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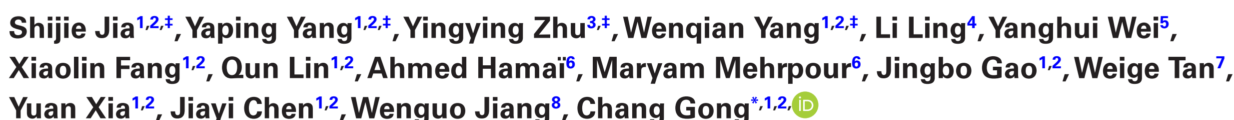
OXFORD

Association of FTH1-Expressing Circulating Tumor Cells With Efficacy of Neoadjuvant Chemotherapy for Patients With Breast Cancer: A Prospective Cohort Study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Abstract

Background: The association between different phenotypes and genotypes of circulating tumor cells (CTCs) and efficacy of neoadjuvant chemotherapy (NAC) remains uncertain. This study was conducted to evaluate the relationship of FTH1 gene-associated CTCs (F-CTC) with/without epithelial-mesenchymal transition (EMT) markers, or their dynamic changes with the efficacy of NAC in patients with non-metastatic breast cancer.

Patients and Methods: This study enrolled 120 patients with non-metastatic breast cancer who planned to undergo NAC. The FTH1 gene and EMT markers in CTCs were detected before NAC (T0), after 2 cycles of chemotherapy (T1), and before surgery (T2). The associations of these different types of CTCs with rates of pathological complete response (pCR) and breast-conserving surgery (BCS) were evaluated using the binary logistic regression analysis.

Results: F-CTC in peripheral blood ≥1 at T0 was an independent factor for pCR rate in patients with HER2-positive (odds ratio [OR]=0.08, 95% confidence interval [CI], 0.01-0.98, P = .048). The reduction in the number of F-CTC at T2 was an independent factor for BCS rate (OR = 4.54, 95% CI, 1.14-18.08, P = .03).

Conclusions: The number of F-CTC prior to NAC was related to poor response to NAC. Monitoring of F-CTC may help clinicians formulate personalized NAC regimens and implement BCS for patients with non-metastatic breast cancer.

Key words: circulating tumor cells; ferroptosis; ferritin heavy chain; neoadjuvant chemotherapy; pathological complete response; breast-conserving surgery.

Implications for Practice

To evaluate the association of ferritin heavy chain (FTH1)-expressing circulating tumor cells (CTCs) with the efficacy of neoadjuvant chemotherapy (NAC) for patients with breast cancer, we dynamically monitored the FTH1 gene and epithelial-mesenchymal transition markers in CTCs during NAC. The status of FTH1 gene-associated CTCs (F-CTC) has predictive value for pathological complete response. Changes in the number of F-CTC can help predict the choice of breast surgery modality. For non-metastatic patients with breast cancer, FTH1, as a potential marker, may facilitate a timely personalized treatment to improve patients' long-term prognosis.

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Introduction

Breast cancer has been the most prevalent cancer worldwide since 2020.[1] Every year, more than 600 000 patients die from breast cancer, mostly due to metastatic recurrence.[2] These patients with successful treatment for early-stage cancer may have minimal residual disease that persists after initial therapy, which is considered as a potential source of metastatic relapse. Circulating tumor cells (CTCs) are cancer cells that shed from the primary tumor into the peripheral blood.[3] The negative impact of CTCs on the long-term survival of non-metastatic breast cancer has been demonstrated.[4-6] However, the relationship between CTCs and pathological complete response (pCR) after neoadjuvant chemotherapy (NAC) is still unclear, mainly due to the relatively low ability in detecting CTCs by previous techniques.[7,8] With the development of high-sensitivity liquid biopsy assays in recent years, the different phenotypes and genomic profiles of CTCs can be identified to help us understand their heterogeneity,[9,10] which are highly desirable to characterize the treatment efficacy of NAC.

CTCs undergo epithelial-mesenchymal transition (EMT) during migration, downregulating epithelial markers, and up-regulating mesenchymal markers. Based on EMT markers, CTCs can be divided into the following subgroups: epithelial CTCs (E-CTC) expressing epithelial markers, mesenchymal CTCs (M-CTC) expressing mesenchymal markers, or biphenotypic epithelial/mesenchymal CTCs (E/M-CTC) expressing both epithelial and mesenchymal markers.[9,11] Several studies have found that CTCs undergoing EMT are associated with tumor progression or long-term survival.[11,12] However, no studies have been conducted to evaluate the impact of E-CTC, M-CTC, E/M-CTC, or total CTCs, or their dynamic changes on the efficacy of NAC in patients with non-metastatic breast cancer.

Apart from phenotypes, the genomic profiles of CTCs have been of increasing interest. Ferroptosis is a non-apoptotic form of cell death that is closely related to iron metabolism and has been found to play an important role in the progression of various malignancies, including breast cancer.[13] One of the important characteristics of ferroptosis is the presence of redox-active iron.[14] Multiple genes including FTH1, GPX4, ACSL4, SLC7A11, and TFRC have been identified to regulate ferroptosis. As a key factor in iron metabolism regulation, FTH1 is involved in regulating cellular redox balance by modulating the storage and release of ferrous ions and has been found to be significantly upregulated in multiple malignant tumors, exerting important effects on ferroptosis. FTH1 mRNA and protein levels were found to be significantly elevated in tumor stem cells from HER2/Neu transgenic mice.[15] In triple-negative breast cancer, nuclear staining of FTH1 was also significantly increased and associated with poor prognosis.[16] Ferroptosis could be activated by toxic level of reactive oxygen species (ROS),[17,18] limiting the progression of tumor cells. The accumulation of iron-dependent lipid peroxides generated by intracellular redox-active iron through the Fenton reaction is an important event in ferroptosis. FTH1 expression in tumor cells is elevated in many types of malignancies, including breast cancer.[19] This may increase their antioxidant capacity by stabilizing high levels of ROS[20] and may also be partially responsible for chemotherapy resistance.[21] Meanwhile, downregulation of FTH1 in cells undergoing EMT induces ferroptosis by increasing ROS production.[22,23] However, whether FTH1-expressing CTCs combined with/without EMT markers or their dynamic changes during NAC are associated with the efficacy of NAC in patients with non-metastatic breast cancer remains unclear.

Herein, we aimed to assess the associations of different types of CTCs and their dynamic changes with the efficacy of NAC in patients with non-metastatic breast cancer.

Materials and Methods

Study Design

This single-center, prospective, observational cohort study was approved by the Clinical Research Ethics Committee of Sun Yat-sen Memorial Hospital (SYSEC-KY-KS-2021-103) and registered in the Chinese Clinical Trials Registry (ChiCTR2100046262). All patients were prospectively enrolled in Sun Yat-sen Memorial Hospital Breast Cancer Center from May 2021 to January 2022 and signed written informed consent. Eligible patients were women (≤70 years) with newly diagnosed, previously untreated non-metastatic breast cancer. Other key inclusion criteria were: (1) patients who met the indications of NAC; (2) Eastern Cooperative Oncology Group performnce status 0–1; (3) sufficient bone marrow reserve capacity, and good liver and renal function. Key exclusion criteria were: (1) inflammatory breast cancer and (2) patients accompanied with other malignant tumors (eg, thyroid cancer).

Data Collection

At baseline, patients underwent physical examinations, breast ultrasound, mammography, magnetic resonance imaging (MRI), tumor biopsy, laboratory tests, and assessments of vital organ function. The NAC regimen based on anthracycline combined with paclitaxel was formulated by the chief physician according to the NCCN guidelines. Dual-targeted therapy was added to patients with human epidermal growth factor receptor 2 (HER2)-positive breast cancer. Physical examinations and laboratory tests were conducted at each cycle. Prior to surgery, breast ultrasound, mammography, MRI were performed to evaluate tumors and calcifications. Breast surgery included breast-conserving surgery (BCS) and mastectomy with or without reconstruction, which was performed by the chief physician. In order to reduce the rate of positive margin and secondary surgery, our center adopted a modified intraoperative method for cavity margin processing.[24] After surgery, recurrence and survival were followed every 3 months (Fig. 1B).

During the study period, 5 mL peripheral venous blood was collected from patients for CTCs detection at 3 different time points: before NAC (T0), after 2 cycles of chemotherapy (T1), and before surgery (T2).

Outcomes

The primary endpoint was pCR rate, which was defined as the absence of residual invasive tumor in the breast and axillary lymph nodes on the operative specimen after NAC.

Secondary endpoints were BCS rate and event-free survival (EFS). The BCS rate was defined as the proportion of patients with BCS among all patients who received surgery. EFS was defined as the time from the first diagnosis to disease progression. Disease progression included local or distant recurrence, occurrence of a second primary cancer, or death from any cause.[25]

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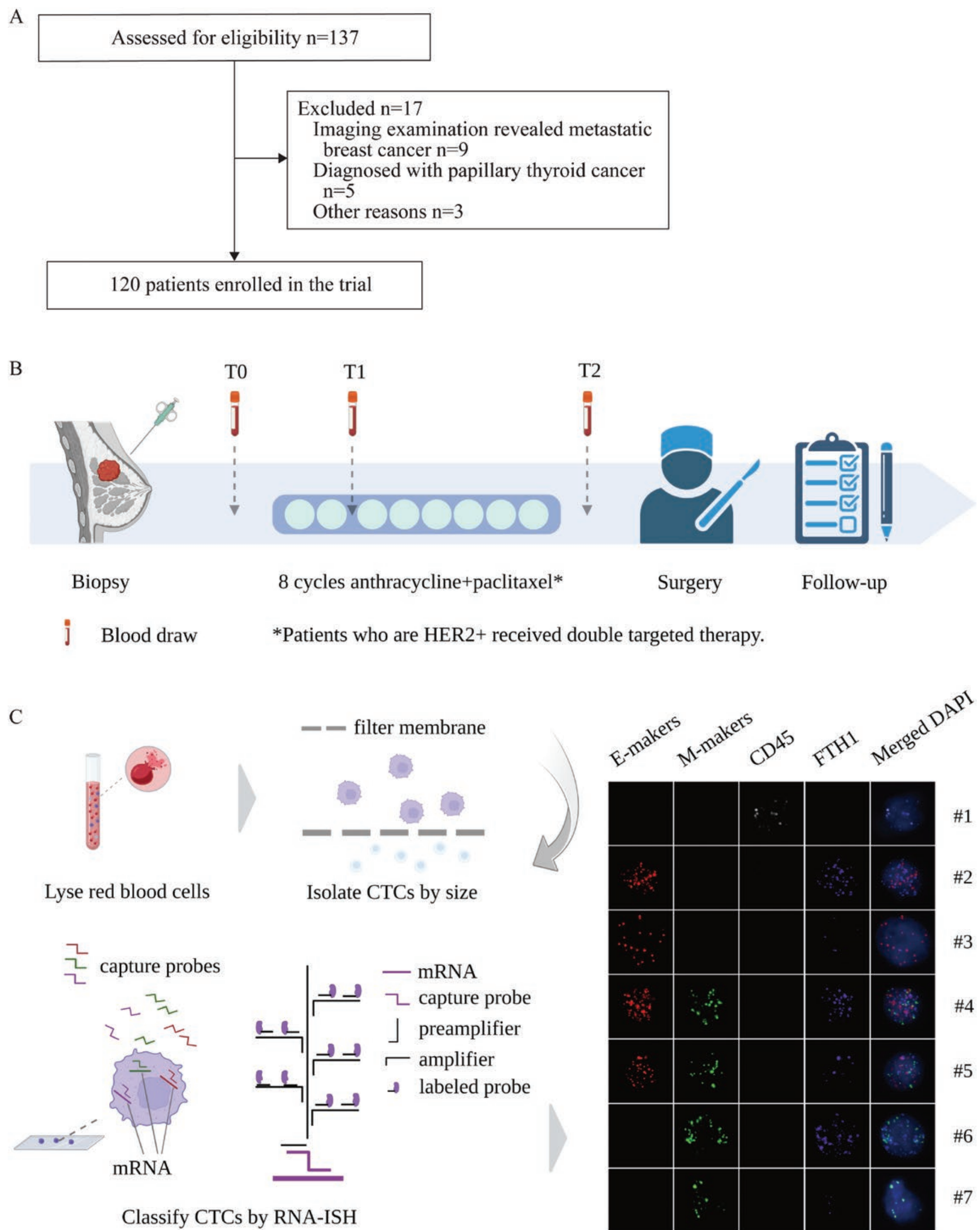

Figure 1. Process for screening enrolled patients, study schema, method of CTCs detection. (**A**) The process of screening enrolled patients. (**B**) The study schema. All patients were confirmed for pathological type by primary tumor biopsy. The neoadjuvant chemotherapy consisted of 8 cycles of anthracyclines and paclitaxel, and dual-targeted therapy was added to patients with HER2-positive. Surgical treatment was performed after completing chemotherapy, and telephone follow-up was conducted regularly after surgery. Blood samples were collected at the following time points: T0, before NAC; T1, after 2 cycles of chemotherapy; T2, before surgery. (**C**) Flowchart of CTCs detection. After lysing erythrocytes, filtering CTCs, and classifying CTCs by RNA-ISH, fluorescent signal spots of the corresponding biomarkers could be observed under fluorescence microscopy. #1 refers to leukocytes, which express only CD45. #2 and #3 refer to E-CTC, which expresses epithelial biomarkers. #4 and #5 refer to E/M-CTC, which expresses both epithelial and mesenchymal markers. #6 and #7 refer to M-CTC, which expresses mesenchymal biomarkers. Among them, the purple FTH1 gene signal points >9 on #2, #4, and #6, which refers to the corresponding CTCs with high-FTH1 gene expression, namely F-CTC^{E}, F-CTC$^{E/M}$, and F-CTC^{M}, respectively. Fig. 1B, 1C were created at BioRender.com and the corresponding authorization has been obtained.

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Isolation and Classification of CTCs

To isolate and classify CTCs, we utilized the CanPatrol system and used the tricolor RNA-ISH assay.

Erythrocyte were removed by adding erythrocyte lysis buffer (154 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA) before isolating CTCs. After centrifuging with a TDZ5-WS centrifuge, the vacuum pump (SurExam, Guangzhou, China) filtered the sample to the 8-μm diameter pores filter membrane (Milli-pore, Billerica, USA).

The assay was performed in a 24-well plate (Corning, NY, USA). A multiplex RNA-in situ hybridization (RNA-ISH) assay was applied to classify and count CTCs based on branched DNA (bDNA) signal amplification, which included capture probes, preamplifier sequence, amplifier sequence, and label probe. Four epithelial biomarkers (EpCAM and CK8/18/19), 2 mesenchymal biomarkers (vimentin and twist), one leukocyte biomarker (CD45), and the FTH1 gene biomarker were used to classify the CTCs. The bDNA signal amplification sequences of those biomarkers are shown in Supplementary Tables S1 and S2. 4′,6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei. Under a fluorescence microscope (100× oil objective, Olympus BX53, Tokyo, Japan), the red signal represented epithelial biomarkers, green showed mesenchymal biomarkers, white meant leukocyte biomarker, and purple indicated the FTH1 gene (Fig. 1C). The mean number of FTH1 gene signal points on CTCs of all patients was 9.4 (±9.5). We defined CTCs with FTH1 gene signal points greater than or equal to 9 as FTH1 gene-associated CTCs (F-CTC). Accordingly, E-CTC, E/M-CTC, and M-CTC that highly expressed the FTH1 gene were defined as F-CTC^{E}, F-CTC$^{E/M}$, and F-CTC^{M}, respectively.

Statistical Analyses

Baseline characteristics of all patients were represented as frequencies and percentages for categorical variables. Chi-square test or Fisher's exact test were performed to detect the difference of characteristics between the pCR and non-pCR groups. The receiver operating characteristic (ROC) curves and the area under the ROC curve (AUC) were used to evaluate the performance of each type of CTCs at T0 for predicting pCR. The highest Youden index was used to calculate the cutoff value of each type of CTCs. The thresholds that divided the number of total CTCs, E-CTC, E/M-CTC, and M-CTC were 4, 2, 3, and 1, respectively. The thresholds that divided the number of F-CTC, F-CTC^{E}, F-CTC$^{E/M}$, and F-CTC^{M} were 1, 1, 1, and 1, respectively. At T1 or T2, compared with T0, we defined a reduction in the number of each type of CTCs below the cutoff value as a decrease and vice versa as no decrease. Chi-square test or Fisher exact test was used to analyze the relationship between the CTCs status and pCR at T0, and the relationship between the change of CTCs status and pCR or BCS at T1 and T2. Univariate and multivariate binary logistic regression analyses were used to explore associated factors of pCR and BCS. A statistical significance level of 0.05 was used to select variables for inclusion in multivariate regression analysis. The Kaplan-Meier method and the log-rank test were used to perform survival analyses and to evaluate the association between the status of different types of CTCs at T0 and the changes in status at T1 and T2 with EFS.

Two-sided $P < .05$ was considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics software version 26.

Results

Clinical Characteristics of Patients

A total of 137 patients were evaluated for eligibility since May 2021, of whom 17 were excluded. Among them, 9 patients had metastatic breast cancer, 5 were diagnosed with papillary thyroid cancer, and 3 rejected the enrollment due to the COVID-19 pandemic. Eventually, 120 patients were prospectively enrolled. The flow chart is shown in Fig. 1A.

In all patients, 39 (32.5%) patients were classified into pCR group, and 81 (67.5%) into non-pCR group based on the postoperative pathological results. The clinical characteristics of all patients and those in different groups are presented in Table 1. The majority of patients were ≤50 years of age (67.5%), premenopausal (69.2%), had tumor size ≤50 mm (75.8%). Most patients had clinical stage IIB-IIIC disease (72.5%), indicating that they had locally advanced breast cancer. The vast majority of patients had a Ki-67 proliferation index ≥14% (96.7%). There were no significant differences in age, menstrual status, tumor size, lymph nodes status, Ki-67 proliferation index, and breast surgery approach between the 2 groups (P>.05). Compared to the non-pCR group, the pCR group had lower proportion of hormone receptor (HR) positive status (84.0% vs 59.0%, P = .003) and higher proportion of HER2-positive status (27.2% vs 76.9%, P < .001).

Association Between CTCs and pCR

Detection of CTCs was performed in 120 patients at T0, T1, and T2. Among them, CTCs were detected in 105 patients (87.5%) at T0, 117 (97.5%) at T1, and 116 (96.7%) at T2. To describe the distribution of CTCs, we plotted ROC curves to predict the pCR rate using the number of each type of CTCs at T0 and calculated the corresponding cutoff values using the highest Youden index, respectively.

At T0, the number of some CTCs correlated with pCR. At T1 and T2, no correlation was found between the status of CTC and pCR. As shown in Table 2 and Fig. 2, the pCR rate of patients with total CTCs ≥4 at T0 was significantly lower than that of patients with total CTCs <4 (25.4% vs 42.9%, P = .044). However, the distribution of E-CTC, E/M-CTC, and M-CTC at T0 was not significantly associated with the pCR rate. Afterwards, we found that patients with F-CTC or F-CTC$^{E/M}$ ≥1 at T0 had significantly lower pCR rate than patients without F-CTC (17.1% vs 59.1%, P < .001) or F-CTC$^{E/M}$ (18.0% vs 47.5%, P = .001). Such a relationship was not observed in terms of F-CTC^{E} and F-CTC^{M}. Similarly, no relationship was found between the distribution of each CTCs type and the pCR rate at T1 and T2 (Table 2).

Changes in the status of each type of CTC did not affect pCR at T1 and T2. We included total CTCs, F-CTC, and F-CTC$^{E/M}$ with differences in pCR rate distribution at T0 in the analysis of dynamic changes. Since we also wanted to explore whether the dynamic change of CTCs screened with EMT markers alone was meaningful, we included it in further analyses even though the distribution of E/M-CTC at T0 was unrelated to pCR rate. As shown in Fig. 3, 69 of 71 patients with total CTCs ≥4 at T0 did not achieve a reduction at T1, and 17 patients subsequently achieved pCR. One of the 2 patients with a reduction in total CTCs at T1 achieved pCR. There was no significant difference in pCR rate between the 2 groups. Change in the total CTCs at T1 did not correlate

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Table 1. Clinical characteristics between pCR and non-pCR patients.

Clinical characteristic	Number of patients (%)			*P* value
	All patients (n = 120)	pCR (= 39)	Non-pCR (n = 81)	
Age(years)				
≤50	81(67.5)	27(69.2)	54(66.7)	0.78
>50	39(32.5)	12(30.8)	27(33.3)	
Menstrual status				
Premenopausal	83(69.2)	29(74.4)	54(66.7)	.39
Postmenopausal	37(30.8)	10(25.6)	27(33.3)	
Tumor size				
cT1-2	91(75.8)	32(82.1)	59(72.8)	.27
cT3-4	29(24.2)	7(17.9)	22(27.2)	
Lymph nodes				
cN0-1	101(84.2)	34(87.2)	67(82.7)	.53
cN2-3	19(15.8)	5(12.8)	14(17.3)	
Clinical tumor stages				
IIA	33(27.5)	9(23.1)	24(29.6)	.75
IIB	51(42.5)	19(48.7)	32(39.5)	
IIIA	25(20.8)	7(17.9)	18(22.2)	
IIIB	1(0.8)	0(0)	1(1.2)	
IIIC	10(8.3)	4(10.3)	6(7.4)	
HR status				
Negative	29(24.2)	16(41.0)	13(16.0)	.003
Positive	91(75.8)	23(59.0)	68(84.0)	
HER2 status				
Negative	68(56.7)	9(23.1)	59(72.8)	<.001
Positive	52(43.3)	30(76.9)	22(27.2)	
Molecular subtype				
HR+/HER2-	51(42.5)	4(10.3)	47(58.0)	<.001
HR+/HER2+	40(33.3)	19(48.7)	21(25.9)	
HR-/HER2+	15(12.5)	11(28.2)	4(4.9)	
TNBC	14(11.7)	5(12.8)	9(11.1)	
Ki-67 proliferation index (%)				
<14	4(3.3)	1(2.6)	3(3.7)	.74
≥14	116(96.7)	38(97.4)	78(96.3)	
Breast surgery				
Mastectomy	54(45.0)	15(38.5)	39(48.1)	.32
Breast-conserving surgery	66(55.0)	24(61.5)	42(51.9)	

Abbreviations: CTCs: circulating tumor cells; HR: hormone receptor; HER2: human epidermal growth factor receptor 2; pCR: pathological complete response.

with the pCR rate. Similarly, changes in E/M-CTC, F-CTC, and F-CTC$^{E/M}$ at T1 were not related to the pCR rate. In the same way, we explored the above-mentioned changes in the number of CTCs at T2, but unfortunately, we did not find an association with pCR rate (Fig. 3).

The status of F-CTC at T0 was an independent factor affecting pCR. As shown in Table 3, patients with positive HR status (odds ratio [OR]=0.28, 95% confidence interval [CI], 0.12-0.66, P = .004), total CTCs ≥4 (OR = 0.45, 95%CI, 0.21-0.99, P = .046), F-CTC ≥1 (OR = 0.14, 95%CI, 0.06-0.33, P < .001), or F-CTC$^{E/M}$ ≥1 (OR = 0.24, 95%CI, 0.11-0.56, P = .001) were not susceptible to pCR. Patients with positive HER2 status were more likely to obtain pCR (OR = 8.94, 95% CI, 3.67-21.80, P < .001). Other clinicopathological features and changes in CTCs status at T1 and T2 were not found to affect the pCR rate. We found that the status of F-CTC at T0 remained meaningful after adjustment for HR and HER2 status, which was an independent factor for pCR (OR = 0.16, 95% CI = 0.03-0.95, P = .043). Considering that HER2 status was an important influencing factor of pCR rate, we performed a multivariate binary logistic regression analysis after stratification by different HER2 status. As demonstrated in Supplementary Table S3, it was not susceptible to obtain pCR in patients with HR-negative, HER2-negative disease (HR = 0.14, 95% CI, 0.03-0.65, P = .01). In patients with HER2-positive, the presence of F-CTC at T0 was an independent factor for pCR (HR = 0.08, 95% CI, 0.01-0.98, P = .048).

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Table 2. Distribution of various types of CTCs in pCR and non-pCR populations at T0, T1, T2.

	Number of patients (%) at T0				Number of patients (%) at T1				Number of patients (%) at T2			
	All patients (n = 120)	pCR (n = 39)	Non-pCR (n = 81)	P-value	All patients (n = 120)	pCR (n = 39)	Non-pCR (n = 81)	P-value	All patients (n = 120)	pCR (n = 39)	Non-pCR (n = 81)	P-value
Total CTCs												
<4	49(40.8)	21(42.9)	28(57.1)	.044	18(15.0)	9(50.0)	9(50.0)	.09	28(23.3)	9(32.1)	19(67.9)	.96
≥4	71(59.2)	18(25.4)	53(74.6)		102(85.0)	30(29.4)	72(70.6)		92(76.7)	30(32.6)	62(67.4)	
E-CTC												
<2	53(44.2)	20(37.7)	33(62.3)	.28	38(31.7)	15(39.5)	23(60.5)	.27	42(35.0)	13(31.0)	29(69.0)	.79
≥2	67(55.8)	19(28.4)	48(71.6)		82(68.3)	24(29.3)	58(70.7)		78(65.0)	26(33.3)	52(66.7)	
E/M-CTC												
<3	63(52.5)	24(38.1)	39(61.9)	.17	40(33.3)	16(40.0)	24(60.0)	.22	54(45.0)	21(38.9)	33(61.1)	.18
≥3	57(47.5)	15(26.3)	42(73.7)		80(66.7)	23(28.8)	57(71.3)		66(55.0)	18(27.3)	48(72.7)	
M-CTC												
<1	89(74.2)	27(30.3)	62(69.7)	.39	66(55.0)	19(28.8)	47(71.2)	.34	74(61.7)	27(36.5)	47(63.5)	.24
≥1	31(25.8)	12(38.7)	19(61.3)		54(45.0)	20(37.0)	34(63.0)		46(38.3)	12(26.1)	34(73.9)	
F-CTC												
<1	44(36.7)	26(59.1)	18(40.9)	<.001	26(21.7)	9(34.6)	17(65.4)	.80	67(55.8)	25(37.3)	42(62.7)	.21
≥1	76(63.3)	13(17.1)	63(82.9)		94(78.3)	30(31.9)	64(68.1)		53(44.2)	14(26.4)	39(73.6)	
F-CTC^{E}												
<1	78(65.0)	28(35.9)	50(64.1)	.28	71(86.7)	28(39.4)	43(60.6)	.051	88(73.3)	33(37.5)	55(62.5)	.052
≥1	42(35.0)	11(26.2)	31(73.8)		49(13.3)	11(22.4)	38(77.6)		32(26.7)	6(18.8)	26(81.3)	
F-CTC$^{E/M}$												
<1	59(49.2)	28(47.5)	31(52.5)	.001	36(30.0)	11(30.6)	25(69.4)	.77	74(61.7)	27(36.5)	47(63.5)	.24
≥1	61(50.8)	11(18.0)	50(82.0)		84(70.0)	28(33.3)	56(66.7)		46(38.3)	12(26.1)	34(73.9)	
F-CTC^{M}												
<1	108(90.0)	37(34.3)	71(65.7)	.33	85(70.8)	26(30.6)	59(69.4)	.49	110(91.7)	36(32.7)	74(67.3)	1.0
≥1	12(10.0)	2(16.7)	10(83.3)		35(29.2)	13(37.1)	22(62.9)		10(8.3)	3(30.0)	7(70.0)	

Abbreviations: CTCs: circulating tumor cells; E-CTC: epithelial circulating tumor cells; E/M-CTC: biphenotypic epithelial/mesenchymal circulating tumor cells; M-CTC: mesenchymal circulating tumor cells; F-CTC: FTH1 gene-associated circulating tumor cells; F-CTC^{E}: FTH1 gene-associated epithelial circulating tumor cells; F-CTC$^{E/M}$: FTH1 gene-associated biphenotypic epithelial/mesenchymal circulating tumor cells; F-CTC^{M}: FTH1 gene-associated mesenchymal circulating tumor cells.

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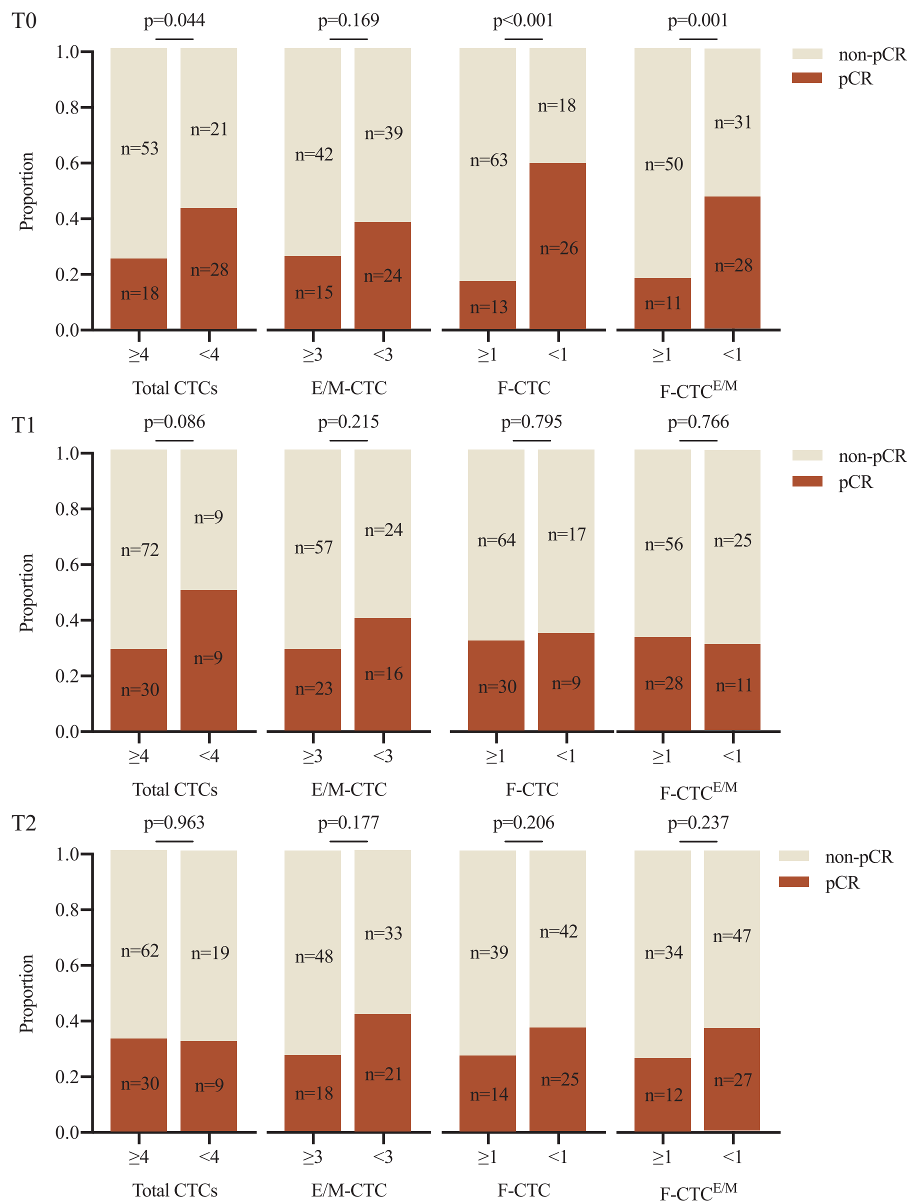

Figure 2. Association between CTCs and pCR rate. The association between the status of total CTCs, E/M-CTC, F-CTC, F-CTC$^{E/M}$ and pCR rate at T0, T1, and T2.

Association Between CTCs and BCS

Among 120 patients, 87 patients (72.5%) were diagnosed as stage IIB-IIIC disease and deemed unsuitable candidates for BCS prior to systemic treatment. These patients were anticipated to require NAC for tumor downstaging. Eighty-seven patients received the appropriate surgical procedure after NAC, of which 43 patients (49.4%) underwent BCS. As shown in Fig. 4, the status changes of total CTCs, E/M-CTC, F-CTC, and F-CTC$^{E/M}$ at T1 were not associated with the BCS rate. We further explored whether the change in CTCs status at T2 affected the BCS rate. A total of 51 patients had F-CTC ≥1 at T0, and 20 patients had decreased F-CTC at T2, 5 of whom underwent BCS. BCS was performed in 19 of 31 patients with no reduction in F-CTC. The BCS rate of patients with decreased F-CTC at T2 was significantly higher than that of patients with no reduction (61% vs 25%, P = .011). Such a relationship was not presented in total CTCs, E/M-CTC, and F-CTC$^{E/M}$.

The results of the univariate analysis suggested that patients with menopause (OR = 0.32, 95% CI, 0.12-0.82, P = .02), tumor size >50 mm (OR = 0.15, 95% CI, 0.05-0.42, P < .001) and clinical lymph node stages 2-3 (OR = 0.28, 95% CI, 0.09-0.87, P = .03) were less likely to receive BCS, while patients with decreased F-CTC at T2 were more likely to receive BCS (OR = 4.75, 95% CI, 1.37-16.47, P = .01). When these 3 factors were included in multivariate analysis, tumor size (OR = 0.18, 95% CI, 0.04-0.75, P = .02) and the change in preoperative F-CTC status (OR = 4.54, 95% CI, 1.14-18.08, P = .03) remained significant (Table 4).

Association Between CTCs and EFS

With a median time of 13 months (interquartile range: 10-16), 3 patients developed chest wall metastases, 2 patients had bone metastases, and 1 patient had adrenal metastases. The Kaplan-Meier survival curves for total CTCs, E/M-CTC, F-CTC, and F-CTC$^{E/M}$ status at T0 and

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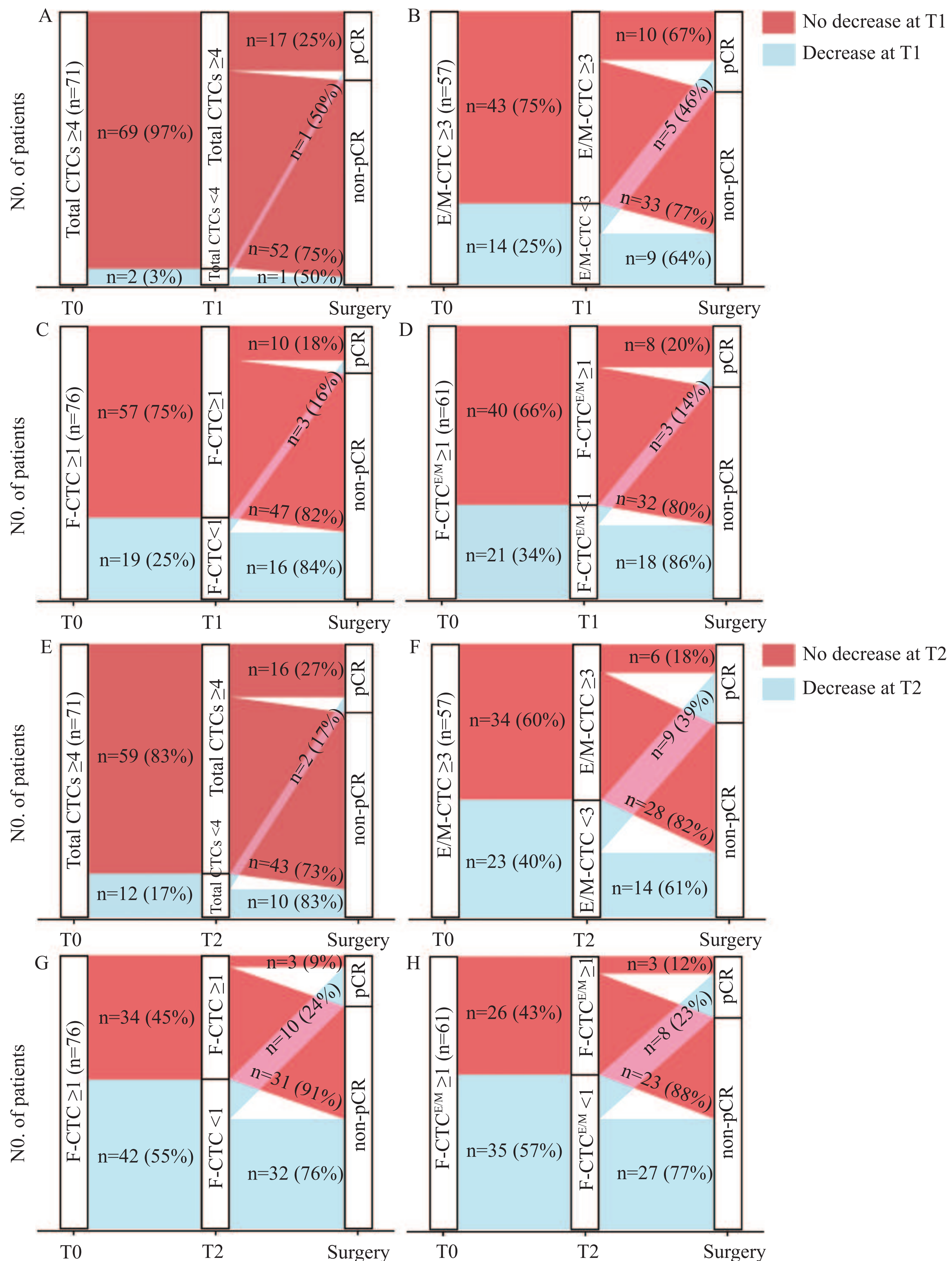

Figure 3. Association between changes in CTCs numbers and pCR rate at T1 and T2. (**A-D**) Differences in pCR rate among quantitative changes in total CTCs, E/M-CTC, F-CTC, F-CTCE/M at T1. (**E-H**) Differences in pCR rate among quantitative changes in total CTCs, E/M-CTC, F-CTC, F-CTCE/M at T2.

the corresponding dynamic changes at T1 and T2 with EFS were presented in Supplementary Fig. S1. We have not yet identified factors affecting EFS, which may be due to the short follow-up time.

Discussion

In this prospective, observational cohort study, we explored the relationship between the F-CTC and the efficacy of NAC in patients with non-metastatic breast cancer. We found that

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Table 3. Univariate and multivariate logistic regression of variables correlated with the rate of pCR.

Variables	Univariable analysis		Multivariable analysis	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Age: ≤50 vs >50	0.89 (0.39-2.02)	.78	/	/
Menstrual status: premenopausal vs postmenopausal	0.69 (0.29-1.62)	.39	/	/
Tumor size: cT1-2 vs cT3-4	0.59 (0.23-1.52)	.27	/	/
Lymph nodes: cN0-1 vs cN2-3	0.70 (0.23-2.12)	.53	/	/
HR status: negative vs positive	0.28 (0.12-0.66)	.004	0.23(0.08-0.68)	.008
HER2 status: negative vs positive	8.94 (3.67-21.80)	<.001	7.53(2.75-20.61)	<.001
Ki-67 level: <14% vs ≥14%	1.46 (0.15-14.52)	.75	/	/
Total CTCs at T0: <4 vs ≥4	0.45 (0.21-0.99)	.046	0.75(0.24-2.35)	.75
E/M-CTC at T0: <3 vs ≥3	0.14 (0.06-0.33)	<.001	0.16(0.03-0.95)	.043
F-CTC at T0: <1 vs ≥1	0.58 (0.27-1.26)	.17	/	/
F-$CTC^{E/M}$ at T0: <1 vs ≥1	0.24 (0.11-0.56)	.001	1.45(0.23-9.14)	.69
Total CTCs at T1: <4 vs ≥4	0.42 (0.15-1.15)	.09	/	/
E/M-CTC at T1: <3 vs ≥3	0.89 (0.35-2.22)	.80	/	/
F-CTC at T1: <1 vs ≥1	0.61 (0.27-1.34)	.22	/	/
F-$CTC^{E/M}$ at T1: <1 vs ≥1	1.14 (0.49-2.64)	.77	/	/
Total CTCs at T2: <4 vs ≥4	1.02 (0.41-2.53)	.96	/	/
E/M-CTC at T2: <3 vs ≥3	0.60 (0.28-1.32)	.21	/	/
F-CTC at T2: <1 vs ≥1	0.61 (0.24-1.52)	.29	/	/
F-$CTC^{E/M}$ at T2: <1 vs ≥1	0.61 (0.27-1.38)	.24	/	/
Dynamic of total CTCs at T1: no decrease vs decrease	3.06 (0.18-51.59)	.44	/	/
Dynamic of E/M-CTC at T1: no decrease vs decrease	0.88 (0.22-3.61)	.86	/	/
Dynamic of F-CTC at T1: no decrease vs decrease	1.83 (0.50-6.74)	.36	/	/
Dynamic of F-$CTC^{E/M}$ at T1: no decrease vs decrease	0.94 (0.28-3.19)	.93	/	/
Dynamic of total CTCs at T2: no decrease vs decrease	0.54 (0.11-2.73)	.45	/	/
Dynamic of E/M-CTC at T2: no decrease vs decrease	3.23 (0.81-12.86)	.10	/	/
Dynamic of F-CTC at T2: no decrease vs decrease	3.00 (0·89-10.12)	.08	/	/
Dynamic of F-$CTC^{E/M}$ at T2: no decrease vs decrease	2.27 (0.54-9.58)	.26	/	/

Abbreviations: pCR: pathological complete response; HR: hormone receptor; HER2: human epidermal growth factor receptor 2; CTCs: circulating tumor cells; E-CTC: epithelial circulating tumor cells; E/M-CTC: biphenotypic epithelial/mesenchymal circulating tumor cells; M-CTC: mesenchymal circulating tumor cells; F-CTC: FTH1 gene-associated circulating tumor cells; F-CTC^{E}: FTH1 gene-associated epithelial circulating tumor cells; F-$CTC^{E/M}$: FTH1 gene-associated biphenotypic epithelial/mesenchymal circulating tumor cells; F-CTC^{M}: FTH1 gene-associated mesenchymal circulating tumor cells; OR: odds ratio; CI: confidence interval.

F-CTC ≥1 before NAC was an independent factor for pCR and decreased F-CTC after NAC was an independent factor for BCS. The effect of FTH1 gene on prognosis will be further explored after extending follow-up time.

Previous studies have demonstrated that CTCs number is an independent factor for the prognosis of patients with breast cancer, but no clear correlation has been found with pCR.[6] Researchers can explore the heterogeneity of MRD by detecting types of CTCs using in situ fluorescence hybridization. In this study, we found the number of F-CTC at T0 was an independent factor for pCR rate. We believe that the presence of F-CTC reflects the high expression of the FTH1 gene in primary breast tumors. The high expression of the FTH1 gene enhances the cell's ability to chelate ferrous iron and blocks the accumulation of lipid ROS, thereby inhibiting iron death,[26,27] which may be part of the reason for drug resistance. Therefore, patients with the presence of F-CTC are less sensitive to chemotherapy and anti-HER2-targeted therapy and have difficulty in achieving pCR.

Studies have shown that CTCs expressing mesenchymal markers has stronger migratory and invasive abilities,[28,29] which led us to wonder if it is related to the efficacy of NAC. Unfortunately, E/M-CTC screened by EMT markers were not found to affect the pCR rate. Further clinical research needs to explore whether it will affect the efficacy of metastatic breast cancer.

In addition, changes in the number of CTCs did not affect pCR rate neither at T1 nor T2. A previous cohort study found elevated numbers of CTCs in patients with early-stage breast cancer who had undergone several cycles of NAC.[30] This can be partly explained that chemotherapy could stimulate the release of tumor cells and cause the fluctuations in CTCs count during NAC. Thus, the number change could not accurately reflect the condition of the primary tumor, suggesting that the baseline CTCs status may have a more significant impact on the efficacy of NAC.

Compared with mastectomy, BCS has better cosmetic effect and less effect on patients' life quality and psychological status.[31] This study found that, in addition to tumor size, change in the number of F-CTC after NAC also significantly affected the BCS rate. It may be explained that primary tumors in patients with decreased F-CTC may release ferrous iron by downregulating FTH1, which promotes the accumulation of ROS and lipoid

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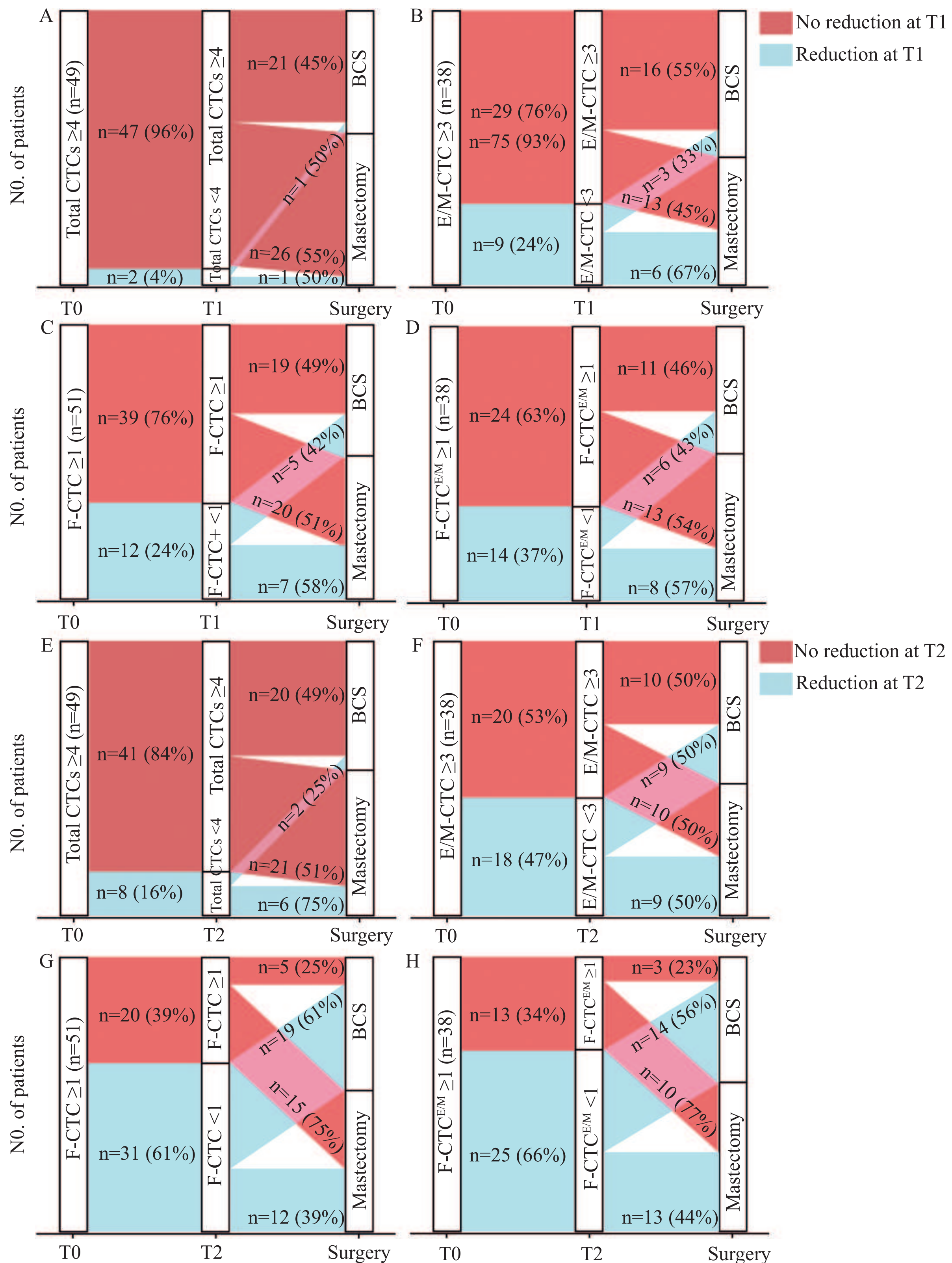

Figure 4. Association between changes in CTCs numbers and BCS rate at T1 and T2 in patients with locally advanced breast cancer. (**A**-**D**) Differences in BCS rate among quantitative changes in total CTCs, E/M-CTC, F-CTC, F-CTCE/M at T1. (**E**-**H**) Differences in BCS rate among quantitative changes in total CTCs, E/M-CTC, F-CTC, F-CTCE/M at T2.

oxidation products via the Fenton reaction, leading to ferroptosis. Therefore, patients with decreased number of F-CTC achieved satisfactory local tumor reduction and higher BCS rate.

In our study, the status of each CTCs at T0 and the dynamic changes at T1 or T2 were not found to affect EFS by log-rank test. This may be due to the relatively short follow-up period.

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Table 4. Univariate and multivariate logistic regression of variables correlated with the BCS rate in patients with locally advanced breast cancer.

Variables	Univariable analysis		Multivariable analysis	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Age: ≤50 vs >50	0.63 (0.27-1.52)	.30	/	/
Menstrual status: premenopausal vs postmenopausal	0.32 (0.12-0.82)	.02	0.60(0.16-2.28)	.45
Tumor size: cT1-2 vs cT3-4	0.15 (0.05-0.42)	<.001	0.18 (0.04-0.75)	.02
Lymph nodes: cN0-1 vs cN2-3	0.28 (0.09-0.87)	.03	0.43 (0.08-2.23)	.31
HR status: negative vs positive	0.42 (0.15-1.12)	.08	/	/
HER2 status: negative vs positive	0.79 (0.34-1.84)	.59	/	/
Total CTCs at T0: <4 vs ≥4	0.66 (0.28-1.55)	.34	/	/
E/M-CTC at T0: <3 vs ≥3	1.04 (0.45-2.43)	.93	/	/
F-CTC at T0: <1 vs ≥1	0.80 (0.34-1.87)	.60	/	/
F-CTC$^{E/M}$ at T0: <1 vs ≥1	0.72 (0.31-1.68)	.44	/	/
Total CTCs at T1: <4 vs ≥4	0.97 (0.33-2.88)	.96	/	/
E/M-CTC at T1: <3 vs ≥3	2.15 (0.88-5.25)	.09	/	/
F-CTC at T1: <1 vs ≥1	0.85 (0.31-2.35)	.75	/	/
F-CTC$^{E/M}$ at T1: <1 vs ≥1	1.66 (0.66-4.17)	.28	/	/
Total CTCs at T2: <4 vs ≥4	1.11 (0.40-3.08)	.84	/	/
E/M-CTC at T2: <3 vs ≥3	1.26 (0.54-2.92)	.59	/	/
F-CTC at T2: <1 vs ≥1	0.44 (0.18-1.05)	.06	/	/
F-CTC$^{E/M}$ at T2: <1 vs ≥1	0.69 (0.28-1.68)	.41	/	/
Dynamic of total CTCs at T1: no decrease vs decrease	1.24 (0.07-21.00)	.88	/	/
Dynamic of E/M-CTC at T1: No decrease *vs*. Decrease	0.58 (0.13-2.51)	.46	/	/
Dynamic of F-CTC at T1: No decrease *vs*. Decrease	0.75 (0.20-2.78)	.67	/	/
Dynamic of F-CTC$^{E/M}$ at T1: No decrease *vs*. Decrease	0.89 (0.24-3.35)	.86	/	/
Dynamic of total CTCs at T2: no decrease vs.s decrease	0.35 (0.06-1.94)	.23	/	/
Dynamic of E/M-CTC at T2: no decrease vs. decrease	1.00 (0.28-3.57)	1.00	/	/
Dynamic of F-CTC at T2: no decrease vs. decrease	4.75 (1.37-16.47)	.01	4.54 (1.14-18.08)	.03
Dynamic of F-CTC$^{E/M}$ at T2: no decrease vs. decrease	4.24 (0.94-19.26)	.06	/	/

Abbreviation: BCS: breast-conserving surgery; HR: hormone receptor; HER2: human epidermal growth factor receptor 2; CTCs: circulating tumor cells; E-CTC: epithelial circulating tumor cells; E/M-CTC: biphenotypic epithelial/mesenchymal circulating tumor cells; M-CTC: mesenchymal circulating tumor cells; F-CTC: FTH1 gene-associated circulating tumor cells; F-CTC^{E}: FTH1 gene-associated epithelial circulating tumor cells; F-CTC$^{E/M}$: FTH1 gene-associated biphenotypic epithelial/mesenchymal circulating tumor cells; F-CTC^{M}: FTH1 gene-associated mesenchymal circulating tumor cells; OR: odds ratio; CI: confidence interval.

Thus, we will extend the duration of follow-up to explore the impact of F-CTC on EFS.

In this study, FTH1 was innovatively selected as a biomarker for CTCs classification, providing a noninvasive detection method to show the relationship with the efficacy of NAC. Whether patients with F-CTC before NAC need an intensive chemotherapy regimen deserves further consideration. For those patients whose number of F-CTC did not decrease after NAC, we may need to pay attention to wider surgical margins in BCS and may need to prepare for reconstruction in advance. Meanwhile, further studies exploring the clear mechanism of these subtypes of CTCs during chemotherapy will also provide new ideas for the development of corresponding targeted drugs.

Several limitations exist in this study. First, this was a single-center clinical study with potential selection bias. Second, the follow-up time is relatively short, and further survival analysis will be carried out in the future. In addition, unlike circulating tumor DNA (ctDNA), which mainly carries information at the DNA level, CTCs also carries information at RNA level, protein level, and cellular characteristics.[32] The information provided by ctDNA and CTCs is complementary. CTCs testing is relatively inexpensive and has a higher degree of patient acceptance in clinical application. There are ongoing ctDNA studies at our center (ChiCTR2100048870) to explore molecular markers that can be used to predict efficacy of breast cancer treatment.

In conclusion, the F-CTC is associated with the efficacy of NAC. It shows promise that early response prediction of NAC treatment by FTH1 gene in patients with non-metastatic breast cancer may facilitate a timely personalized treatment to improve patients' long-term prognosis.

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Ethics Approval

The study was approved by the ethics committee of Sun Yat-sen Memorial Hospital (SYSEC-KY-KS-2021-103). Written informed consent was obtained from all participants in the study.

Conflict of Interest

The authors indicated no financial relationships.

Author Contributions

Conception/design: S.J., Y.Y., Y.Z., C.G. Provision of study material or patients: W.Y., X.F., J.G., W.T., Y.X., Y.C. Collection and/or assembly of data: S.J., W.Y. Data analysis and interpretation: S.J., Y.Y., Y.Z. Manuscript writing: S.J., Y.Y., Y.Z., L.L., Y.W., A.H., M.M., W.J., C.G. Final approval of manuscript: All authors.

Data Availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Supplementary material

Supplementary material is available at *The Oncologist* online.

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