Circulating Tumor Cells and Cancer Stem Cells: Clinical Implications in Nonmetastatic Breast Cancer

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ABSTRACT

PURPOSE: Despite the therapeutic advances, disease recurrence remains an ever-present threat to the health and well-being of breast cancer survivors. Assessment of circulating tumor cells (CTCs) and cancer stem cells (CSCs) during and after treatment may be of value in refining treatment.

METHODS: Three 5 mL blood samples were taken from each patient: the first, at diagnosis; the second, after completion of neoadjuvant anthracyclin-based chemotherapy; and the third, a month after surgery and completion of adjuvant radiotherapy. The absolute numbers of CTCs were identified as CD45$^-$cytokeratin$^+$ cells. CTCs per 5 mL of blood were determined by recording all events in the whole suspension. CSCs were identified as cytokeratin$^+$CD44$^+$CD24$^-$/CD45$^-$ cells. The CSCs were expressed as a percentage of CTCs.

RESULTS: Univariate analysis identified the measurements of baseline CTCs and CSCs, taken after chemotherapy and one month after the cessation of radiotherapy, as prognostic factors for both four-year disease-free survival and four-year overall survival. Multivariable analysis identified the third measurement of CSCs, taken one month after the completion of radiotherapy, as the only independent prognostic factor for the four-year disease-free survival ($P < 0.002$, hazard ratio [HR] = 1.231, 95% CI 1.077–1.407). The initial CTC measurement was the one factor that reached significance on multivariate analysis ($P < 0.03$, HR 1.969, 95% CI 1.092–3.551) for the four-year overall survival. Correlation was higher between CTC and CSC counts at diagnosis ($r = 0.654$, $P < 0.001$) than after chemotherapy ($r = 0.317$, $P < 0.03$), because of the more rapid decrease in the mean CTC count with chemotherapy.

CONCLUSION: The CTC count could be suitable as one of the measures for monitoring response to chemotherapy, while persistence of CSC after cessation of the treatment of nonmetastatic breast cancer, except hormonal therapy when indicated, may be a reason to consider additional therapy in the future. These findings need confirmation in larger randomized trials.

KEYWORDS: nonmetastatic breast cancer, CTC, CD44$^+$CD24$^-$ CSC

Introduction

Breast cancer (BC) is the most common cancer among women.[1] Although the prognosis of nonmetastatic BC is generally good, a significant proportion (20%–40%) of chemotherapy-treated BC patients develop metastatic disease.[2] The identification of BC patients who will relapse is not yet well established. It is becoming increasingly accepted that metastatic disease is initiated by circulating tumor cells (CTCs) that originate from the primary tumor and spread the cancer in the body via the blood circulatory system.[3] The presence of CTCs is commonly associated with an increased risk of metastases.[4,5] The detection of CTCs in BC has been performed in patients with diseases ranging from ductal carcinoma in situ to metastatic BC,[6–11] and their detection is generally associated with a poor prognosis. Previous studies have shown that the detection of CTCs after the completion of treatment is a prognostic marker.[9,12–15] However, it must be noted that considerable variation has been reported in CTC detection rates and correlation with prognosis,[16] and, to date, this limits the routine use of CTC count as a prognostic marker.

CTCs can exist in intermediate states expressing both epithelial and mesenchymal markers to varying degrees.[17–21] Subpopulations of these tumor cells at any point may acquire cancer stem cell (CSC) attributes such as quiescence, self-renewal, asymmetric division, drug resistance,[19,22] and radiation resistance.[23] After radiation therapy, a greater fraction of CSCs is observed[24] in BC cell lines; thus, it is proposed that hypofractionated irradiation may be more effective for the control of tumors containing these cells.[25] CSC markers have been identified in BC CTC populations by a number of researchers.[26–29] It was concluded in a recent review article that further studies assessing the clinical utility of CTCs at different stages of treatment as well as looking at CTC subpopulations, including the presence of CSC populations and their alterations with treatment, are needed.[30] In addition, the link between CTC subsets and the clinical outcome warrants

further evaluation.[31] The aim of this study was to assess the clinical impact of the detection of CTCs and CSCs in the peripheral blood of nonmetastatic BC patients measured before and at different stages of treatment.

Methods

This prospective study was conducted from January 2011 to January 2015. Eligible patients were women with nonmetastatic BC stages II to III (T2–T4, N0–N3, and M0) and were aged 18 years or older who have not previously received chemotherapy or hormonal therapy. The patients were required to have an Eastern Cooperative Oncology Group performance status (ECOG PS) of 0–2. The research was approved by the Institutional Review Board of the South Egypt Cancer Institute and conducted in accordance with the principles of the Declaration of Helsinki. Patients gave their written, informed consent to participate in this research.

Flow cytometric detection of CTCs and CSCs. The CSC and CTC counts were assessed in three blood samples from each patient. They were detected by fluorescein isothiocyanate-conjugated pan-cytokeratin, phycoerythrin-conjugated CD24, peridinium-chlorophyll-protein-conjugated CD45, and allophycocyanin-conjugated CD44. All monoclonal antibodies were purchased from BD Biosciences. After discarding the first 1 mL of blood to avoid potential contamination with skin epithelial cells, 5 mL of blood were collected. Following lysis

of erythrocytes in the 5 mL blood sample, the cell suspension was incubated for 20 minutes in the dark with 10 µL of CD24, CD45, and CD44. After that, the cells were washed with phosphate-buffered saline (PBS), followed by the addition of fixative solution to fix the cells and incubation for 15 minutes. The cells were then washed with PBS, followed by the addition of permeabilizing solution and 10 µL of pan-cytokeratin and incubation for 15 minutes at room temperature. After washing with PBS, the cells were ready for analysis. Flow cytometric analysis was done using the FACSCalibur flow cytometry with CellQuest software (BD Biosciences). Antihuman IgG was used as an isotype-matched negative control for each sample. The absolute numbers of CTCs were identified as CD45⁻ cytokeratin⁺ cells. CTCs per 5 mL of blood were determined by recording all events in the whole suspension. CSCs were identified as cytokeratin⁺CD44⁺CD24⁻/CD45⁻ cells (Fig. 1). The CSCs were expressed as a percentage of CTCs.

Treatment. Patients received six cycles of neoadjuvant anthracycline-based chemotherapy in one of the following two regimens: FAC (fluorouracil 500 mg/m², adriamycin 50 mg/m², and cyclophosphamide 500 mg/m² every 21 days) and FEC (fluorouracil 500 mg/m², epirubicin 100 mg/m², and cyclophosphamide 500 mg/m² every 21 days).

All patients received adjuvant radiotherapy for one or more of the following indications: conservative breast surgery, lymph node positive disease and or tumor 5 cm or more.

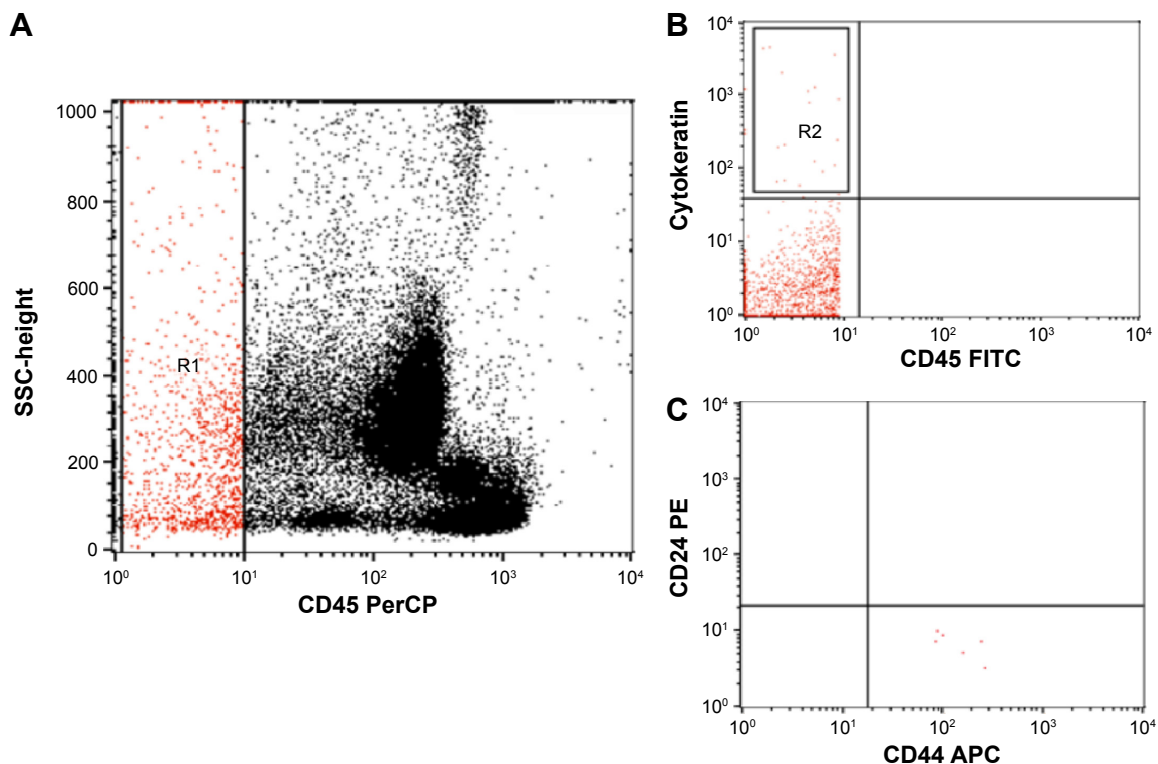**Figure 1.** Flow cytometric detection of circulating tumor cells (CTCs) and circulating CSCs. (**A**) CD45 and side scatter histogram were used to select the CD45⁻ cells (R1). (**B**) The expression of cytokeratin in CD45⁻ cells (R1) was detected. CTCs were defined as cytokeratin⁺CD45⁻ (R2). (**C**) The CTCs were further gated to detect CSCs, which are identified as cytokeratin⁺CD44⁺CD24⁻ CD45⁻ cells.

A hypofractionated accelerated schedule was used, in which the whole breast received 42.5 Gy in 16 fractions of 2.66 Gy each and the lumpectomy site received a 12 Gy electron boost divided in 16 fractions of 0.75 Gy, each given concomitantly over 3.2 weeks. At simulation, all patients underwent computed tomography to generate a three-dimensional plan. The planning target volume (PTV) included the extent of the breast volume as identified on computed tomography, excluding a 0.5-cm skin thickness. The boost PTV was identified using the lumpectomy cavity seroma and/or surgical clips. If the tumor bed seroma was not easily palpated and the surgical clips were not found, a 3- to 4-cm margin was placed parallel to the surgical scar with a 1-cm margin at the ends of the scar to define the boost PTV. The heart and lung were also contoured. Two tangential wedged fields for the whole breast and a matched supraclavicular field was added when indicated. An en face electron field for the boost volume prescribed at the 90% isodose line was given to all patients.

Postmastectomy radiotherapy was similarly given in two tangential fields and a matching supraclavicular field when needed for 42.5 Gy in 16 fractions. The radiation therapy plan was evaluated using a dose–volume histogram. V95 and V107 were defined as the volumes that received 95% and 107% of the prescribed dose, respectively. After the completion of radiotherapy, hormonal therapy was started by either tamoxifen or aromatase inhibitors or a combination for hormonal-positive tumors. Follow-up was every three months for the first year, then every six months for two years, and then yearly thereafter.

Statistical analysis. Data analysis was performed using the statistical package for social sciences, version 17. The Kaplan–Meier method for the generation of actuarial survival curves was used to assess progression free survival (PFS) and overall survival (OS). The effect of each clinicopathological factor on survival was compared using the log-rank test. Multivariable analysis for PFS and OS included the statistically significant factors by the Kaplan–Meier method and was performed using the Cox regression proportional hazards model. Receiver operating characteristic analysis was used to define the optimum cutoff for each group of CTC and CSC measurements. The two-tailed paired T test was used to compare each CTC and CSC measurement with the subsequent one and correlate them. Pearson's correlation was used to assess the relation between CTC and CSC counts during the course of treatment. The P value was considered as statistically significant if <0.05.

Results

This study included 51 eligible patients. The characteristics of patients and tumors are shown in Table 1. Twenty-one (41.2%) patients were under the age of 50 years, while the rest were 50 years or older. The majority of the patients, 34 (66.7%), had an ECOG PS of 1. Almost half of the patients, 24 (47.1%), had T2 tumors. Grade 2 was the most common tumor grading with 38 patients (74.5%) having it. Thirty-two patients

Table 1. The characteristics of patients and tumors.

CHARACTERISTIC	NO. (%)
Age	
<50	21 (41.2)
≥50	30 (58.8)
ECOG PS	
0	7 (13.7)
1	34 (66.7)
2	10 (19.6)
T stage	
1	None
2	24 (47.1)
3	12 (23.5)
4	15 (29.4)
Grade	
1	2 (3.9)
2	38 (74.5)
3	11 (21.6)
Lymph node metastases	
Negative	32 (62.7)
Positive	19 (37.3)
Estrogen receptor	
Negative	22 (43.1)
Positive	29 (56.9)
Progesterone receptor	
Negative	32 (62.7)
Positive	19 (37.3)
Her2-neu	
Negative	34 (66.7)
Positive	12 (23.5)
Unknown	5 (9.8)
Menopausal status	
Premenopausal	21 (41.2)
Postmenopausal	30 (58.8)

(62.7%) were lymph node negative. Estrogen, progesterone, and Her2-neu receptor status were positive in 29 (56.9%), 19 (37.3%), and 12 patients (23.5%), respectively.

The range, mean, and median of CTC and CSC counts for each sample are shown in Table 2. By receiver operating

Table 2. The CTC and CSC counts.

CELL COUNT	RANGE	MEAN ± SEM	MEDIAN
CTC baseline	0.5–10.0	3.9157 ± 0.34897	3.4
CTC after chemotherapy	0.2–8.0	2.2608 ± 0.29766	1.6
CTC after radiotherapy	0.1–9.40	2.0784 ± 0.2985	1.0
CSC baseline	4.5–67.0	27.2941 ± 2.4179	23.0
CSC after chemotherapy	4.0–60.0	24.0294 ± 2.258	19.0
CSC after radiotherapy	3.9–61.0	21.984 ± 2.3285	17.0

characteristic analysis, the optimal cutoff for high and low values for the measurements of CTCs in the 5 mL of blood were 4.5, 3.1, and 2.75 for the first, second, and third measurements, respectively, while for the measurements of CSCs, the values were 26.5%, 24%, and 21.75% for the first, second, and third measurements respectively. The four-year PFS for the entire group was 60.7%, while the four-year OS was 75.7%. A univariate analysis of factors possibly affecting the four-year disease-free survival (DFS) and the 4-year OS is shown in Table 3. A statistically significant difference in the four-year DFS was found according to the age ($P < 0.004$), progesterone receptor positivity ($P < 0.02$), menopausal status ($P < 0.004$), baseline CTC count, CTC count after chemotherapy, CTC count after surgery and chemotherapy, baseline CSC count, CSC count after chemotherapy, and CSC count after surgery and chemotherapy ($P < 0.001$). Factors affecting the four-year OS were progesterone receptor positivity ($P < 0.03$), baseline CTC count, CTC count after chemotherapy ($P < 0.001$), CTC count after surgery and radiotherapy ($P < 0.02$), baseline CSC count, CSC count after chemotherapy, and CSC after surgery and radiotherapy ($P < 0.001$). By multivariate analysis, however, the third measurement of CSCs, taken one month after the cessation of radiotherapy, was the only independent prognostic factor for DFS ($P < 0.002$, HR = 1.231, 95% CI 1.077–1.407; Fig. 2). For OS, the one independent prognostic factor was the initial CTC count ($P < 0.03$, HR 1.969, 95% CI 1.092–3.551; Fig. 3).

Correlation was done to assess the relations between every two consecutive measurements of CTCs and CSCs (Table 4). The first and second measurements of CTCs were significantly correlated ($r = 0.730$, $P < 0.001$); however, they were less closely correlated than the second and third measurements ($r = 0.927$, $P < 0.001$), indicating that the greatest change in CTC count occurred after chemotherapy, more than that which occurred after radiotherapy and surgery. Serial CSC counts were also highly correlated with r being 0.986 for the first and second measurements and 0.978 for the second and third measurements ($P < 0.001$), indicating less of a change between measurements. When correlation was done to determine how closely related the CTC and CSC counts were, it was found that they were most closely related initially ($r = 0.654$, $P < 0.001$; Fig. 4), more than in the sample after chemotherapy ($r = 0.317$, $P < 0.03$; Fig. 5), or after surgery and radiotherapy ($r = 0.368$, $P < 0.008$), which may be due to that the CTC count decreased more noticeably than the CSC count with treatment, especially after chemotherapy.

Discussion

Conventional therapeutic approaches (chemotherapy and radiotherapy) as well as most of the current targeted therapies are based on the intention to target tumor cells using maximum tolerated doses. The failure of these treatments to eradicate the disease, in a substantial number of patients,

Table 3. Univariate analysis for the effect of clinicopathological factors on DFS and OS.

FACTOR	4-YEAR PFS, %	P VALUE	4-YEAR OS, %	P VALUE
Age				
<50	38.1	0.004	71.4	0.304
≥50	76.7		80.0	
ECOG PS				
0	80	0.708	57.1	0.137
1	58.3		85.3	
2	60.0		60.0	
T stage				
2	54.2	0.228	75.0	0.792
3	83.3		83.3	
4	53.3		76.5	
Grade				
1	100	0.372	100	0.656
2	55.3		73.7	
3	72.7		81.8	
Lymph node metastases				
Negative	72.7	0.415	90.9	0.246
Positive	57.5		72.5	
Estrogen receptor				
Negative	54.5	0.409	63.6	0.075
Positive	65.5		86.2	
Progesterone receptor				
Negative	46.9	0.014	65.6	0.023
Positive	84.2		94.7	
Her2-neu				
Negative	67.6	0.290	85.3	0.116
Positive	50.0		58.3	
Unknown	40.0		60	
Menopausal status				
Premenopausal	38.1	0.004	71.4	0.304
Postmenopausal	76.7		80.0	
Baseline CTC count				
Low	81.8	0.001	90.9	<0.001
High	22.2		50.0	
CTC count after chemotherapy				
Low	79.5	<0.001	93.9	<0.001
High	0.0		44.4	
CTC count after surgery and radiotherapy				
Low	71.4	<0.001	91.9	<0.02
High	11.1		25.0	
Baseline CSC count				
Low	87.1	<0.001	93.4	<0.001
High	20.0		47.7	
CSC count after chemotherapy				
Low	80.8	<0.001	94.2	<0.001
High	18.8		32.8	
CSC count after surgery and radiotherapy				
Low	84.8	<0.001	97.0	<0.001
High	16.7		31.8	
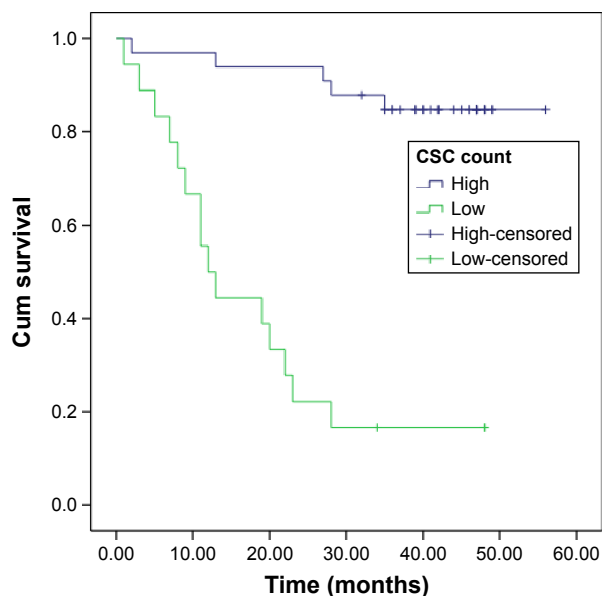
Figure 2. DFS according to the final CSC count.

Table 4. Correlation between serial measurements of CTCs and CSC and between measurements of CTC and CSC.

	R VALUE	*P* VALUE
Correlation for CTC counts		
Baseline and second measurements	0.730	<0.001
Second and third measurements	0.927	<0.001
Correlation for CSC counts		
Baseline and second measurements	0.986	<0.001
Second and third measurements	0.978	<0.001
Correlation for CTC and CSC counts		
Baseline measurements	0.654	<0.001
Second measurements	0.317	<0.03
Third measurements	0.368	<0.008

has renewed the interest in CSCs.[30] By definition, recurrence originates from residual treatment-resistant cells, which regenerate the BC phenotype. The existence of radiation-resistant subpopulations of tumor cells has been long proposed by radiobiologists.[22] How important understanding the tumor cell origin will be in improving BC outcome is still debatable, so further studies of these cells at different stages of treatment and their correlation with the outcome may aid in clarifying how they could possibly be incorporated in BC treatment and follow-up. In this study, we measured CTCs and CSCs before, during, and after the completion of treatment, except continuing hormonal treatment.
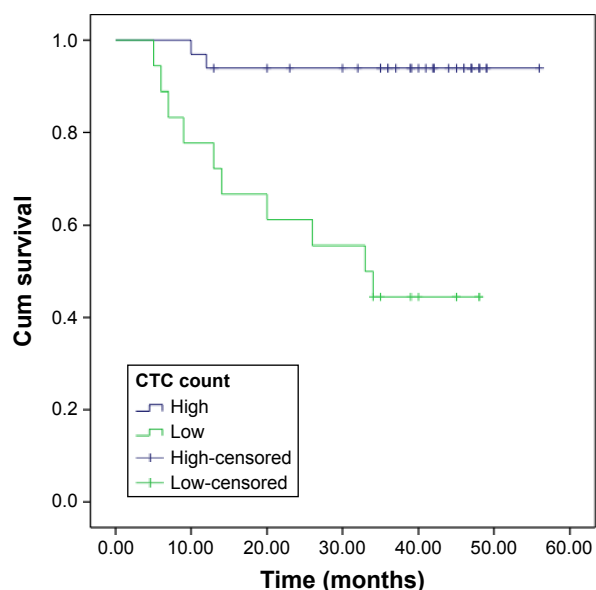
The prognostic value, observed in this study, of the CTC count at diagnosis being an independent prognostic factor for OS in nonmetastatic BC has been reported in numerous trials, and many of them were included in a meta-analysis by Zhang et al.[5] The pooled HR, for patients considered positive for CTCs, was reported to be 2.78 (95% CI 2.22–3.48, $P = 0.00$). In this study, the CTC count at diagnosis was a significant prognostic factor for DFS as well. This was similarly found in the meta-analysis in which the pooled HR, in 19 studies, for patients considered to have high CTCs, was 2.86 (95% CI, 2.19–3.75, $P = 0.00$). Therefore, the prognostic significance of the CTC count before treatment has been proven repeatedly; however, fewer trials have assessed its value during and after treatment.

In this study, the CTC count measured after chemotherapy was of prognostic significance for DFS and OS. Among the most important studies that reported the CTC count after chemotherapy, as well as before chemotherapy, and its relation with survival, is the Success trial[11] in which 2026 early BC patients participated. This trial proved that the persistence
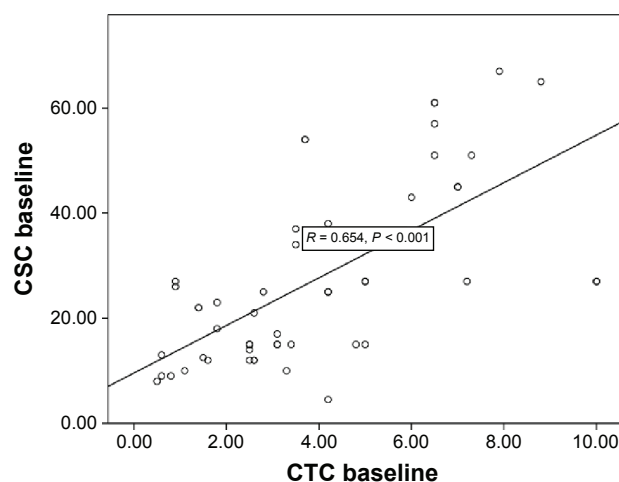

Figure 3. OS according to the initial CTC count.

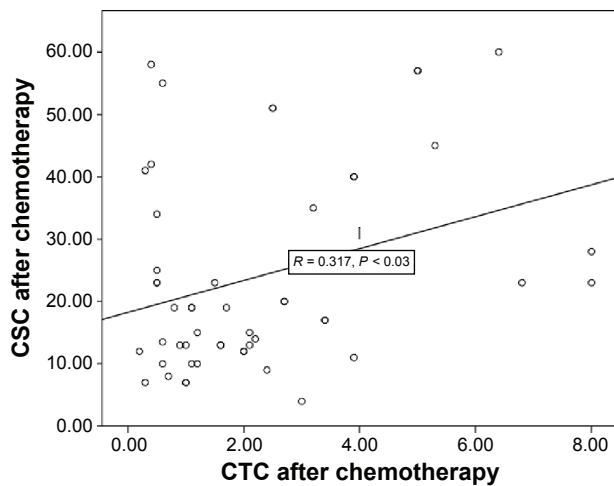**Figure 4.** Correlation between CTC and CSC counts at diagnosis.

Figure 5. Correlation between CTC and CSC counts after chemotherapy.

of CTCs after chemotherapy showed a negative influence on DFS (HR = 1.12, 95% CI 1.02–1.25, P = 0.02) and on OS (HR = 1.16, 95% CI 0.99–1.37) as well. The multivariable analysis conducted in the Success trial also proved the CTC count before chemotherapy to be an independent prognostic factor for both DFS (P < 0.0001) and OS (P < 0.002), similar to the present study.

Studies evaluating the effect of CD44$^+$/CD24$^-$ CSCs on clinical outcome are less common, especially those assessing them in the peripheral blood of the patients. These cells have been linked to worse BC-specific survival when studied in BC tissue in 1342 patients (P = 0.001).[32] The presence of CSC in the bone marrow was found to be an independent prognostic factor for DFS (HR = 15.8, P = 0.017) in a study conducted in MD Anderson.[33] In this study, three peripheral blood samples were taken from each patient and the presence of high CD44$^+$/CD24$^-$ CSC was associated with a decreased DFS and OS every time. The third sample taken in this study (after surgery and radiotherapy) was the factor that reached statistical significance on multivariate analysis for DFS. So the CSCs, which persisted after treatment, were the strongest factor linked to recurrence. Assessment of CSC after the completion of all treatments of BC, except for hormonal, may be one of the methods of identifying patients in need of further treatment.

The measured counts of CTCs and CSCs were the most highly correlated initially in this study, more than after chemotherapy or after surgery and radiotherapy. This could be due to that, in these patients, the mean change in the CTC count with treatment was greater than the mean decrease in the CSC count, most noticeable in the sample after chemotherapy, so CTC count may be more useful for response assessment, during and after chemotherapy because it decreased more rapidly than the CSC count. Consideration of switching to a different chemotherapy may be a future direction upon lack of response to a chemotherapy regimen assessed during

treatment. This strategy was tested in the SWOG0500 trial,[34] but for patients with metastatic BC, and although it showed no OS advantage for the patients who switched to a different cytotoxic regimen upon lack of decrease in the CTC count, this may not be true for nonmetastatic tumors and it may also not be true for all alternative chemotherapy regimens, so it should be tested during neoadjuvant chemotherapy for early-to-advanced BC with different cytotoxic regimens.

Conclusion

In the early- to high-risk nonmetastatic BC patients, involved in this study, the only independent prognostic factor for OS was the initial CTC count, and it was concluded to be more appropriate for monitoring response to therapy than the CSC count due to its more rapid decrease with chemotherapy. Persistent CSCs, however, were the independent prognostic factor for DFS, so their assessment may be used for the consideration of additional treatment in the future. These results need to be confirmed in larger trials.

Author Contributions

Conceived and designed the experiments: AMZ and MS. Analyzed the data: MS. Wrote the first draft of the article: MS. Contributed to the writing of the article: AMZ. Agreed with the article results and conclusions: MS, AMZ, MSFH, and DOM. Jointly developed the structure and arguments for the article: MS and AMZ. Made critical revisions and approved the final version: MS and AMZ. All authors reviewed and approved the final article.

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