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Aneuploid Circulating Endothelial Cells with Prognostic Value in Locally Advanced Breast Cancer Patients After Neoadjuvant Chemotherapy

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Background: Aneuploid circulating endothelial cells (CECs) are an indicator in breast cancer (BC). Significant changes of aneuploid CECs occurred during neoadjuvant chemotherapy (NCT). This study aimed to explore the predictive and prognostic values of aneuploid CECs in locally advanced breast cancer (LABC) patients with different NCT responses.

Methods: Breast cancer patients received an EC4-T4 NCT regimen. A novel subtraction enrichment and immunostaining fluorescence in situ hybridization (SE-iFISH) strategy was applied for the detection of CECs (CD45–/CD31+/DAPI+). Receiver operating characteristic (ROC) curves were plotted to evaluate the predictive value of aneuploid CEC counts in distinguishing NCT-resistant patients from sensitive ones. All patients were observed for progression-free survival (PFS) and overall survival (OS).

Results: The CEC counts at any time point did not show the ability to predict the efficacy of NCT. The difference in the CECs between post-chemotherapy levels and baseline could be sufficient to distinguish chemotherapy-resistant cases from other cases in Hormone+Her-2-/+ (HR+) BC patients. Patients with reduction of CECs after all courses of NCT were associated with higher probability of PFS.

Conclusion: Variations in aneuploid CECs during NCT may predict chemotherapy response in patients with HR+ breast cancer. The decrease in the number of aneuploid CECs after all courses of NCT indicates better treatment outcomes in patients with LABC.

Keywords: circulating endothelial cells, aneuploidy, neoadjuvant chemotherapy, prognosis, locally advanced breast cancer

Introduction

With the rapid development of the liquid biopsy technology in medical oncology, the field of its application is extended. As a classic indicator, CEC has been shown to be increased in neoplastic diseases.[1] And then several researches demonstrated the treatment of tumor could affect CEC numbers.[2] Our previous study took an innovative approach to detect aneuploid CECs, which were directly related to primary tumor, in patients with LABC receiving standard NCT.[3] Significant changes of aneuploid CECs occurred during NCT. However, there is a lack of definite conclusions for the predictive and prognostic values of aneuploid CECs in the neoadjuvant setting currently.

Previous study merely described the dynamic change of aneuploid CECs in NCT and analyzed the different trends according to the treatment response criteria of the primary lesion; however, the predictive value of aneuploid CEC to NCT response has not been evaluated.[3] Furthermore, although surrogate endpoints, such as pathologic complete response (pCR) of the primary lesion, were used in NCT, this may not be the exact evidence of long-term efficacy. Therefore, in the present study, our follow-up outcomes were used to evaluate the prognosis of patients with different aneuploid CEC numbers. To our knowledge, there were no direct clinical evidence

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yet that the aneuploid CECs were related to tumor metastasis or recurrence in LABC patients. In the present study, we followed up with the enrolled patients and clarified the predictive and prognostic value of aneuploid CECs in NCT.

Methods

Patients and Sample Collection

This study included patients with LABC treated at the First Affiliated Hospital of Nanjing Medical University between October 2016 and November 2017. This study was approved by the institutional review board of the First Affiliated Hospital of Nanjing Medical University (SR-171).

Patients were treated according to the National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology. The chemotherapy regimens of the enrolled patients were identical (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). Before the start of, after the first and eighth NCT cycles, 6mL blood samples were collected. All the patients with BC underwent surgery. Estrogen receptor (ER), progesterone receptor (PgR) status, Ki-67 value, and human epidermal growth factor receptor 2 (HER2) status were determined locally by routine pathologic evaluation according to standard guidelines.

Quantification of CECs and Group Determination

SE-iFISH (iFISH®) platforms were used for CEC detection and characterization. Experiments were performed in strict accordance with the manufacturer's instructions (Cytelligen, San Diego, CA, USA). The operating manual is based on previous studies.[3,4]

Aneuploid CECs were counted at three time points: pre-NCT, post-1st NCT and post-NCT. The dynamic change of aneuploid CEC numbers was indicated by the comparison between any two means. Δvalue1, Δvalue2 and Δvalue3 were defined as the difference between CEC counts of three measurements: Δvalue1=post-1st NCT – pre-NCT; Δvalue2= post-NCT – pre-NCT; Δvalue3= post-NCT – post-1st NCT.

The Ki-67 index values from the postoperative and preoperative biopsy pathology reports were compared and used to evaluate the primary lesions response, as previously described.[3,5] In the prognostic analysis, on the basis of the CEC numbers, patients were classified into two categories, that is, CEC-greater than median and CEC- less than median at three time points, respectively. In dynamic analysis, 41 patients were divided into two groups, CEC reduction group and CEC stabilization or elevation group. Here the cut-off was the medians of Δvalue1, Δvalue2 and Δvalue3.

Statistical Analysis

To evaluate the predictive value of aneuploid CEC counts in distinguishing NCT-resistant patients from sensitive ones, we plotted ROC curves and calculated the area under the curves (AUCs) at three time points. All patients were observed for PFS and OS. Survival analysis was performed using Log rank test with GraphPad Prism version 8.3.0. ROC curves and calculated AUCs were performed with SPSS version 21.0.

Results

Patient Characteristics

A total of 41 patients were enrolled into the study and received identical NCT treatment. The mean age was 51.7 (range: 26–59 years). Most patients (75.6%) were HR+ (Table 1). The positive CEC detection rates were 92.7%, 97.6%, and 100% at the three time points, respectively. The number of CECs (mean ± SD) was 6.780 ± 5.833 before the NCT, 46.32 ± 57.73 after the first NCT course, and 25.46 ± 26.89 after NCT completion. According to the therapeutic response of the primary breast lesions evaluated after surgery, twenty patients belonged to the chemotherapy resistance group, while the other twenty-one patients were in chemosensitive group. Of the 41 patients, 14 had local recurrence or distant metastasis, and a total of 5 patients died by the end of follow-up. In the cases of recurrence and death, the numbers of euploid CEC (mean ± SD) were 7.71±4.93 before the NCT, 31.36 ±41.36 after the first NCT course, 34.86±34.48 after NCT completion, respectively, and in the cases without recurrence, the numbers of the three tests were 6.30±6.09 before the NCT, 54.07±62.25 after the first NCT course, 20.59±19.64 after NCT completion, respectively.

Table 1 Baseline Demographics and Clinical Characteristics

Characteristic	Patients (N=41)
Age, mean (SD), y	51.7 (5.9)
Range, y	26–69
Age group, n (%), y	
<50	21 (51.2)
≥50	20 (48.8)
Histologic type, n (%)	
Ductal carcinoma	41 (100)
Molecular status	
Hormone+Her-2-/+	31 (75.6)
TNBC	8 (19.5)
Hormone-Her-2+	2 (4.9)
Lymph node	
≤1	15 (36.6)
>1	26 (63.4)
Primary tumor response to NCT	
High-response	21
Low-response	20

Different Changing Trends of the Aneuploid CECs Differentiates Low-Response and High-Response Primary Tumor Lesions

ROC analyses predicted the ability of the CECs to differentiate chemotherapy-resistant cases from chemosensitive cases with AUCs. The Figure 1A showed the CEC counts at any time point did not show the ability to predict the efficacy of NCT. Changes in the quantity of CECs among three measurements (Δvalues) were also considered to be inefficient to distinguish the chemotherapy-resistant patients (Figure 1B).

Considering the heterogeneity of different molecular types of breast cancer, thirty-one HR+ breast cancer patients were analyzed separately as a subgroup. With an equal sensitivity and specificity as the models (79% and 57%, respectively), the CEC numbers after all courses of NCT might provide helpful insights in identifying chemotherapy-resistant patients (Figure 2A).

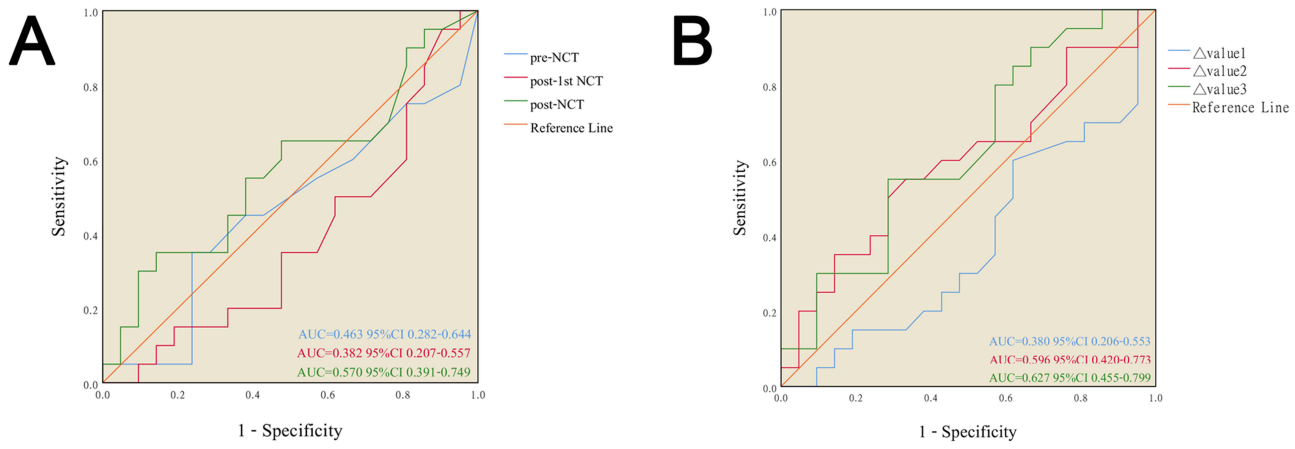

Figure 1 Receiver operating characteristic (ROC) curves of CEC numbers for discriminating low response from high-response in all subtypes of BC. (**A**) Number of aneuploid CECs at different timepoints of NCT in patients with all subtypes of BC. (**B**) Δvalue1 (difference between the second and the first timepoints), Δvalue2 (difference between the third and the first timepoints), Δvalue3 (difference between the third and the second timepoints) of aneuploid CECs in patients with all subtypes of BC.
Abbreviation: AUC, area under the curve.

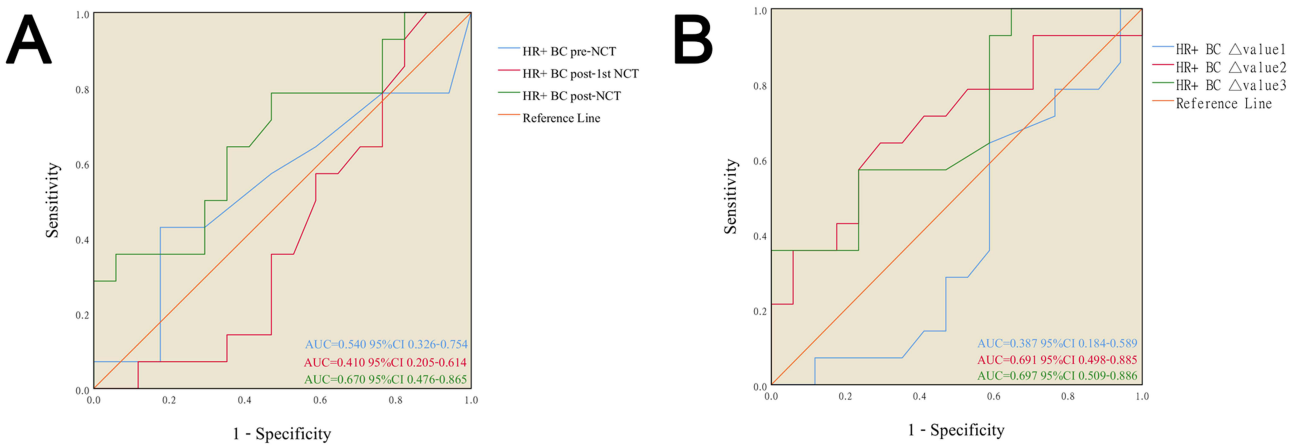

Figure 2 Receiver operating characteristic (ROC) curves of CEC numbers for discriminating low response from high-response in HR+ BC. (**A**) Number of aneuploid CECs at three timepoint of NCT in patients with HR+ BC. (**B**) Δvalue1 (difference between the second and the first timepoints), Δvalue2 (difference between the third and the first timepoints), Δvalue3 (difference between the third and the second timepoints) of aneuploid CECs in patients with HR+ BC.
Abbreviation: AUC, area under the curve.

In addition, Δvalue2 and Δvalue3 had large AUCs in HR+ BC patients (Figure 2B, 0.691 for Δvalue2, 95% confidence interval (CI) 0.498–0.885 and 0.697 for Δvalue3, 95% CI 0.509–0.886). Therefore, we recon the difference of the CECs between post-chemotherapy level and baseline might be sufficient for distinguishing chemotherapy-resistant cases from other cases in HR+ BC patients.

Reduction of Aneuploid CECs After NCT Indicates a Favorite Survival

Fourteen (34.15%) patients experienced locoregional/distant recurrence of the primary breast cancer. Five patients (12.19%) died from any cause. Cox proportional hazards regression analysis did not reveal an association between CEC status and time-to-event at any of the three time points (Figure 3A). In the model considering of the CEC counts changing trends in NCT, patients with reduction of CECs after all courses of NCT were associated with higher

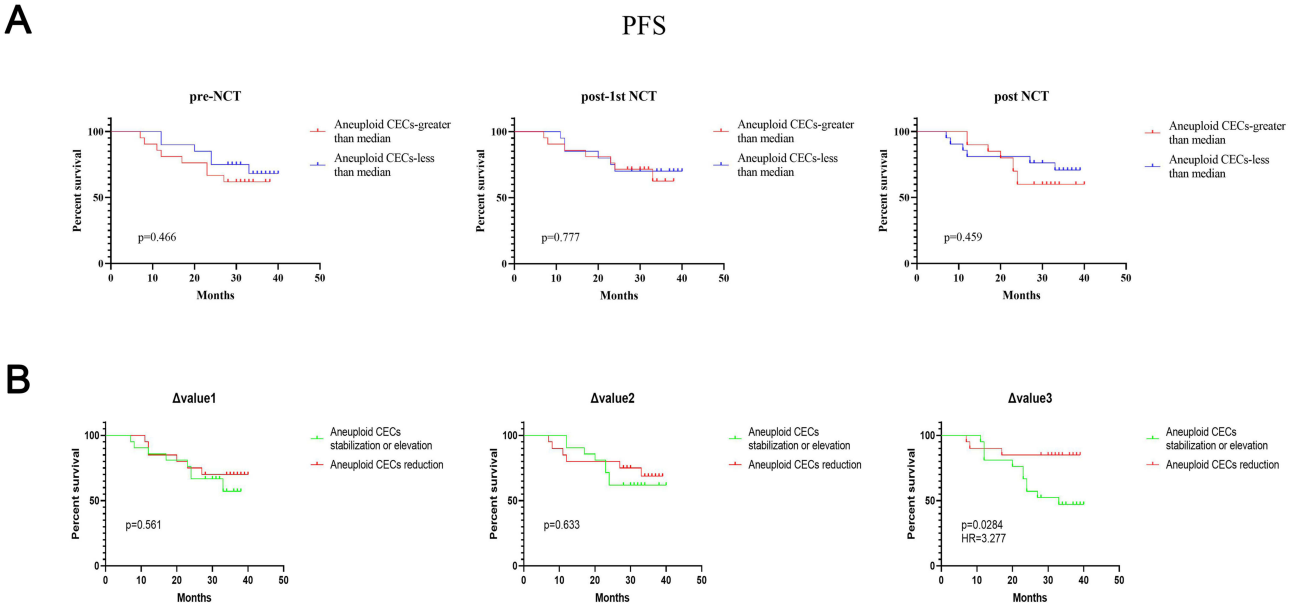

Figure 3 Kaplan-Meier plots of PFS according to aneuploid CEC numbers at three time points (**A**) and the CEC counts changing trends in NCT (**B**). (**A**) Progression-free survival analysis for LABC patients receiving NCT. Median PFS for patients with CEC counts greater or less than median at both blood draws were not defined as the survival curve does not fall to 50% (n.d.). (**B**) Progression-free survival analysis for LABC patients receiving NCT according to Δvalue1, Δvalue2 and Δvalue3. For Δvalue3, median PFS was n.d. for patients with Aneuploid CECs reduction and was 33 months for patients with Aneuploid CECs stabilization or elevation. (p=0.0284).

probability of PFS ($p < 0.05$, Figure 3B). Overall survive was not estimable because only a small number of patients (5 patients [12.19%]) experienced an event by the end of the study.

Discussion

In our series of studies focusing on liquid biopsy in LABC patients receiving NCT, aneuploid CECs were one of the most innovative indicators.[3] It is now widely accepted that circulating tumor cells (CTC) can be used as an evaluation index for tumor prognosis, although methodological flaws still exist in CTC detection. CEC, another rare circulating cell type, may reflect tumor heterogeneity. It is of great significance to detect its prognostic ability and evaluate the function in tumor metastasis.[6,7]

Several other studies have investigated the predictive and/or prognostic value of CECs in the neoadjuvant setting and showed inconsistent conclusions. Mancuso et al demonstrated the increase in CEC numbers was related to clinical benefit in terms of tumor response in diverse malignant neoplasms including breast cancer.[8] However, some studies on different tumors have shown that an increase in CEC is associated with a worse outcome or is not prognostic.[9–11] These discrepancies might be explained by the different assays, the timing of CEC detection and different treatment regimens. In the present study, the CD45-/CD31+/DAPI+ CECs aneuploid for chromosome 8 were tested by SE-iFISH. This technique has been used successfully in circulating rare cells detection.[4,12] Unlike previous studies, this study focused more on the relationship between prognosis and aneuploid CECs.

Three time points for repeated measurements were designed. In cross-sectional study, we attempted to determine the relationship between the NCT response and CEC numbers at different time points respectively. However, it turned out that the model had little predictive power. One possibility is that CEC do not have the capacity to predict NCT response, the elevation of CEC number in breast cancer patients had more relevance to tumor recurrence and metastasis. However, it is more likely that strong heterogeneity of breast cancer affected the results. Although the patients enrolled in the study were at the same stage and received uniform NCT regimen, the distant micrometastasis states of different patients were not identical at the beginning of NCT, which may generate from biological behavior of different subtypes of breast cancer. Therefore, we carried out subgroup analysis. In patients with HR+ breast cancer, the difference of aneuploid CEC number in High-response groups approached but failed to achieve a customary level of statistical significance.

We reviewed several studies of CEC in patients with BC receiving NCT (Table 2).[13–17] There is little agreement about the relationship between CEC numbers and pCR rates in NCT. But in agreement with our findings, the same rising trend of CEC counts after NCT was showed in these studies. In the series studies, dynamic changes of CECs during NCT were monitored. Various liquid biopsy markers showed different variation trends, bidirectional or unidirectional in three time points among different groups.[3,5] In SWOG0500 trial, the researchers also took the changes of CTCs between before and after one course of chemotherapy as evidence to select the next step treatment.[18] As the results, the difference among three repeated measurements was independently used as a variable to evaluate the diagnostic effects. In HR+ breast cancer patients, the Δvalue 2 and Δvalue 3 showed certain ability to predict the response to NCT, which suggested it may be of great significance to compare CEC numbers with baseline level during NCT. At the same time, we believe a larger sample study is still needed to prove whether it has exact predictive value. The present study utilized the same cohort of samples for examination as another project within our research group that focused on CTCs.[19] In the aforementioned research, CTCs were identified as having a certain degree of correlation with the efficacy of NCT. Our findings revealed a strong concordance between CECs and CTCs, indicating that the dissemination process of tumors is multi-sourced, not solely confined to single tumor cells entering the bloodstream. The correlation between CECs and CTCs emerges as one of the most intriguing focal points for future research, suggesting a complex interplay beyond the traditional view of tumor dissemination. The consistency between CECs and CTCs not only sheds light on the multifaceted nature of tumor spread but also opens up new avenues for exploring the mechanisms and dynamics behind tumor cell dissemination.

There is a general consensus that pCR is a clear indication of a favorable outcome in NCT, but could not completely replace the prognosis evaluation. There were obvious limitations regarding local therapeutic response as an alternative endpoint of prognosis of NCT.[20] Hence, we compared the PFS in patients with different CEC counts. Similar to previous results, no difference in survival was observed between the patients with different aneuploid CECs at any time point. However, the difference of CECs between the third and second time points (post-NCT and post-1st NCT) was proven to have the ability to

Table 2 Overview of Relevant Literature Concerning the Variation Trend of CECs in with BC Treated with NCT

Reference	Trial	CEC Marker	Detection Platform	CEC Numbers at Baseline	CEC Numbers After Chemo therapy	Variation Trend	Correlation with pCR
Wendy Onstenk et al[11]	NEOZOTAC	CD34+/DNA+/CD146+/CD45-	Flow cytometric assay	31.5	144.5	Increase	No
J.-Y. Pierga et al[12]	BEVERLY-1 and BEVERLY-2	CD146+CD105+	CellSearch System	15		Increase	No
F.-C. Bidard et al[13]	MO19391	CD146+CD105+CD45-DAPI+	CellSearch System	17	26	Increase	Yes
Arwa M. Ali et al[14]		CD146+CD105+CD45-DAPI+	CellSearch System			Increase	Yes
Jeanine M. Roodhart et al[15]		CD45-/CD31+/CD146+	Flow cytometry analysis	100%	275%	Increase	Yes

assess prognosis. The data revealed aneuploid CEC number increased after one cycle of NCT in most patients, but once there was a significant decrease after all NCT courses, patients had a longer life expectancy. In the treatment of LABC, the purpose of systemic therapy is to eliminate distant micrometastases. The increasing or stable CEC numbers may predict micrometastasis purge failure and lead to tumor recurrence eventually.

A limitation of this study was that it was designed as a small sample single center study, which led to some differences falling marginally short of significance. Due to the limitation of the follow-up time, the long prognostic efficacy of CECs requires a longer follow-up time.

Conclusions

This study proposes that variations in aneuploid CECs during NCT may have the ability to predict chemotherapy response in patients with HR+ breast cancer. The decrease in aneuploid CECs number after all courses of NCT indicates better treatment outcomes in LABC patients.

Abbreviations

CECs, Circulating endothelial cells; BC, Breast cancer; NCT, Neoadjuvant chemotherapy; LABC, Locally advanced breast cancer; SE-iFISH, Immunostaining fluorescence in situ hybridization; ROC, Receiver operating characteristic; PFS, Progression-free survival; OS, Overall survival; HR+, Hormone+Her-2-/+; pCR, Pathologic complete response; ER, Estrogen receptor; PgR, Progesterone receptor; HER2, Human epidermal growth factor receptor 2; AUCs, Area under the curves; CI, Confidence interval; CTC, Circulating tumor cell.

Data Sharing Statement

All remaining data and materials are available from the authors upon reasonable request. This paper has been uploaded to ResearchSquare as a preprint: https://www.researchsquare.com/article/rs-2553063/v1.

Ethics approval and consent to participate

Samples were collected at the First Affiliated Hospital of Nanjing Medical University. Ethical approval was obtained from the Institutional Ethics Committee of the First Affiliated Hospital of Nanjing Medical University (approval number: SR-171), and written informed consent was obtained from all participants. This study complies with the Declaration of Helsinki.

Consent for Publication

All authors have given consent for publication.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declared no potential conflicts of interest.

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