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Dynamic monitoring of CD45-/CD31+/DAPI+ circulating endothelial cells aneuploid for chromosome 8 during neoadjuvant chemotherapy in locally advanced breast cancer

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Abstract

Background: Neoadjuvant chemotherapy (NCT) is the standard treatment for patients with locally advanced breast cancer (LABC). The aim of this study was to verify this relationship, and to estimate the clinical value of aneuploid circulating endothelial cells (CECs) in LABC patients with different NCT responses.

Methods: Breast cancer patients received an EC4-T4 NCT regimen. Peripheral blood mononuclear cells were obtained before NCT, and after the first and last NCT courses. A novel subtraction enrichment and immunostaining fluorescence *in situ* hybridization (SE-iFISH) strategy was applied for detection of circulating rare cells (CRCs). CECs (CD45–/CD31+/DAPI+) and circulating tumor cells (CTCs) with different cytogenetic abnormalities related to chromosome 8 aneuploidy were analyzed in LABC patients subjected to NCT.

Results: A total of 41 patients were enrolled. Firstly, CD31+/EpCAM+ aneuploid endothelial-epithelial fusion cells were observed in LABC patients. Further, aneuploid CECs in the peripheral blood showed a biphasic response during NCT, as they initially increased and then decreased, whereas a strong positive correlation was observed between aneuploid CECs and CTC numbers.

Conclusion: We determined that aneuploid CEC dynamics vary in patients with different response to chemotherapy. Elucidating the potential cross-talk between CTCs and aneuploid CECs may help characterize the process associated with the development of chemotherapy resistance and metastasis.

Keywords: aneuploidy, breast cancer, circulating endothelial cells, circulating tumor cells, liquid biopsy, neoadjuvant therapy

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Introduction

Breast cancer is the most common malignant tumor in females worldwide. Although adequate treatments have led to favorable outcomes in early-stage patients, metastasis remains a major challenge, especially for locally advanced breast cancer (LABC). Neoadjuvant chemotherapy (NCT), the standard treatment for LABC patients, may also cause metastasis.[1] Cancer metastasis is a multi-step process involving many factors. In a previous study, we investigated the impact of NCT on circulating tumor cells (CTCs), as direct dissemination of these cells is a key step in cancer metastasis. The present study focused on another key factor to tumor metastasis, circulating endothelial cells (CECs).

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Increased CEC numbers are observed in patients with tumors and other diseases, including, but not limited to, vasculitis, septic shock, and peripheral vascular disease.[2] In neoplastic diseases, the pathogenetic role of CECs is thought to be related to angiogenesis.[3] Karyotype analysis indicates that tumor endothelial cells contain multiple chromosomal aneuploidies, whereas normal endothelial cells are strictly diploid.[4] The presence of aneuploid CECs is considered a hallmark of cancer, albeit the specific role of these cells remains to be defined.[5] CECs are indicators of progressive disease in cancer patients.[6–8] Moreover, several preclinical studies have demonstrated that CECs may be extremely useful in identifying the optimal dosage of anti-angiogenic drugs.[9,10] However, the clinical value of CEC counts in relation to chemotherapy response remains to be established.

Both CECs and CTCs are rare in the peripheral blood. Several studies have demonstrated that subtraction enrichment and immunostaining fluorescence *in situ* hybridization (SE-iFISH) is a suitable method for the determination of CTCs and CECs.[11] By using this approach, we quantified the number of CD45–/CD31+/DAPI+ CECs during NCT. Based on a stringent selection of clinical cases, we attempted to elucidate the relationship between CEC and CTC variations during NCT. The purpose of this study was to explore the value of CEC determination in liquid biopsies of LABC patients as a marker of response to chemotherapy.

Materials and methods

Patients and sample collection

All patients enrolled in this study provided written informed consent (Supplemental file 1). All procedures were approved by the Institutional Review Boards of the First Affiliated Hospital with Nanjing Medical University (SR-171). From October 2016 to November 2017, a total of 41 patients diagnosed with LABC were enrolled at the First Affiliated Hospital with Nanjing Medical University. All patients were evaluated to meet the standard of preoperative systemic therapy and were diagnosed with breast cancer *via* core biopsy, and histological type, hormone receptors, Her-2 status, and Ki-67 index were included in the pathological report. All patients were staged as LABC and received an EC×4 –T×4 NCT regimen (epirubicin 90 mg/m^2 iv D1, cyclophosphamide 600 mg/m^2 iv D1 on a 21-day cycle for four cycles, then docetaxel 80 mg/m^2 iv D1, on a 21-day cycle for four cycles). Blood samples (6 mL) were collected prior to commencing chemotherapy (at the time of biopsy) as well as after the first and eighth chemotherapy courses. All breast cancer patients underwent surgery. Both the Miller-Payne system and the Ki-67 index value were provided from the postoperative and preoperative biopsy pathology reports. The results were used to evaluate the response to NCT. Patients with Miller-Payne grade 1–3 tumors were classified as the Low-Response group (Low-R), while patients with Miller-Payne grades 4 and 5 represented the High-Response group (High-R). Compared with the 66.67% basal Ki-67 value prior to NCT, a higher Ki-67 index after NCT was considered a Low-R and a lower Ki-67 index as a High-R.

Immunofluorescence staining and SE-iFISH

SE-iFISH (iFISH®) platforms were applied for CEC detection and characterization. The experiments were performed in strict accordance with the operations manual (Cytelligen, San Diego, CA, USA). Briefly, peripheral blood was collected into Cytelligen tubes containing ACD anticoagulant (Becton Dickinson, Franklin Lakes, NJ, USA), and centrifuged at 450 × *g* for 5 min. All deposited cells were loaded immediately onto 3 mL of non-hematopoietic cell separation matrix for density gradient centrifugation.

Supernatants above the erythrocyte layer were collected and combined with anti-leukocyte antibody (CD45) immunomagnetic beads. The cocktail was incubated at room temperature for 15 min with gentle shaking. Subsequently, the solution was magnetically separated. The bead-free solution was centrifuged at 500 × *g* for 2 min and mixed thoroughly with cell fixative. The precipitated cells were applied to coated CEC slides for subsequent iFISH analysis. Air-dried samples on coated CTC slides were hybridized with centromere probe 8 (CEP8) (Abbott Laboratories, Abott Park, IL, USA) for 3 h, followed by antibody staining by incubation with Alexa Fluor (AF) 594-anti-CD45, Cy5-anti-EpCAM, Cy7-anti-vimentin, 4′,6-diamidino-2-phenylindole (DAPI), and AF488-anti-CD31 at room temperature for 30 min.

Automated CRCs 3D scanning and image analysis

The identification of CRCs was performed using automated Metafer- iFISH® CRC 3D scanning and

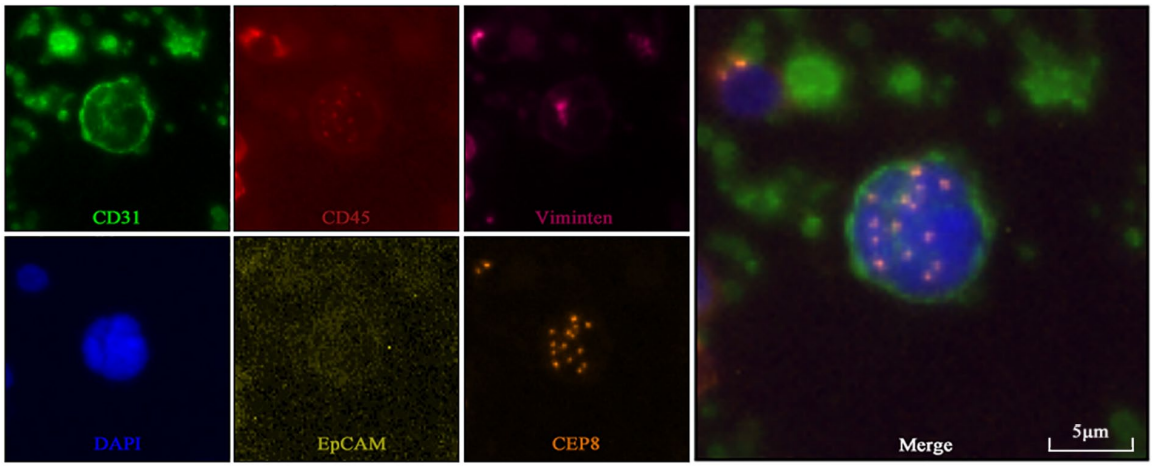

Figure 1. Detection of CECs by SE-iFISH. A CEC with Chr8 multiploidy (greater than pentaploid).
CECs, circulating endothelial cells; Chr8, chromosome 8 ; SE-iFISH, subtraction enrichment and immunostaining fluorescence *in situ* hybridization.

an analyzing system (Carl Zeiss, Oberkochen, Germany; MetaSystems, Altlussheim, Germany; and Cytelligen, San Diego, CA, USA). Briefly, CRC slides loaded onto a fluorescence microscope (AXIO Imager Z2) stage were subjected to automated full X-Y plane scanning with cross Z-sectioning of all cells, performed at a 1-mm step depth, with fluorescence signal acquisition of all color channels. Classification and statistical analysis were performed through automated image processing to comprehensively evaluate cell size, cell cluster, tumor biomarker expression, and chromosome ploidy. A cell was classified as CEC if it had the DAPI+/CD45–/CD31+ phenotype and exhibited chromosome 8 (Chr8) diploidy or polyploidy. A cell was defined as a CTC if it met one of the following criteria: (1) DAPI+/CD45−/CD31−/EpCAM+/−/vimentin+/−/aneuploid chromosome 8 (Chr8) or Chr8 polyploidy; (2) DAPI+/CD45–/CD31−/diploid chr8/at least one tumor biomarker+.

Statistical analysis

The results were expressed as the mean ± standard deviation (SD). The CEC number and subtypes were analyzed by repetitive measurement deviation analysis between High-R and Low-R patients. Multiple comparative analysis, corrected by Tukey's test, was used to analyze the differences between groups. The *Chi*-square test was used to analyze the positive rates of CECs in patients with different clinicopathological characteristics. Correlation analysis was used to verify the relationship between CECs, CTCs, and other circulating cells or tumor markers. All statistical analyses were performed by SPSS version 21.0 (SPSS, IBM; Chicago, IL, USA) and GraphPad Prism 8.0 software (San Diego, CA). All p values were two-tailed with 5% significance levels.

Results

Establishment of SE-iFISH for in situ *phenotype and karyotype identification of CECs from breast cancer patients*

SE-iFISH was developed and optimized to monitor breast cancer CECs with chr8 aneuploidy, and expressing CD31. Chr8 was detected by a specific centromeric probe (CEP8). The cells were stained with different fluorescent markers. Figure 1 shows a CEC with Chr8 multiploidy (greater than pentaploidy).

Analysis of CEC Chr8 aneuploidy in relation to patient classification

Before NCT and after the first NCT cycle, the positive CEC detection rate was 38/41 cases (92.7%) and 40/41 cases (97.6%), respectively. After eight rounds of NCT, the positive rate reached 100%. The clinicopathological characteristics of breast cancer patients and their correlation with CECs are shown in Table 1. The 41 patients were divided in groups by age, Her-2 status, lymph node status, and molecular subtype. Significant differences were observed at different time points and with distinct grouping methods (all p values < 0.05), while the differences between groups were not statistically significant.

Table 1. The number of aneuploid CECs for Chr8 in patients with different clinical characteristics.

Factors	Number	aneuploid CEC numbers				
		pre-NCT	post-first NCT	post-NCT	*p* value[1]	*p* value[2]
Total	41					
Age					0.0002	0.215
<50	20	6.40 ± 5.60	55.60 ± 56.55	28.35 ± 28.80		
⩾50	21	7.14 ± 6.17	37.48 ± 58.83	22.71 ± 25.34		
Her-2 status					0.0007	0.999
Negative	27	6.70 ± 5.47	48.59 ± 63.11	21.93 ± 22.13		
Positive	14	6.93 ± 6.70	41.93 ± 47.52	32.29 ± 34.20		
Molecular subtype					0.0274	0.471
Hormone+Her-2–/+	31	6.52 ± 6.03	42.94 ± 53.24	23.19 ± 23.43		
TNBC	8	7.38 ± 4.57	65.63 ± 78.26	21.38 ± 29.78		
Hormone-Her-2+	2	8.50 ± 10.60	21.50 ± 28.99	77.00 ± 25.46		
Lymph node					0.0003	0.842
⩽1	15	7.07 ± 5.18	52.27 ± 61.21	21.87 ± 29.07		
>1	26	6.62 ± 6.27	42.88 ± 56.58	27.54 ± 25.92		

[1]*p* value different timepoints.
[2]*p* value different groups.
CECs, circulating endothelial cells; NCT, neoadjuvant chemotherapy; TNBC, triple-negative breast cancer.

Heteroploid CECs exhibit biphasic trend

In patients undergoing NCT, CECs exhibited a biphasic trend, with an initial increase followed by a decrease (Figure 2A). The numbers of CECs (mean ± SD) were 6.78 ± 5.83 before NCT, 46.31 ± 57.73 after the first NCT course, and 25.46 ± 26.89 after NCT completion. The number of CECs increased significantly after the first NCT course, compared with the baseline level. Notably, after eight courses of chemotherapy, the number of CECs was significantly lower than after the first course.

Further, aneuploid CECs were predominant over diploid CECs in all patients, and their proportion increased during chemotherapy ($p < 0.0001$, *Chi*-square test). After the first course of NCT, both diploid and aneuploid CECs were increased ($p = 0.036$ and $p < 0.0001$, respectively), with respect to their pre-NCT levels, and aneuploid CECs were significantly increased after NCT ($p < 0.0001$). Alternatively, no significant differences were observed in diploid CECs, before and after NCT (Figure 2B).

The proportions of CECs with different karyotypes are presented in Figure 2C. The CECs with Chr8 triploidy were 10%, 20%, and 15% to all CECs at the three consecutive time points, respectively, while CECs with Chr8 tetraploidy were 9%, 18%, and 15%, respectively. The triploid and tetraploid fractions were found to increase after the first course of NCT. Notably, the increased proportion of CTC with triploidy and tetraploidy Chr8 was observed with CTCs (data not shown).

Vimentin+ aneuploid CECs and aneuploid endothelial-epithelial fusion cells

SE-iFISH analysis in CECs showed significant intracellular staining of EpCAM and of the

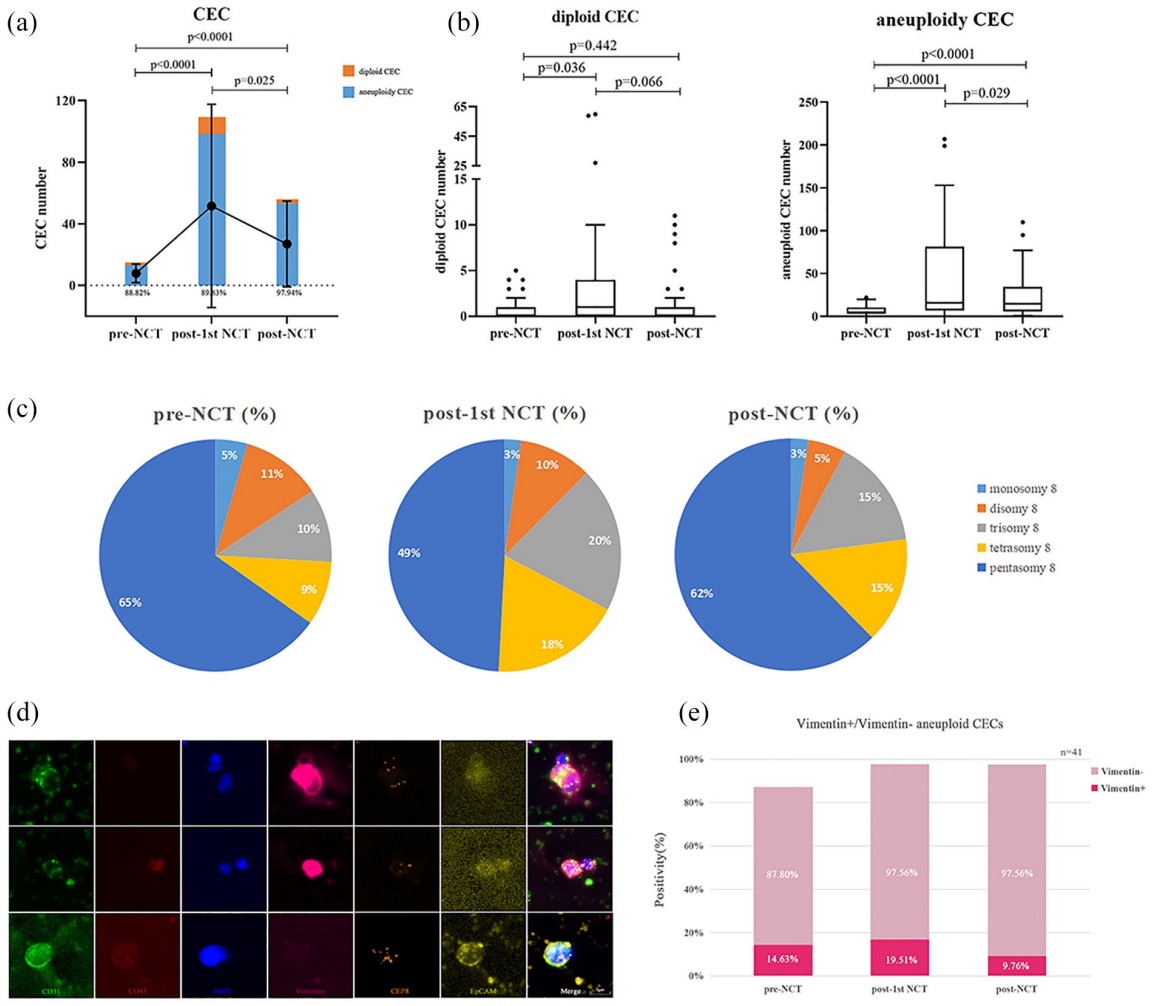

Figure 2. The trends of diploid and aneuploid CECs. (A) Total CEC number tended to increase and then decreased significantly; CEC number was higher after than before NCT. The proportion of aneuploid CECs was on the rise. (B) The number of diploid CECs increased significantly after the first course of NCT, while aneuploid CECs increased significantly after the first and the eighth NCT course. (C) Proportion of CECs with different karyotypes. (D) Aneuploid chromosome and expression of multiple biomarkers in CECs. The picture was obtained by merging *in situ* CD31, CD45, DAPI, EpCAM, and vimentin immunostaining with karyotypic iFISH. (E) The positive rate of aneuploid CECs (Vim+ and Vim–) at the three time points was 87.80%, 97.56%, and 97.56%, respectively. The positive rate of vimentin+ CECs was 14.63%, 19.51%, and 9.76%, respectively.
CECs, circulating endothelial cells; NCT, neoadjuvant chemotherapy; iFISH, immunostaining fluorescence *in situ* hybridization.

mesenchymal marker, vimentin (Vim) (Figure 2D). EpCAM−Vim+ and EpCAM+Vim− CECs are shown in the merged picture.

We found that the endothelial marker, CD31, and Vim were co-localized in the CTCs of LABC patients. Statistical analyses were also performed on the different phenotypes of CD31+/Vim− *versus* CD31+/Vim+ aneuploid CECs at the three time points. The positive incidence of CD31+/Vim+ were 14.63%, 19.51% and 9.76%, respectively (Figure 2E). In addition, the existence of endothelial-epithelial aneuploid tumor cells was observed in breast cancer patients. CD31+/EpCam+ aneuploid CECs were detected in four samples: one sample collected before NCT and three samples collected after the first course of NCT.

Relationship between aneuploid CECs and circulating cancer (and non-cancer) cells during NCT

The number of different kinds of cells changed significantly during NCT. We also quantified the number of CTCs in all samples. A strong positive correlation was observed between aneuploid

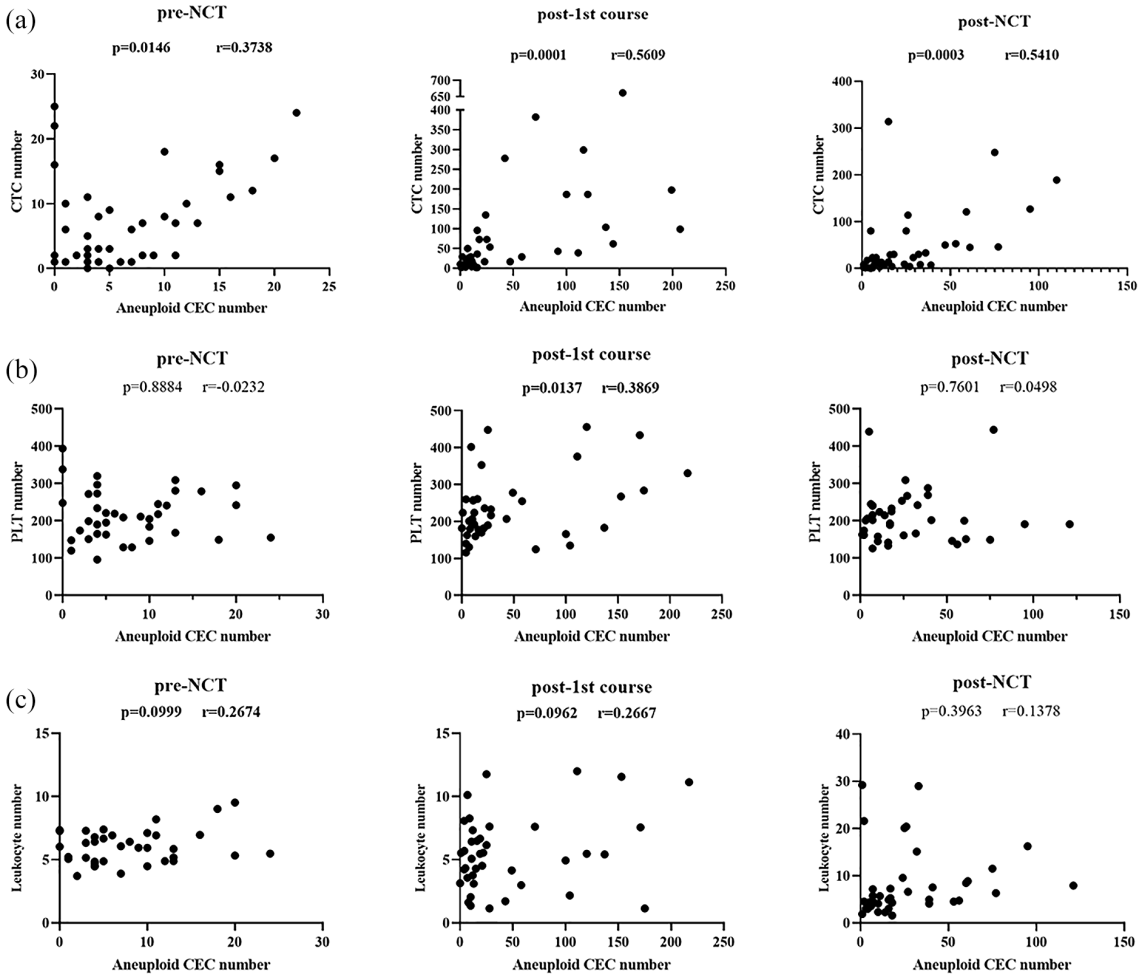

Figure 3. Correlation between aneuploid CECs, CTCs, and non-cancer cells. Correlation between aneuploid CEC and CTC (A), PLT (B), and leukocyte (C) numbers at three time points.
CECs, circulating endothelial cells; CTCs, circulating tumor cells; PLT, platelet.

CECs and CTCs at all time points ($p = 0.015$, $p < 0.001$, and $p < 0.001$, respectively).

The relationship between aneuploid CECs and non-cancer cells [platelet (PLT) and leukocyte] is shown in Figure 3. A positive correlation was observed between CECs and PLTs after the first course of treatment ($p = 0.014$, $r = 0.387$). However, the correlation between leukocytes and aneuploid CECs was not statistically significant ($p = 0.096$, $r = 0.277$).

Correlation of CECs with plasma VEGF and VEGFR2

Vascular endothelial growth factor (VEGF) and VEGF receptor 2 (VEGFR2) were the most important indicators related to tumor angiogenesis. We also examined the relationship between aneuploid CECs and relevant indicators of angiogenesis, VEGF and VEGFR2 levels. The number of aneuploid CECs, although negatively correlated with the concentration of VEGF after the first course of NCT, did not show any significant correlation with the concentration of VEGFR2 (Figure 4). Moreover, no correlation was observed between aneuploid CECs and the tumor markers CEA, CA12-5, and CA15-3 (Supplemental file 2).

Comparison of aneuploid CEC numbers in different patient groups: correlation with the response to NCT

Patients with different Miller-Payne grades. Based on pathological reports after surgery, patients were divided into two groups according to the

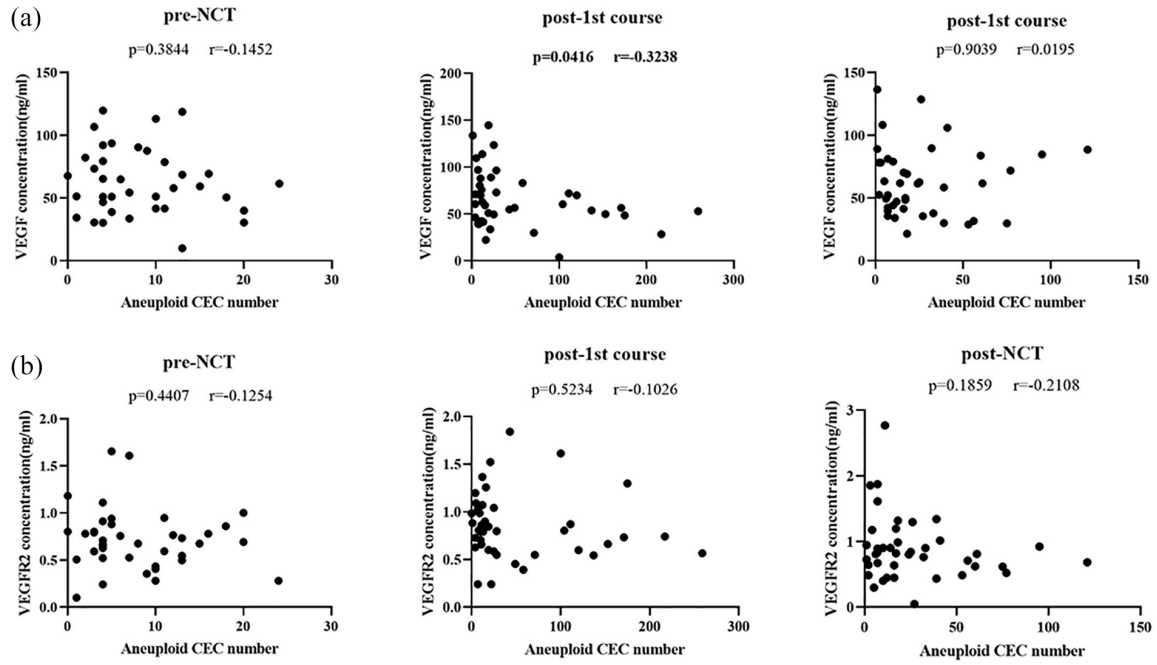

Figure 4. Correlation analysis between aneuploid CEC numbers and VEGF/VEGFR2 concentration. (A) Correlation between aneuploid CEC number and VEGF concentration at three different times. (B) Correlation between aneuploid CEC number and VEGFR2 concentration at three different time points.
CEC, circulating endothelial cell; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2.

Miller-Payne system. Six patients exhibited >90% tumor cell loss and were classified as High-R (Miller-Payne grades 4 and 5), while the other 35 were defined as Low-R (Miller-Payne grades 1–3) patients.

A *Chi*-square test showed no significant differences in the clinical characteristics of patients (Table 2). No significant differences were observed in the number of aneuploid CECs between Miller-Payne grades 1–3 and Miller-Payne grades 4 and 5 patients at any time point. Aneuploid CECs remained stable in the six patients with Miller-Payne 4 and 5 grade, yet increased continuously during NCT in Miller-Payne grade 1–3 patients. Moreover, in the Low-R group, aneuploid CECs increased significantly after the first round of NCT compared with before chemotherapy ($p = 0.001$), and further increased after the eighth NCT course ($p = 0.001$). In Low-R patients, no differences were observed between the measurements performed after the first NCT course and after NCT completion ($p = 0.235$), while the High-R group showed no differences at any time point. In the diploid CECs, no differences were observed within each group at any time point (Figure 5A and B). Diploid CECs showed no difference at any time point in either patient group (Figure 5E and F).

CEC dynamics in patients with different Ki-67 index variations during NCT. Patients were also compared according to the tumor Ki-67 index, before and after NCT. Of the 41 patients, 20 (48.8%) showed a decline of up to 33.33% in the Ki-67 index (Low-R group), while in 21 patients (51.2%) this index declined by more than 33.33%, compared with the biopsy sample after surgery (High-R group). The response to chemotherapy between groups according to clinical characteristics was not statistically significant (Table 3). In the High-R group, aneuploid CECs increased after the first course and decreased after the eighth course of therapy. In contrast, in the Low-R group, aneuploid CECs increased after the first course, after which point they remained stable until the end of treatment (Figure 5C and D). Diploid CECs showed no difference at any time point in either patient groups (Figure 5G and H).

Changes in Chr8 triploid and tetraploid CECs in patients with different NCT response

CECs triploid and tetraploid for Chr8 were analyzed separately (Figure 6), and were found to

Table 2. The number of aneuploid CECs in patients with different clinical characteristics (Miller-Payne system).

Factors	Total	High-R	Low-R	*p* value
Total	41	6	35	
Age				0.948
<50	20	3	17	
⩾50	21	3	18	
Her-2 status				0.375
Negative	27	3	24	
Positive	14	3	11	
Molecular subtype				0.575
Hormone+Her-2−/+	31	4	27	
TNBC	8	2	6	
Hormone-Her-2+	2	0	2	
Lymph node				0.413
⩽1	15	4	17	
>1	26	2	18	

CECs, circulating endothelial cells; High-R, high response; low-R, low response; TNBC, triple-negative breast cancer.

exhibit a similar trend to that of general aneuploid CECs according to both grouping strategies. Generally, at the three considered time points, no significant difference were observed between the two different response groups. However, at the completion of NCT, triploid and tetraploid CECs tended to be more abundant in Miller-Payne grade 1–3 compared with grade 4–5 patients ($p = 0.087$). Further, Miller-Payne grade 1–3 patients showed a significant increase in triploid and tetraploid CECs after the first and eighth NCT, compared with pre-NCT values ($p = 0.003$ and $p < 0.001$, respectively). However, no significant changes were observed in Miller-Payne grade 4–5 patients.

With respect to the Ki-67 index, triploid and tetraploid Chr8 CECs exhibited variations similar to those observed in aneuploid CECs. In particular, a biphasic profile, with an initial increase followed by a decrease, was observed in the High-R group but not in the Low-R group.

Discussion

In patients with neoplastic disease, CTCs and CECs constitute the primary non-hematologic CRCs. In a previous study, we demonstrated a correlation between the number of CTCs and the response to NCT in LABC patients. In the present study, we addressed the impact of NCT on the dynamics of another major subpopulation of circulating cells, CECs.

In neoplastic diseases, CECs originate from destabilized vessels at tumor sites and from chemotherapy-induced vessel injury.[12] However, technical issues have thus far hindered the study of CECs. Specifically, CECs of different subtypes express distinct biological markers. As such, the lack of consensus on CEC phenotypes has led to a discrepancy in CEC counting of more than 1000-fold. CD31 is one of the molecules shared by all CEC subtypes.[2] However, conventional testing based on immunophenotypic criteria ($CD45^{-}CD31^{high}$) can result in false-positive signals due to the presence of large platelets.[13] Alternatively, SE-iFISH is a novel system coordinating tri-elements of cell morphology, tumor protein markers, and nucleic acids for detection of CRCs. DAPI and CEP8 were used to confirm the shape of the nucleus and the karyotype of the target cells. Absence of a nucleus is the most

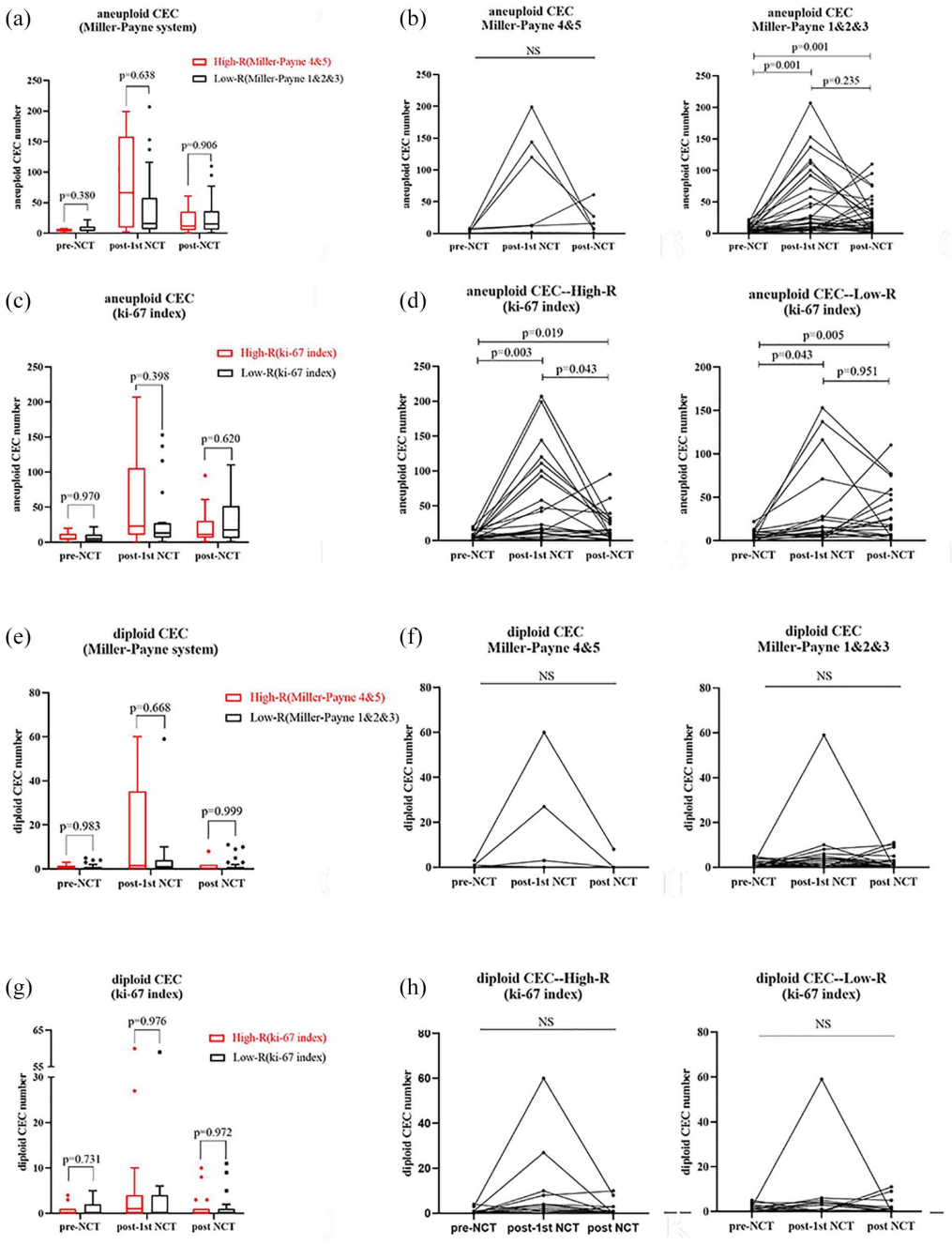

Figure 5. Aneuploid CEC numbers analyzed by patients with different NCT responses. (A) Comparison of aneuploid CECs between two response groups according to the Miller-Payne classification. No significant differences were observed between High-R and Low-R patients at three time points. (B) Comparison of aneuploid CECs in different response groups during NCT. The number of aneuploid CECs in the Low-R group (Miller-Payne grades 1, 2, and 3) after the first NCT course and after NCT completion compared with the pre-NCT period. The number of aneuploid CECs in the High-R group (Miller-Payne grades 4 and 5) did not show any significant difference. (C) Comparison of aneuploid CECs between the two response groups, as defined by the Ki-67 index. No significant differences were observed between High-R and Low-R patients at the three time points. (D) Comparison of aneuploid CECs between the response groups over the course of NCT. The number of aneuploid CECs increased significantly in both groups after the first NCT course and after NCT completion compared with the pre-NCT period. However, in the High-R group, but not in Low-R group, aneuploid CECs was significantly lower after NCT completion than after the first NCT course. (E and F) Comparison of diploid CEC numbers in the two response groups based on the Miller-Payne classification. No significant differences were observed between High-R and Low-R patients at three time points. (G and H) No significant differences were observed between High-R and Low-R patients defined on the basis of the Ki-67 index at any of the time points.
CEC, circulating endothelial cell; High-R, high response; Low-R, low response; NCT, neoadjuvant chemotherapy.

Table 3. The number of aneuploid CECs in patients with different clinical characteristics (ki-67 index).

Factors	Total	High-R	Low-R	*p* value
Total	41	21	20	
Age				0.272
<50	20	12	8	
⩾50	21	9	12	
Her-2 status				0.585
Negative	27	13	14	
Positive	14	8	6	
Molecular subtype				0.682
Hormone+Her-2–/+	31	17	14	
TNBC	8	3	5	
Hormone-Her-2+	2	1	1	
Lymph node				0.031
⩽1	15	11	4	
>1	26	10	16	

CEC, circulating endothelial cells; High-R, high response; low-R, low response; TNBC, triple-negative breast cancer.

important character of platelets. The application of this method avoids confounding factors, such as platelets, and improves the specificity of CEC detection.

In our study, CECs exhibited hallmarks of chromosomal instability. Individual CECs had different cytogenetic profiles, indicating that aneuploid CECs were heterogeneous and not clonal. Tumor endothelial cells (TECs) are important components of tumor blood vessels and TEC abnormalities are related to cancer progression.[14] It has been shown that aneuploidy is associated with highly metastatic TECs.[15] The chromosomal abnormalities in CECs strongly suggest their origin from TECs. The present study focused on dynamic changes in the number of aneuploid CECs during NCT. Previous studies have reported contradictory conclusions. One study found that mature CECs were significantly elevated in breast cancer patients and decreased during chemotherapy.[16] Other investigators reported that CEC counts increased after chemotherapy in responding patients, and attributed this phenomenon to the release of apoptotic CECs from tumor vessels.[17] Furthermore, another study reported an increase in the number of CEC following treatment with paclitaxel, attributing it to chemotherapy.[18,19] Hence, the existence of a relationship between CECs and chemotherapy response has been questioned.[20]

In this study, a highly homogeneous patient cohort was used to monitor changes in the number of diploid and aneuploid CECs in LABC patients. Our results can be summarized as follows.

First, total CECs increased after one cycle of chemotherapy in nearly all patients, and then decreased. Diploid and aneuploid CECs exhibited the same trend.

Second, our study is the first to demonstrate the expression of Vim and EpCAM in aneuploid CECs. Vim is a cytoskeletal component crucial for cell morphology. Some aneuploid CECs exhibited a high level of Vim expression. Intravasation and extravasation of cancer cells both require the disruption of endothelial junctions for the cancer

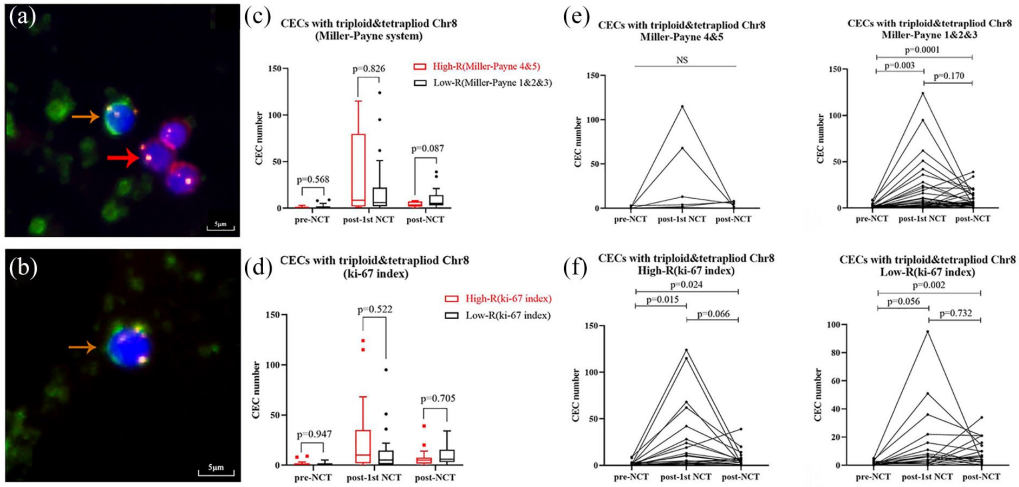

Figure 6. Changes in tetraploid and triploid Chr8 CEC numbers in patients with different response to NCT. (A and B) Typical fluorescence images of tetraploid and triploid Chr8 CECs. (WBC: red arrow). (C and E) Comparison of triploid and tetraploid Chr8 CECs between the two response groups. No significant differences were observed at three time points. No significant differences were observed in High-R patients (Miller-Payne grades 4 and 5). In the Low-R group (Miller-Payne grades 1–3) the number of triploid and tetraploid Chr8 CECs was significantly higher after the 1st-course of NCT, as well as after NCT completion, compared with the pre-NCT period. (D and F) Comparison of triploid and tetraploid Chr8 CECs between the two response groups based on the Ki-67 grouping scheme. No significant differences were observed between High-R and Low-R patients at any time point. In both groups, the number of triploid and tetraploid Chr8 CECs was significantly higher after the first NCT course, as well as after NCT completion, compared with pre-NCT patients. In High-R, but not in Low-R patients, triploid and tetraploid Chr8 CECs were found to be significantly decreased after NCT completion.
CEC, circulating endothelial cell; Chr8, chromosome 8; High-R, high response; Low-R, low response; NCT, neoadjuvant chemotherapy; WBC, white blood cell.

cells to cross the endothelium — a process known as transendothelial migration. The change of cell morphology is one of the essential requirements in the transendothelial migration of primary tumor cells.[21] Notably, a strong expression of Vim in endothelial cells may favor transendothelial migration.[22] Vim+ aneuploid CECs significantly increased after NCT. High expression of Vim in endothelial cells may increase the probability of transendothelial migration of primary tumor cells and of their conversion to CTCs. Another rare cell population, aneuploid CD31+/EpCam+ CECs, was found in breast cancer patients undergoing chemotherapy. This cell type was defined as an 'aneuploid endothelial-epithelial fusion cluster'.[5] To date, the biological significance of this cell population is unknown. However, the interaction between tumor and stromal cells may induce abnormalities in the latter cells, such as those characterizing cancer-associated fibroblasts. The heterogeneity of CECs may suggest that TECs originate from the transdifferentiation of cancer stem cells (CSCs) or from fusion events between tumor and normal endothelial cells.[23] Chemotherapy may promote such transformation.

In addition, an interesting and strong positive correlation was found during NCT, between aneuploid CECs and CTCs. Both CTCs and CECs derive from the primary tumor. The correlation suggested that cell heterogeneity, which is known to characterize the primary tumor, is also present among tumor-derived CRCs. The view that chemotherapy can induce CSC characteristics and epithelial-to-mesenchymal transition, in addition to promoting metastasis, is increasingly accepted among investigators.[24] Tumor angiogenesis is a key step in metastasis, and aneuploid CECs are strongly implicated in this process. The elevation of Vim+ aneuploid CECs after chemotherapy may suggest the interaction between primary tumor and CTCs. Positive correlations between aneuploid CECs and blood cells (leukocytes and platelets) were also found after the first course of NCT. During the metastatic process, cancer cells encounter many other circulating cells, including other cancer cells, that can modulate the

way and efficiency of their extravasation. Several studies have shown that circulating platelets and leukocytes contribute to the binding of cancer cells to the endothelium and to their extravasation across the endothelial barrier.[21,25,26] The impact of chemotherapy on these events is still largely obscure, and elucidating the potential cross-talk between circulating non-cancer and cancer cells (CTCs and aneuploid CECs) may help dissect tumor angiogenesis, progression, and metastasis.

In addition to the overall analysis, the number of diploid and aneuploid CECs was compared in patients with different NCT responses. As in the previous study, two different grouping schemes were adopted, that is, the Miller-Payne system and the Ki-67 index, before and after NCT.[27] The Miller-Payne system is an accepted standard for the assessment of NCT efficacy. The Ki-67 index is a classic indicator of tumor cell proliferation. Both grouping strategies reflected differences in NCT responses, highlighting similar variations in CEC numbers. There were no significant differences observed in diploid CECs between the different response groups at any time point and by any grouping strategy. Alternatively, in the grouping scheme based on the Ki-67 index, aneuploid CECs initially increased in both High-R and Low-R patients, but displayed strikingly different profiles in the two groups after NCT. Specifically, in the High-R group, the number of aneuploid CECs was significantly lower following NCT completion than after the first round of therapy. However, this change was not observed in the Low-R group. When grouping was based on the Miller-Payne system, aneuploid CECs significantly increased after NCT in patients with tumor grades 1, 2, or 3, yet remained stable in patients with tumor grades 4 and 5. However, it should be considered that, in this grouping scheme, the sample size was unbalanced between groups (6 *versus* 35).

Based on the available results, we reasoned that the increase in diploid CECs may have been related primarily to chemotherapy-induced vascular damage, and had no relevance to chemotherapy response. Similar results have been previously reported.[20] In addition, we hypothesized that chemotherapy-induced apoptosis of aneuploid CECs could substantially contribute to their increase after the first course of NCT. The negative correlation between plasma VEGF and aneuploid CECs likely reflected anti-angiogenic effects of chemotherapy. The decreased expression of the VEGF–VEGF receptor signaling pathway loosens the tight junctions that interconnect endothelial cells.[28] In the absence of VEGF, TECs shed from tumor blood vessels and gave rise to CECs. At later stages of NCT, apoptotic aneuploid CECs were eliminated, which would explain the decrease observed in the final measurement. However, the 3-week intervals between successive cycles of therapy reduce the anti-angiogenic effects of conventional chemotherapy,[10] and some of the patients may have become resistant to chemotherapy, while the correlation between VEGF and aneuploid CECs disappeared.

Alternatively, in Low-R patients, the increase in CEC number after NCT cannot be attributed completely to apoptotic cells. In patients resistant to chemotherapy, the primary tumors exhibited drug resistance. The corollary of this phenomenon is that CECs possess proliferative capacity. The CEC elevation observed after the first course of NCT in this group of patients may be unrelated to apoptosis, and active CECs may be predominant. Although direct evidence was not provided, the biphasic trend in CEC number (initial increase followed by decrease) was evident. Our results may partly explain the above-mentioned conflicting results.

By utilizing the SE-iFISH platform, we analyzed chr8 karyotype in CECs. Aneuploidy of chr8 is a common biological phenomenon in several neoplastic diseases.[29–32] And the CEP8 used in the SE-iFISH® platform has been validated for detection of various rare tumor cells including circulating tumor cells.[33–35] A number of recent studies showed that triploid and tetraploid Chr8 CTCs exhibit intrinsic drug resistance in gastric cancer, nasopharyngeal carcinoma, and rectal cancer.[33,36] To date, no studies have addressed the clinical significance of CECs with triploid and tetraploid Chr8. When the latter cells were analyzed separately, they showed changes similar to those of total aneuploid CECs in the different response groups. The role of CECs with triploid and tetraploid Chr8 in NCT resistance remains to be elucidated.

In previous studies, metronomic chemotherapy (MCT) with the cyclophosphamide analog ifosfamide decreased the CEC levels of cancer patients,[37] suggesting that metronomic treatment of anticancer drugs inhibits tumor angiogenesis by decreasing CECs. Studies demonstrated that

the MCT regimen functionally impaired circulating endothelial cells.[38] The present study monitored aneuploid CECs changes with conventional chemotherapy. In the future, randomized controlled trials could be designed to compare the chemotherapy response and the number of CECs during NCT between different drug administrations.

In summary, in patients undergoing NCT, the number of aneuploid CECs in the peripheral blood exhibited a biphasic trend, characterized by an initial increase followed by a decrease. The number of aneuploid CECs was closely related to that of CTCs during NCT. The results of this study indicate that continuous release of tumor-derived cells into the circulation could be presented as the NCT resistance of primary tumor, supporting liquid biopsy examination as an effective method to monitor NCT response. Overall, our data demonstrated that, in addition to CTCs, further attention must be paid to other circulating tumor-related cell populations when evaluating patient response to chemotherapy.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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Supplemental material

Supplemental material for this article is available online.

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