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Article *in* Journal of chemotherapy (Florence, Italy) · June 2020

DOI: 10.1080/1120009X.2020.1774207

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ISSN: 1120-009X (Print) 1973-9478 (Online) Journal homepage: https://www.tandfonline.com/loi/yjoc20

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To cite this article: Zhaoqing Cui, Fengbo Su, Yang Li & Dawei Yang (2020): Circulating tumour cells as prognosis predictive markers of neoadjuvant chemotherapy-treated breast cancer patients, Journal of Chemotherapy, DOI: 10.1080/1120009X.2020.1774207

To link to this article: https://doi.org/10.1080/1120009X.2020.1774207

Published online: 05 Jun 2020.

Anticancer Original Research Paper

Circulating tumour cells as prognosis predictive markers of neoadjuvant chemotherapy-treated breast cancer patients

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ABSTRACT

In this study, we detected and measured the count of circulating tumour cells (CTCs) in breast cancer (BC) patients who were treated by neoadjuvant chemotherapy (NAC) in order to assess the clinical validity of CTCs. A total of 96 patients with locally advanced BC and who were treated by NAC were enrolled in this study. The CTC count in the peripheral blood was estimated by negative enrichment-fluorescence *in situ* hybridization before and after NAC. The clinicopathological data of the patients were recorded. CTCs were detected in 59 of the 96 patients with BC before NAC. Particularly, the detection rate of CTCs was significantly lower in human epidermal growth factor receptor-2 (HER-2)-negative patients than in HER-2-positive patients. CTCs were significantly fewer after NAC than before NAC. The CTC-detection sensitivity in the NAC efficacy evaluation was 75.5% (40/53), while the specificity was 72.1% (31/43). The CTC consistency analysis with clinical effects (Response Evaluation Criteria in Solid Tumors Version 1.1 Standard) was described as moderate (kappa = 0.476, $P < 0.001$). Thus, our findings suggest that CTC detection is a potential new approach to assess the efficacy of NAC.

Keywords: Breast cancer; circulating tumour cells; neoadjuvant chemotherapy; negative enrichment; fluorescence in situ hybridization; HER-2

Introduction

Breast cancer (BC) is among the most common cancers affecting women in China. In 2018, the International Agency for Research on Cancer (IARC) found that BC is the most common cancer affecting women worldwide, with the incidence demonstrating an upward trend.[1] BC is highly heterogeneous in genetic, clinical, and phenotypical perspectives, which has immensely complicated treatment and diagnosis approaches for BC. For instance, the clinical outcomes of similar treatments may differ for patients with similar clinical or pathological presentations. It is believed that personalized BC therapy will be greatly facilitated by identifying biomarkers that can predict accurate treatment response and prognosis of BC.

Neoadjuvant chemotherapy (NAC) was first applied for BC in the 1970s, and, since then, it has become an important part of the comprehensive BC treatment.[2] NAC is an important treatment for localized advanced BC with the potential to improve the outcomes of successful and breast-conserving operations.[3] Recently, pathological complete response (pCR) after NAC was confirmed as an outstanding prognosis predictive factor.[4,5] NAC has 2 main functions: primary tumour shrinkage and eradication of blood-borne tumour cell dissemination.[6] However, the present NAC assessments typically ignore the methods of evaluating tumour cell dissemination despite the presence of disseminated tumour cells in the bone marrow showing significant independent predictive ability for poor prognosis in metastatic BC.[7] However, in patients with BC, it is impossible to repeatedly perform bone marrow aspiration for diagnostic purpose. Therefore, research is actively ongoing to assess whether circulating tumour cells (CTCs) are clinically valuable for monitoring the therapeutic efficacy of BC, and several studies has reported the

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DOI 10.1080/1120009X.2020.1774207

clinical validity of CTCs in several cancer types.[8,9] In BC patients, the presence of CTCs, which is detected using the FDA-approved CellSearch assay, has been demonstrated to be associated with worse prognosis.[10–12] The CellSearch Assay has been developed to detect CTCs on the basis of epithelial cell adhesion molecule (EpCAM) expressed on the plasma membrane of the tumour cells.[13] However, recent studies have questioned this method's suitability owing to the dynamic nature of the EpCAM expression,[14,15] which depends on the type or stage of cancer cell development, particularly in cells that undergo epithelial to mesenchymal (EMT) transition,[16,17] as this transition lower the expression of epithelial markers such as cytokeratin and EpCAM.[18] Recently, we used a detection platform with integrated immunostaining-fluorescence *in situ* hybridization (FISH) and EpCAM-independent subtraction that demonstrated a remarkable potential for the detection of CTCs in diverse cancer types in routine clinical examinations.[19,20]

In this study, we evaluated the predictive prognosis capability of CTCs after NAC in Chinese patients with BC based on the negative enrichment (NE)-FISH approach.

Materials and methods

Study patients

Between March 2016 and June 2018, the Breast Surgery Department of Liaocheng People's Hospital admitted 96 patients who were histologically confirmed with locally advanced BC (LABC). The patients were treated with the NAC regimen TEC comprising 6 cycles of 100 mg/m^2 docetaxel (T), 100 mg/m^2 of epirubicin (E) hydrochloride, and 500 mg/m^2 of cyclophosphamide (C). Each TEC cycle constituted 21 days. The study patients were not allowed to receive any other treatments. For immunohistochemical examination, adequate core biopsy samples were collected from all patients for the detection of the expression of human epidermal growth factor receptor-2 (HER-2). This study was approved by the institutional ethics committee of the Liaocheng People's Hospital (Number: 201626). In addition, all patients provided their written informed consent before their participation in the study.

CTC detection

In this study, we performed the negative enrichment-fluorescence *in situ* hybridization (NE-FISH) assay to detect CTCs in patients with BC [19]. Briefly, 3.2 mL of blood sample was collected from patients, washed once with CS1 buffer, lysed with CS2 buffer, and then spun down. Next, the cells were re-suspended and incubated with immunomagnetic particles conjugated with an anti-leukocyte monoclonal antibody (anti-CD45; Cyttel, Jiangsu, China) under gentle shaking condition. The depleted CD45 positive cells were then placed over CS3 (Cyttel)—a special gradient centrifugation liquid—and separated by gradient centrifugation. On a magnetic stand, the resultant CTC-containing solution was mixed with a cell fixative and smeared on a slide. The CTCs were then fixed and dried for subsequent analyses. The enriched CTCs were identified by subjecting the slides to FISH with the chromosome centromere probe (CEP) 8 and 17, immunostaining with Alexa Fluor 594-conjugated anti-human CD45 (Cyttel), and staining with 4′,6-diamidino-2-phenylindole (DAPI). The slides were scanned under a microscope (BX63; Olympus) and subjected to image analysis using an automated image analysis system, the IMSTAR high-content screening device equipped with Pathfinder^{TM} software (IMSTAR S.A., Paris, France). CTCs were identified as CD45 negative DAPI positive and CEP positive

Response evaluation criteria

CTC evaluation criteria

The CTCs in the peripheral blood of BC patients were quantified before and after NAC regime. CTC count $\geq$1 was classified as a positive count. Reducing count of CTCs after one or more NAC regime round indicated effectiveness of the chemotherapy (valid).

Clinical effect criteria

To evaluate the clinical response, Response Evaluation Criteria in Solid Tumors (RECIST) guideline (Version 1.1) was referred.[21] Based on the primary tumour diameter and the axillary lymph node status, the clinical response was classified as follows: complete response (CR), stable disease (SD), partial response (PR), or progressive disease (PD). PR and CR were indicative of clinical efficacy (valid).

Statistical analysis

The SPSS 15.0 software was used for statistical analysis. The χ^2 test was used to analyze the association between the presence of CTC and the clinicopathological characteristics of BC patients. The McNemar's test was applied to compare the positive rate of CTC before and after NAC. Kappa and McNemar's tests were used to conduct consistency analysis between the CTC counts and the clinical effects (RECIST Version 1.1 standard).

Table 1. Relationship of CTCs with clinical characteristics and patient demographics before NAC.

Clinicopathological characters	Cases	CTC		χ^2	*P*
		Positive	Negative		
Age					
>35 years	57	32	25	0.283	0.595
≤35 years	39	27	12		
Tumour size					
>5 cm	34	22	12	1.021	0.312
≤5 cm	62	37	25		
ER					
Positive	64	38	26	0.777	0.378
Negative	32	21	11		
PR					
Positive	57	34	23	1.331	0.249
Negative	39	25	14		
HER-2					
Positive	49	40	9	11.126	<0.001
Negative	47	19	28		
Ki67					
≥15%	54	40	14	0.169	0.681
<15%	42	19	23		

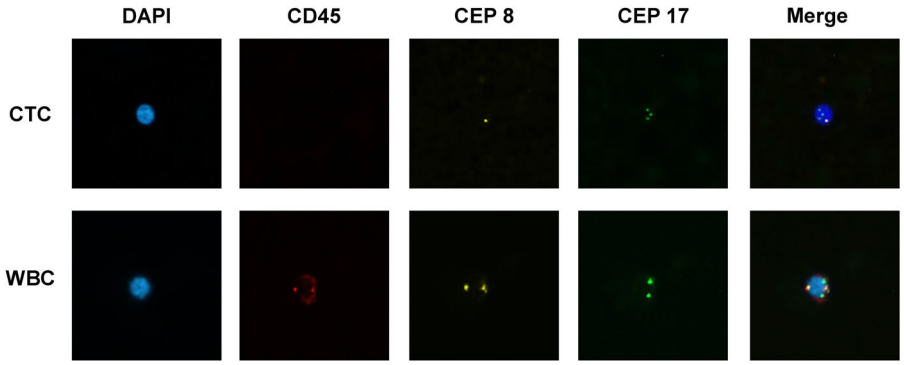

Figure 1. Representative image of CTC by NE-FISH in breast cancer patients. CD45: red; DAPI: blue; CEP8: orange; CEP17: green. DAPI: (4′,6-diamidino-2-phenylindole); CEP: centromere probe; CD45: cluster of differentiation 45; CTC: circulating tumour cell; WBC: white blood cell.

Results

A total of 96 patients (mean age: 44.7 ± 12.2 years) were enrolled in this study. The baseline characteristics of these patients along with the detected biomarkers are listed in Table 1. All patients were treated with the NAC regimen comprising six TEC cycles.

Immunostaining markers were used for the identification of CTCs, including leukocyte common antigen 45 (CD45), DAPI, and CEP 8 and 17. CD45+ and DAPI + were used to stain white blood cells, whereas CD45− and DAPI + were used to stain CTCs because of the presence of the specific markers on the WBC surface. Therefore, CTCs were identified as DAPI+/CD45−/chromosome multiploid (CEP 8+ and/or CEP17+) in BC (Figure 1).

Using the NE-FISH assay, CTCs were detected in the BC patients following the definition of DAPI+/CD45−/CEP+ (Figure 1). The detection rate of CTC in the peripheral blood before NAC was 61.5% (59/96) (CTCs ≥1). In particular, the detection rate of CTC was significantly lower in HER-2-negative patients as compared to that in HER-2-positive patients [40.4% (19/47) *vs.* 81.6% (40/49), $\chi^2 = 11.126$, $P < 0.001$] (Table 1). The detection rate of CTC was found to be significantly low after NAC than before NAC [25.0% (24/96) *vs.* 61.5% (59/96), $\chi^2 = 34.102$, $P < 0.001$] (Table 2).

Reducing counts of CTCs after 1 round of NAC regime was indicative of the effectiveness of chemotherapy, as assessed based on the clinical effects described by the revised RECIST guideline (Version 1.1). The CTC count clearly decreased in 52 patients after NAC, whereas, 53 patients indicated the effectiveness of NAC as per the revised RECIST standard (Table 3). No significant difference was noted between CTC counts and the clinical outcomes. The consistency of CTC counts with the clinical effects (as per RECIST standard) was described as moderate (kappa = 0.476, $P < 0.001$) (Table 3).

Table 2. CTC status of breast cancer patients before and after NAC.

	CTC after NAC		
CTC before NAC	Positive	Negative	Total
Positive	21	38	59
Negative	3	34	37
Total	24	72	96

Note: McNemar's test was used, $\chi^2 = 34.102$, $P < 0.001$.

Table 3. Evaluation of the consistency of CTCs with the clinical effects.

	Clinical effects (RECIST Version 1.1)		
CTC	Valid	Invalid	Total
Valid	40	12	52
Invalid	13	31	44
Total	53	43	96

Note: McNemar's test was used to evaluate the two methods, $\chi^2 = 0.040$, $P = 1.000$.
Kappa test was used to evaluate the consistency, kappa = 0.476, $P < 0.001$.

Discussion

NAC regimens commonly use docetaxel, doxorubicin, and cyclophosphamide (TAC); docetaxel plus cyclophosphamide (TC); and docetaxel, epirubicin, and cyclophosphamide (TEC). Among these, TEC regimen is considered as a well-tolerated and effective NAC regimen for LABC.[22]

CTCs are tumour cells that dissociate from the primary solid tumour and enter the circulation through either the blood or lymph.[23] Detection of CTCs in the peripheral blood may allow the relatively non-invasive acquisition of tumour tissue specimens, also called liquid biopsy.[24] The use of peripheral blood samples for CTC counting and the detection of molecular biology characteristics can assist in monitoring the disease progression, evaluation of treatment effectiveness, and providing evidence for personalized treatment for breast tumours.[25] For NAC, CTCs also offer a unique advantage of helping predict the sensitivity of the cells to therapeutic drugs and guide personalized treatment approach for patients.[26]

A recent study reported that the detection of CTC before NAC acted as an independent prognostic factor and that the combination of CTC and pCR could better stratify patients for treatment intervention.[27] A second-phase clinical trial demonstrated that the detection rates of CTC before and after NAC was 23% and 17%, respectively, which are consistent with our findings.[28] A past meta-analysis detected $\geq$ CTCs in 25.2% of the patients before NAC,[29] which is lower than that in the present study. In this study of 96 BC patients administered with the standard treatment of NAC regimen comprising of 6 cycles of TEC, the detection rate of CTC in the peripheral blood before NAC was 61.5%; the rate after NAC was significantly lower than that before NAC. Hence, the detection rate of CTCs acted as a sensitive predictor of NAC effectiveness (Table 2).

Furthermore, we investigated the association between CTC detection and the clinicopathological characteristics of BC patients and found that CTC detection was related to the HER-2 expression. The CTC-positive rates were lower in HER-2-negative than in HER-2-positive BC patients. This finding may be attributed to the highly invasive and malignant tumours in high HER-2 expression patients.[30] However, this observation needs further clarification through detailed investigation (Table 1). Moreover, the detection of the HER-2 status of CTCs could be a strategy with potential clinical application, considering that HER-2 status may vary among different metastatic sites and also during the treatment.[7,31]

Recently, several in-depth research on the comparison of CTC counting methods and the related clinical outcomes have been conducted in metastatic BC. For instance, a prospective longitudinal clinical study was conducted to ascertain the association between CTC counting with clinical assessment and disease status.[32] In this study, the advantages of employing CTCs instead of imaging alterations as an indicator of treatment success were applied to determine a statistically significant correlation between the two in the disease progression. In the course of chemotherapy, the efficacy of the treatment was evaluated, which suggested that CTC counting was statistically significant for 7–9 weeks before the changes were observed on imaging. After chemotherapy, CTC counts were decreased in 52 BC patients. This finding suggests that the status of CTC count may serve as an indicator in predicting the short- and long-term efficacy of chemotherapy in BC.[33] Another study reported that the CTC count undergoes significant changes after the first cycle itself, which shortens the time for judging the effect based on imaging after 2–3 cycles and hence avoids medical delays.[34] The changes in the CTC count during the course of treatment are influenced by the long-term remission. We compared CTC counting with clinical effects and found that CTC counts and impact detection have basically been changing. There is no significant difference between the statistical analyses of the CTC counting and clinical effects, suggesting that CTC counting is one of the effective evaluation indicators of chemotherapy effectiveness (Table 3). Furthermore, CTC detection is relatively more repeatable, non-radioactive, and economical

than imaging tests.[35] Therefore, monitoring changes in the CTC count offers an advantage in predicting the development of diseases in patients with advanced BC.

We identified a few limitations of this study. First, this study assessed a small sample size, which restricts the representation of the patient population, warranting cautious interpretation of the conclusions of this study. In addition, the small sample size of only 96 patients made it difficult to assess whether the effects observed in them could translate into prognostic factors.

Conclusions

In conclusion, NAC-induced alteration of CTC is related to tumour reduction. Therefore, the status of CTCs is a potential marker to help predict the response to NAC. We believe that further studies are warranted to differentiate among biomarkers on the basis of their ability to better predict the prognosis and treatment responses.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

All participants consented to the publication.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the China Postdoctoral Science Foundation (2015M580595), Shandong Natural Science Foundation of China (ZR2017LH050 and ZR2019PH084).

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