underwent neoadjuvant chemotherapy (NCT) following surgery and adjuvant therapy (Table 1).

In order to detect CTCs and CTECs, 7.5 mL of peripheral blood was collected by acid citrate dextrose (ACD) anticoagulant tube (Becton Dickinson, Franklin Lakes, NJ, USA) from 20 noncancer controls and 32 breast cancer patients. There are 72 patients after surgery who received CTCs and CTECs tests, while 23 of them received twice, after surgery and postoperative adjuvant chemotherapy. The design of this study is shown in Figure 1.

This study involves human participants and was approved by the Ethical Committee of Department of Oncological Surgery, 3201 Hospital Affiliated to Xi’an Jiaotong University and Quanzhou First Hospital Affiliated to Fujian Medical University. The experiments were conducted after collecting the informed consent of each subject, and the study conforms with The Code of Ethics of the World Medical Association (Declaration of Helsinki) in the British Medical Journal (18 July 1964).

#### Table 1: Clinical characteristics of all 89 breast cancer patients in this study.

| Clinical characteristics | Total | Proportion (%) |
|--------------------------|-------|----------------|
| Age (years) ≤50          | 46    | 51.69          |
| Age (years) >50          | 43    | 48.31          |
| BMI <24.0                | 52    | 58.43          |
| BMI ≥24.0                | 37    | 41.57          |
| Molecular classification  |       |                |
| Luminal A                | 8     | 8.99           |
| Luminal B                | 46    | 51.69          |
| HER2+                    | 16    | 17.98          |
| TNBC                     | 19    | 21.35          |
| Stage                    |       |                |
| 0                        | 2     | 2.25           |
| I                        | 12    | 13.48          |
| II                       | 53    | 59.55          |
| III                      | 19    | 21.35          |
| IV                       | 3     | 3.37           |
| Lymph node metastasis    |       |                |
| Yes                      | 52    | 58.43          |
| No                       | 37    | 41.57          |
| Therapy                  |       |                |
| Surgery following adjuvant therapy | 75 | 84.27 |
| NCT following surgery and adjuvant therapy | 14 | 15.73 |

Stage 0, pTisN0M0.

#### Figure 1: Flowchart. A total of 89 breast cancer patients were enrolled.

#### 2.2. Subtraction Enrichment (SE). CTCs and CTECs were enriched with a Human Circulating Rare Cell Subtraction Enrichment kit (Cytelligen, San Diego, CA, USA) according to the manufacturer’s instructions and previous studies’ suggestions [22]. In brief, 7.5 mL of peripheral blood was centrifuged at 800 × g for 8 min at room temperature to remove plasma. Then, the lower layer of cells was transferred into centrifuge tubes containing 3 mL...
of hCTC separation matrix, and red blood cells were discarded by centrifugation at 450 × g for 8 min at room temperature. Buffy coat cells were collected in new tubes and then incubated with an immunomagnetic particle-conjugated anti-CD45 antibody for 20 min with gentle shaking. WBCs bound to immunobeads were depleted using a magnetic separator. The solution without beads was centrifuged at 450 × g for 8 min following rinsed twice by hCTC buffer at room temperature. Finally, the cell pellet was completely mixed with cell fixative, and the mixture was coated on glass slides and dried overnight at 30°C, which would be identified by immunostaining-fluorescence in situ hybridization (iFISH).

2.3. Immunostaining-Fluorescence In Situ Hybridization (iFISH). For CTCs and CTECs identification, the dried monolayer cells on the coated and formatted CTC slides were rinsed and incubated with saline-sodium citrate buffer for 10 min following dehydration in ethanol for 2 min. Centromere probe 8 (CEP8) spectrum orange (Cytelligen, San Diego, CA, USA) was added to the CTC slides, which were denatured at 76°C for 10 min and hybridized for 4 h at 37°C. Then, the hybridization slides were subsequently darkly incubated with AlexaFluor® 594-conjugated anti-CD45 IgG (spectrum red) and AlexaFluor® 488-conjugated anti-CD31 IgG (spectrum green) for 2 h at room temperature. Finally, DAPI (spectrum blue) was added to the CTC slides and was subjected to fluorescence microscope scanning and analyses.

2.4. CTC and CTEC Identification. The CTC were confirmed by CD31−/CD45−/DAPI+/CEP8 > 2 (Figure 2(a)), and the CTEC was defined as CD31+/CD45−/DAPI+/CEP8 > 2 (Figure 2(b)). The interference by leukocyte should be excluded using CD45+ (Figure 2(c)) [26], which was defined as CD31−/CD45+/DAPI+/CEP8 = 2.

2.5. Statistical Analysis. Statistical analysis and graphical plots were performed using SPSS 22 (IBM Corp.), GraphPad Prism 6 (La Jolla, CA, USA), and R project. The differences of categorical variables in distribution among groups were analyzed using the chi-square test or the Fisher exact test. Differences of continuous variables with normal distribution among groups were compared by the t-test, while nonparametric tests (Mann–Whitney U test or Kruskal–Wallis H test) were used if they are not consistent with normal distribution. Receiver-operating characteristic (ROC) curve was used to assess the diagnostic efficacy of variables between controls and patients. Kaplan–Meier survival curves and log-rank tests were used to compare the differences in progression-free survival (PFS) rate between the two groups. Cox proportional hazards regression analyses were carried out to identify risk factors of poor prognosis. P < 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. Analysis of CTCs and CTECs in Breast Cancer Patients before Any Therapy. Table 2 provides the clinical characteristics and the CTC test results of 32 patients who received CTC and CTEC tests before any therapy when newly diagnosed. In addition, we also compared the levels of CTCs and CTECs according to the stage, lymph node metastasis (LNM) status, and molecular classifications (Figure 3). We found that CTEC-positive rate (Table 2; P = 0.033) and level (Figure 3(b); P = 0.043) were significantly higher in breast cancer patients with LNM than those without LNM, while there was no significant difference in CTC-positive rate (Table 2; P = 0.325) and level (Figure 3(b); P = 0.6005). CTC was detected in all triple-negative breast cancer (TNBC) patients (100%, 7/7), and its positive rate was much higher than that in HER2+ (40%, 2/5) and luminal A/B (65%, 13/20) classification. Figure 3(c) also show that TNBC patients had a higher level of CTC than other classifications (P = 0.0188). However, there was no significant difference in the level of CTC and CTEC between stage 0-II and III-IV patients. A novelty of the results was that elder patients (> 50 years) had a significantly higher CTEC-positive rate than younger patients (P = 0.050) and overweight patients (BMI ≥ 24.0) had a higher CTC-positive rate (P = 0.024) than other patients (BMI < 24.0).

3.2. Characteristics of CTCs and CTECs in Breast Cancer Patients. Figure 4 shows the distribution of CTC and CTEC and their ploidy ratio. The results showed that CTEC accounts for a greater proportion than CTC in blood of breast cancer patients (Figures 4(a), 4(c), and 4(e)). In 32 newly diagnosed breast patients, 62 CTCs (34.64%) and 117 CTECs (65.36%) were detected (Figure 4(a)). The CTC and CTEC proportions of patients after surgery (Figure 2(c)) and adjuvant chemotherapy (Figure 3(e)) were similar to the patients before any therapy. During the first two times of tests before adjuvant chemotherapy, the ratio of triploid, tetraploid, and multiploidy of CTCs was approximately equal (Figures 4(b), 4(d)), while multiploidy (≥ 5) accounted for the majority of CTECs (Figures 4(b), 4(d)). We found that the multiploidy (≥ 5) proportion of CTC and CTEC had obviously increased to 52.44% and 84.73%, respectively, after adjuvant chemotherapy (Figure 4(f)).

To comprehensively understand the relationship and difference between novel and traditional cancer biomarkers, we calculated Spearman’s rank correlation coefficient of CTC, CTEC, CEA, CA12-5, CA19-9, and CA15-3 (Figure 5). CTC was significantly and positively correlated with CTEC (r = 0.28, P = 0.01). Meanwhile, CEA had markedly positive correlations with CA19-9 (r = 0.22, P = 0.04) and CA15-3 (r = 0.33, P < 0.01), and CA19-9 was positively correlated with CA15-3 (r = 0.24, P = 0.03). No significant correlation was found between novel biomarkers (CTC and CTEC) and traditional biomarkers (CEA, CA12-5, CA19-9, and CA15-3). These results indicated CTC and CTEC were independent of traditional cancer biomarkers.
3.3. Clinical Screening Value of CTC and CTEC in Breast Cancer Patients. To evaluate the clinical application value of CTC and CTEC for breast cancer screening, 20 noncancer participants were recruited to detect CTC and CTEC as controls. CTC was not detected in 18 of 20 (90%) controls, and all the 20 controls’ CTEC (100%) was negative. For breast cancer patients, the positive rate of CTC and CTEC was 68.75% (22/32) and 71.88% (23/32), respectively. The mean value of CTC and CTEC was significantly higher in breast cancer patients than those in controls (Figures 6(a) and 6(b), \( P < 0.0001 \)). Results of ROC curve analysis showed that CTEC (AUC = 0.859) had better performance than CTC.

![Figure 2: Identification of CTC, CTEC, and WBC by SE-iFISH.](image)

(a) CTC (CD31−/CD45−/DAPI+/CEP8 > 2); (b) CTEC (CD31+/CD45−/DAPI+/CEP8 > 2); (c) WBC (CD31−/CD45+/DAPI+/CEP8 = 2). Scale bars, 5 μm; CD45, red; CD31, green; CEP8, orange; DAPI, blue.

| Clinical characteristics | Total CTCs | CTECs | \( P \) value | Total CTECs | \( P \) value |
|-------------------------|------------|-------|---------------|-------------|---------------|
| Age (years)             |            |       |               |             |               |
| \( \leq 50 \)           | 19         | 12    | 7             | 11          | 8             |
| \( >50 \)               | 13         | 10    | 3             | 12          | 1             |
| BMI                     |            |       |               |             |               |
| \( <24.0 \)             | 19         | 10    | 9             | 13          | 6             |
| \( \geq 24.0 \)         | 13         | 12    | 1             | 10          | 3             |
| Molecular classification|            |       |               |             |               |
| Luminal (A/B)           | 20         | 13    | 7             | 14          | 6             |
| HER2+                   | 5          | 2     | 3             | 4           | 1             |
| TNBC                    | 7          | 7     | 0             | 5           | 2             |
| Stage                   |            |       |               |             |               |
| 0–II                    | 26         | 17    | 9             | 18          | 8             |
| III-IV                  | 6          | 5     | 1             | 5           | 1             |
| Lymph node metastasis   |            |       |               |             |               |
| Yes                     | 20         | 15    | 5             | 17          | 3             |
| No                      | 12         | 7     | 5             | 6           | 6             |

\( P < 0.05 \), \( ** P < 0.01 \), \( *** P < 0.001 \).

Table 2: CTC and CTECs status according to clinical characteristics of newly diagnosed breast cancer patients before any therapy.
Figure 3: CD31$^-$ CTC and CD31$^+$ CTEC in newly diagnosed breast cancer patients. Comparison of CTC and CTEC abundance according to tumor stage (a), lymph node metastasis (b), and molecular classification (c).

Figure 4: Continued.
(AUC = 0.795) for breast cancer screening. After summing the aneuploid cells (CTC plus CTEC), the AUC was widely promoted to 0.914. This result suggested aneuploid cells from peripheral blood were an ideal biomarker for breast cancer screening.

3.4. Clinical Prognostic Significance of CTCs and CTECs in Breast Cancer Patients. All the patients were followed-up until March 2022, using progression (recurrence or metastasis) as the end of follow-up. The median follow-up time was 37 months (3–62 months). During the follow-up period, 14 patients had recurrence, 1 patient was lost of follow-up, and 74 patients were progression-free. We separately plotted the progression-free survival curves according to the CTC and CTEC values before and after the surgical operation. Preoperative CTEC count ≥2 was a significant risk factor for reducing the PFS of breast cancer patients (*P < 0.0091, Figure 7(d)). However, preoperative CTC enumeration showed no significant impact on PFS.
and postoperative quantity of CTEC also had no significant influence on PFS (Figure 8). Surprisingly, the increased postoperative CTC count (≥2 cells/7.5 mL) can even predict a better prognosis (Figure 8).

4. Discussion

The tumor microenvironment (TME) is a complex system comprised of cancer cells and their surrounding cells such as...
endothelial cells, cancer-associated fibroblasts, immune cells, and so on [27]. CTCs were cancer cells shed into peripheral blood, and similarly, CTECs were the tumor-derived endothelial cells disseminated to the blood circulation [28]. In the last decade, numerous studies have verified that the CTCs, as the main biomarker in liquid biopsy, have been used for screening and monitoring treatment efficacy, as well as predicting the prognosis of many cancers [18–21]. CTECs mostly harbor mixed properties of both endothelial vascularization and cancerous malignancy [28]. In most studies, the CTC detecting methods only identify CTC using the following markers such as cellular size, EpCAM and CK expression, and aneuploid chromosomes, which cannot distinguish CTECs from CTCs.

In this study, we used an improved SE-iFISH method, which could codetect and distinguish aneuploid CTEC by adding an anti-CD31 antibody from aneuploid CTC. Then, 89 breast cancer patients and 20 noncancer controls were enrolled to receive CTC and CTEC tests of peripheral blood. First, we exhibited the cytogenetic characteristics of CTCs and CTECs during the course of breast cancer. We found that higher CTECs quantity and positive rate were significantly correlated with LNM of breast cancer, but no significant difference was found in CTC level. Since endothelial cells make up the lining of the tumor vasculature and lymphogenous cancer metastases are mainly impacted by lymphatic vessel-related lymphangiogenesis [28], an active cross-talk between blood and lymphatic vessel endothelial cells in the TME had been thought to impact cancer cells’ trend to lymphogenous or hematogenous metastasis pathway [29]. Hence, our results provided evidence for CTECs’ relevance to the process of tumor lymphangiogenesis, which approved that an increased CTEC count gave a reminder of LNM. In addition, our results showed the level of CTECs was higher than the level of CTCs in breast cancer patients. Inversely, other studies reported that CTCs occupied the majority of circulating aneuploid cells in colorectal cancer [26] and nonsmall cell lung cancer [30]. A potential mechanism is that epithelial-mesenchymal transition (EMT) and endothelial-to-mesenchymal transition (EndoMT) are generally recognized to be related to cancer cell metastasis, which may proceed diversely in different cancers due to diverse characteristics of the respective blood and lymphatic vessel systems [29].

In fact, many studies have shown that CTECs are associated with the development of cancers. CTECs counts

Figure 8: Kaplan–Meier survival curve analysis of PFS in breast cancer patients according to CTC and CTEC counts after surgery. (a) CTC = 0 vs. CTC ≥ 1; (b) CTC < 2 vs. CTC ≥ 2; (c) CTEC = 0 vs. CTEC ≥ 1; (d) CTEC < 2 vs. CTEC ≥ 2.
were found to be increased in nonsmall cell lung cancer (NSCLC) patients compared with controls and were considered as the high-risk factor of recurrence [24]. CTECs have previously been verified to be effective biomarkers for colorectal cancer screening [26]. The previous studies of CTECs in breast cancer mostly focused on monitoring treatment efficacy [31, 32], especially antivascular endothelial growth factor-A (anti-VEGF-A) antibody bev-acizumab [33–35]. Only a few studies reported its clinical value on breast cancer screening. In this study, we examined the potential of CTC and CTEC for breast cancer screening using a small size of sample involving 20 controls and 32 newly diagnosed breast cancer patients. The results showed the capability of CTEC (AUC = 0.859) was superior to CTC (AUC = 0.795), and the combination of aneuploid CD31+ CTEC and CD31− CTC exhibited the best performance (AUC = 0.914). Especially, CTEC has 100% of specificity in controls. The origin of tumor-derived endothelial cells is formed based on two processes: cancerization of stromal cells and endothelialization of cancer cells. In addition, it is considered that endothelialization of malignant cancer cells may constitute the primary pathway for the formation of tumor-derived endothelial cells [36]. Increasing evidence suggests that cancer results from altered organ homeostasis rather than deregulated control of a single cell or a group of cells [37]. This may give theoretical support for our results on why CTEC has no false-positive in noncancer controls. Comprehensive consideration of CTC and CTEC status may be the optimal strategy for breast cancer screening or diagnosis, which needs to be validated in larger size cohorts in future studies.

In this study, we found surgery had no obvious effect on the heteroploid distribution of CTCs and CTECs. However, preoperative and postoperative CTC/CTEC count had different influences on PFS of breast cancer. Preoperative CTEC count equal to or greater than 2 cells/7.5 mL was the risk factor of shorter PFS of breast cancer, while no similar phenomena appeared on postoperative CTC or CTEC number. Previous studies have proved that the level of CTC or CTEC will rise within a short period when patients were receiving treatment such as surgery or chemotherapy [31, 38]. Ma et al. reported the aneuploid CTECs in peripheral blood of locally advanced breast cancer patients initially increased and then decreased during neoadjuvant chemotherapy [31]. Therefore, it is not suggested to detect CTC and CTEC during or shortly after treatment at a single time point to assess the recurrent risk of breast cancer. Increased preoperative CTEC is a potential risk factor for recurrence or distant metastasis, and it may provide instruction on the selection of operational ways to reduce recurrent risk, in advance.

There are a few limitations to this study. First, the findings are limited because of the small size of breast cancer patients and controls. Second, since the follow-up period is not long enough, less than half of the patients reached the follow-up endpoint. In addition, the clinical pathways of our patients were diverse, which may disturb the results. A prospective study with a larger size of the cohort and a longer follow-up period is necessary to validate the findings.

5. Conclusions

In summary, we used an improved SE-iFISH method to codetect CTC and CTEC in peripheral blood of 89 breast cancer patients and 20 controls. The CTEC level was positively correlated with CTC, and both of them were independent of traditional cancer biomarkers such as CA15-3, CA19-9, and CA12-5. CTECs are a more effective biomarker for breast cancer screening and prognosis prediction than CTCs.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors’ Contributions

Tuo Han and Juanjuan Zhang contributed equally to this study.

Acknowledgments

This work was supported by the Quanzhou City Science and Technology Program of China (2019N038S).

References

[1] R. L. Siegel, K. D. Miller, and A. Jemal, “Cancer statistics, 2020,” CA: A Cancer Journal for Clinicians, vol. 70, no. 1, pp. 7–30, 2020.
[2] F. Cardoso, A. Costa, E. Senkus et al., “3rd ESO–ESMO international consensus guidelines for advanced breast cancer (ABC 3),” Annals of Oncology, vol. 28, no. 1, pp. 16–33, 2017.
[3] J. Ferlay, I. Soerjomataram, R. Dikshit et al., “Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012,” International Journal of Cancer, vol. 136, no. 5, pp. E359–E386, 2015.
[4] S. Braun, F. D. Vogl, B. Naume et al., “A pooled analysis of bone marrow micrometastasis in breast cancer,” New England Journal of Medicine, vol. 353, no. 8, pp. 793–802, 2005.
[5] W. J. Janni, B. Rack, L. W. Terstappen et al., “Pooled analysis of the prognostic relevance of circulating tumor cells in primary breast cancer,” Clinical Cancer Research, vol. 22, no. 10, pp. 2583–2593, 2016.
[6] F. C. Bidard, D. J. Peeters, T. Fehm et al., “Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data,” The Lancet Oncology, vol. 15, no. 4, pp. 406–414, 2014.
[7] M. Bany-Paluchowski, N. Krawczyk, and T. Fehm, “Potential role of circulating tumor cell detection and monitoring in breast cancer: a review of current evidence,” Frontiers in Oncology, vol. 6, p. 255, 2016.
[8] W. Janni, A. Schneeweiss, V. Muller et al., “Update breast cancer 2019 Part 2 - implementation of novel diagnostics and therapeutics in advanced breast cancer patients in clinical practice,” Geburtshilfe und Frauenheilkunde, vol. 79, no. 3, pp. 268–280, 2019.
[9] J. B. Smerage, W. E. Barlow, G. N. Hortobagyi et al., "Circulating tumor cells and response to chemotherapy in metastatic breast cancer: SWOG S05000," *Journal of Clinical Oncology*, vol. 32, no. 31, pp. 3483–3489, 2014.

[10] S. Li, W. Yan, X. Yang et al., "Less micrometastatic risk related to circulating tumor cells after endoscopic breast cancer surgery compared to open surgery," *BMC Cancer*, vol. 19, no. 1, p. 1070, 2019.

[11] R. Ma, P. Zhu, S. Liu, B. Gao, and W. Wang, "miR-496 suppresses tumorigenesis via targeting BDNF-mediated PI3K/Akt signaling pathway in non-small cell lung cancer," *Biochemical and Biophysical Research Communications*, vol. 518, no. 2, pp. 273–277, 2019.

[12] B. Rack, C. Schindlbeck, J. Juckstocket al., "Circulating tumor cells predict survival in early average-to-high risk breast cancer patients," *Journal of the National Cancer Institute*, vol. 106, no. 5, Article ID dju066, 2014.

[13] W. Janni, F. D. Vogl, G. Wiedswang et al., "Persistence of disseminated tumor cells in the bone marrow of breast cancer patients predicts increased risk for relapse—a European pooled analysis," *Clinical Cancer Research*, vol. 17, no. 9, pp. 2967–2976, 2011.

[14] M. Banys-Paluchowski, T. Fehm, W. Janni, E. F. Solomayer, and A. Hartkopf, "Circulating and disseminated tumor cells in breast carcinoma: report from the consensus conference on tumor cell dissemination during the 39th annual meeting of the German society of senology, berlin, 27 June 2019," *Geburtshilfe und Frauenheilkunde*, vol. 79, no. 12, pp. 1320–1327, 2019.

[15] M. Martin, S. Custodio, M. L. M. Casas et al., "Circulating tumor cells following first chemotherapy cycle: an early and strong predictor of outcome in patients with metastatic breast cancer," *The Oncologist*, vol. 18, no. 8, pp. 917–923, 2013.

[16] P. P. Lin, "Integrated EpCAM-independent subtraction enrichment and iFISH strategies to detect and classify disseminated and circulating tumor cells," *Journal of Translational Medicine*, vol. 4, no. 1, p. 38, 2015.

[17] Y. Zhang, J. Li, L. Wang et al., "Clinical significance of detecting circulating tumor cells in patients with esophageal squamous cell carcinoma by EpCAMindependent enrichment and immunostaining-fluorescence in situ hybridization," *Molecular Medicine Reports*, vol. 20, no. 2, pp. 1551–1560, 2019.

[18] W. J. Allard, J. Matera, M. C. Miller et al., "Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases," *Cancer Research*, vol. 10, no. 20, pp. 6897–6904, 2000.

[19] S. Riethdorf, H. Fritsche, V. Muller et al., "Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the CellSearch system," *Clinical Cancer Research*, vol. 13, no. 3, pp. 920–928, 2007.

[20] M. Cristofanilli, G. T. Budd, M. J. Ellis et al., "Circulating tumor cells, disease progression, and survival in metastatic breast cancer," *New England Journal of Medicine*, vol. 351, no. 8, pp. 781–791, 2004.

[21] W. Wu, Z. Zhang, X. H. Gao et al., "Clinical significance of detecting circulating tumor cells in colorectal cancer using subtraction enrichment and immunostaining-fluorescence in situ hybridization (SE-iFISH)," *Oncotarget*, vol. 8, no. 13, pp. 21639–21649, 2017.

[22] P. P. Lin, O. Gires, D. D. Wang, L. Li, and H. Wang, "Comprehensive in situ co-detection of aneuploid circulating endothelial and tumor cells," *Scientific Reports*, vol. 7, no. 1, p. 9789, 2017.

[23] K. Hida and M. Klagsbrun, "A new perspective on tumor endothelial cells: unexpected chromosome and centrosome abnormalities," *Cancer Research*, vol. 65, no. 7, pp. 2507–2510, 2005.

[24] F. Najjar, M. Alammar, M. Bachour, and G. Al-Massarani, "Circulating endothelial cells as a biomarker in non-small cell lung cancer patients: correlation with clinical outcome," *International Journal of Biological Markers*, vol. 29, no. 4, pp. e337–344, 2014.

[25] T. Ueno, N. Masuda, S. Kamigaki et al., "A multicenter phase II trial of neoadjuvant letrozole plus low-dose cyclophosphamide in postmenopausal patients with estrogen receptor-positive breast cancer (JBCRG-07): therapeutic efficacy and clinical implications of circulating endothelial cells," *Cancer Medicine*, vol. 7, no. 6, pp. 2442–2451, 2018.

[26] S. Luo, Y. Ou, T. Zheng et al., "Optimal strategy for colorectal cancer patients’ diagnosis based on circulating tumor cells and circulating tumor endothelial cells by subtraction enrichment and immunostaining-fluorescence in situ hybridization combining with CEA and CA19-9," *Journal of Oncology*, vol. 2021, Article ID 1517488, 9 pages, 2021.

[27] T. L. Whiteside, "The tumor microenvironment and its role in promoting tumor growth," *Oncogene*, vol. 27, no. 45, pp. 5904–5912, 2008.

[28] P. P. Lin, "Aneuploid circulating tumor-derived endothelial cell (CTEC): a novel versatile player in tumor neo-vascularization and cancer metastasis," *Cells*, vol. 9, no. 6, p. 1539, 2020.

[29] R. Paduch, "The role of lymphangiogenesis and angiogenesis in tumor metastasis," *Cellular Oncology*, vol. 39, no. 5, pp. 397–410, 2016.

[30] H. H. Zhu, Y. T. Liu, Y. Feng et al., "Circulating tumor cells (CTCs)/circulating tumor endothelial cells (CTECs) and their subtypes in small cell lung cancer: predictors for response and prognosis," *Thorac Cancer*, vol. 12, no. 10, pp. 2749–2757, 2021.

[31] G. Ma, Y. Jiang, M. Liang et al., "Dynamic monitoring of CD45-/CD31+/DAPI+ circulating endothelial cells aneuploid for chromosome 8 during neoadjuvant chemotherapy in locally advanced breast cancer," *Therapeutic Advances in Medical Oncology*, vol. 12, Article ID 175883592091847, 2020.

[32] G. Furstenberger, R. von Moos, R. Lucas et al., "Circulating endothelial cells and angiogenic serum factors during neoadjuvant chemotherapy of primary breast cancer," *British Journal of Cancer*, vol. 94, no. 4, pp. 524–531, 2006.

[33] A. Vasseur, L. Cabel, O. Tredan et al., "Prognostic value of CEC count in HER2-negative metastatic breast cancer patients treated with bevacizumab and chemotherapy: a prospective validation study (UCBG COMET)," *Angiogenesis*, vol. 23, no. 2, pp. 193–202, 2020.

[34] A. Calleri, A. Bono, V. Bagnardi et al., "The role of lymphangiogenesis and angiogenesis in tumor metastasis," *Cancer Research*, vol. 39, no. 5, pp. 397–410, 2016.

[35] A. Vasseur, L. Cabel, O. Tredan et al., "Prognostic value of CEC count in HER2-negative metastatic breast cancer patients treated with bevacizumab and chemotherapy: a prospective validation study (UCBG COMET)," *Angiogenesis*, vol. 23, no. 2, pp. 193–202, 2020.

[36] A. Calleri, A. Bono, V. Bagnardi et al., "Predictive potential of angiogenic growth factors and circulating endothelial cells in breast cancer patients receiving metronomic chemotherapy plus bevacizumab," *Clinical Cancer Research*, vol. 15, no. 24, pp. 7652–7657, 2009.

[37] F. C. Bidard, C. Mathi0et, A. Degeorges et al., "Clinical value of circulating endothelial cells and circulating tumor cells in metastatic breast cancer patients treated first line with bevacizumab and chemotherapy," *Annals of Oncology*, vol. 21, no. 9, pp. 1765–1771, 2010.
[36] K. Lapis, S. Paku, and L. A. Liotta, “Endothelialization of embolized tumor cells during metastasis formation,” Clinical & Experimental Metastasis, vol. 6, no. 1, pp. 73–89, 1988.

[37] G. P. Dotto, “Multifocal epithelial tumors and field cancerization: stroma as a primary determinant,” Journal of Clinical Investigation, vol. 124, no. 4, pp. 1446–1453, 2014.

[38] J. J. Yu, W. Xiao, S. L. Dong et al., “Effect of surgical liver resection on circulating tumor cells in patients with hepatocellular carcinoma,” BMC Cancer, vol. 18, no. 1, p. 835, 2018.