The role of circulating tumor cells as a prognostic marker in the adjuvant setting of patients with breast cancer

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Received 23 December 2018
Revised 03 January 2019
Accepted 06 January 2019
Published 10 August 2020

Journal of Current Medical Research and Practice
2020, 5:241–247

Background
Still, there is no clinically reliable marker to detect micrometastasis or breast cancer relapse. This study aimed to evaluate the role of circulating tumor cells (CTCs) as a biomarker in patients with nonmetastatic breast cancer.

Patients and methods
CTC quantification was carried out using flow cytometry for 50 patients with breast cancer postoperatively: before starting, after three cycles, and at the end of adjuvant chemotherapy. The relationship between CTCs and other tumor characteristics and outcomes were studied.

Results
The median follow-up duration was 35 months. Before starting adjuvant chemotherapy, CTCs were positive (cutoff point ≥5/7.5 ml) in 36% of the patients and decreased to 20% after finishing chemotherapy (P = 0.04). CTCs were detected in 88.9% (n = 16 of 18) of node-positive patients and in 11.1% of node-negative patients (n = 2 of 18, P = 0.04). No significant association was found with tumor size, grading, or hormone receptor status. Distant metastasis was detected in 20% (n = 10 of 50) of patients and was significantly associated with CTCs more than or equal to 5 in 80% of them (n = 8 of 10) (P = 0.01). The presence of more than or equal to 5 CTCs at baseline was associated with a reduction in both the disease-free survival and overall survival (P < 0.001 and P = 0.003, respectively). Baseline CTCs more than or equal to 5/7.5 ml were confirmed as an independent prognostic factor in multivariate Cox hazard regression analysis for disease-free survival (hazard ratio = 3.71; 95% confidence interval = 1.62–8.48; P = 0.002) and overall survival (hazard ratio = 3.14; 95% confidence interval = 1.34–7.37; P = 0.009).

Conclusions
The current work suggested that the presence of more than or equal to 5 CTCs/7.5 ml at baseline would predict early disease recurrence and reduce the overall survival in patients with nonmetastatic breast cancer receiving adjuvant chemotherapy.

Keywords:
circulating tumor cells, early breast cancer, prognostic factors in breast cancer

J Curr Med Res Pract 5:241–247

2357-0121

Background

Breast cancer is the first cause of tumor-related death in women worldwide, which occurs primarily because of the onset of distant metastases [1]. Despite surgical and chemotherapeutic treatments, ~30%of patients with lymph node-negative axilla and ~50% of patients with positive axilla may relapse within 5 years [2]. There is no clinically useful method to detect micrometastases suitable for reliable monitoring, predicting relapse, and guiding drug selection. Current markers, for example, the CA 15–3, are not recommended for routine follow-up in an asymptomatic patient with no particular findings in clinical examinations [3]. It is therefore pivotal to look for new clinical prognostic and predictive tests that can help early identification of patients who are at a higher risk of relapse [4]. A sensitive and easily reproducible test to support clinicians in monitoring disease and treatment response with a stricter follow-up and in better clinical decision making could have a major effect on mortality and disease-free survival (DFS). The so-called liquid biopsy, a noninvasive, possibly periodical peripheral blood test, with the aim of finding cancer-related factors, has been considered capable of meeting all these clinical needs [5]. Circulating tumor cells (CTCs) are the spearhead of metastatic dissemination, a crucial element of the metastatic process, and potentially the starting point for liquid biopsy employment [6]. CTCs are detected in 10–60% of patients with stages 1–3 nonmetastatic breast cancer, suggesting that occult dissemination can happen early in disease progression and more

frequently in patients with metastatic disease, with a prevalence of 70% [7,8].

This study is designed to prospectively investigate the role of CTCs as a potential biomarker in nonmetastatic breast cancer and to find the correlation between baseline CTCs count before the receipt of adjuvant chemotherapy and other prognostic and predictive factors and the clinical outcome.

Patients and methods

Study design

A prospective study was conducted at Clinical Oncology Department, Assiut University Hospital, together with the collaboration of South Egypt Cancer Institute regarding laboratory work.

A total of 50 female patients with nonmetastatic breast cancer were enrolled between February 2014 and February 2015 and were followed up till February 2018 with a median follow-up time of 35 months.

This study took the approval of the local ethics committee, and all patients gave a written consent.

Inclusion criteria

The following were the inclusion criteria: female patients aged more than or equal to 18 years with histologically proven invasive breast cancer postoperatively with free metastatic workup, good performance status, and within normal laboratory functions and normal ejection fraction.

Exclusion criteria

The following were the exclusion criteria: a history of other malignancy, bilateral breast cancer, pregnant women, and the presence of a severe uncontrolled chronic disease.

Workup

Routine diagnostic workup was done in the form of baseline bilateral breast ultrasound to exclude contralateral breast cancer. Chest imaging and abdominal ultrasound were done to exclude metastasis, and if results are suspicious, MSCT of chest and pelviabdomen was done. Bone scan as done in case of bone pain or elevated alkaline phosphatase and mandatory in stage III disease. Baseline evaluation should include echocardiography to exclude ischemic changes and restricted ejection fraction. Hormonal receptor assays (estrogen receptor and progesterone receptor) and assessment of human epidermal growth factor receptor 2 (HER-2) neu status of the tumor were performed.

Treatment schedule

All patients had undergone either breast conservative surgery with axillary evacuation or modified radical mastectomy and were candidates for adjuvant treatment. All patients received adjuvant chemotherapy either: FEC chemotherapy regimen (fluorouracil 500 mg/m^2 intravenously, pharmorubicin 100 mg/m^2 intravenously, and cyclophosphamide 750 mg/m^2) every 3 weeks for six cycles or FAC (fluorouracil 500 mg/m^2, doxorubicin 50 mg/m^2, and cyclophosphamide 750 mg/m^2 intravenously) every 3 weeks for six cycles.

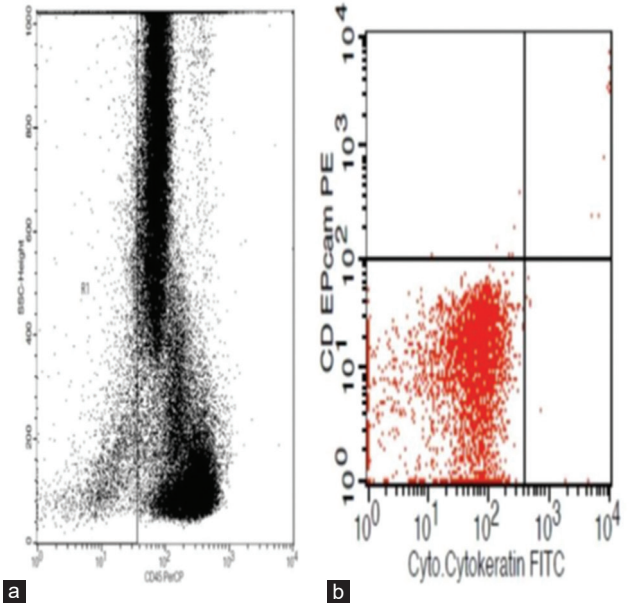
Enumeration of circulating tumor cells

Modification of the method by Hristozova *et al.* [9], was used for CTC identification, and counting was done by flow cytometry. Quantification of CTCs was done on three intervals before starting adjuvant chemotherapy, after three cycles, and after finishing adjuvant treatment (six cycles).

After discarding the first 1 ml of blood to avoid potential contamination with skin epithelial cells, peripheral blood samples (7.5 ml) were drawn from the patients, and EDTA was added (Fig. 1).

This was followed by lysis of the erythrocytes of the 7.5-ml blood, followed by incubation of

Figure 1



Flow cytometric detection of circulating tumor cells (CTCs). (a) CD45 and side scatter histogram was used to select the CD45 cells (R1). (b): The expressions of EPCAM and cytokeratin in CD45 and 8722 cells (R1) were detected. CTCs were defined as EPCAM + cytokeratin + CD45.

the cell suspension for 20 min in the dark with fluorescein isothiocyanate-labeled pan-cytokeratin and monoclonal antibody to CD326/EPCAM and peridinium chlorophyll-protein-labeled CD45. All monoclonal antibodies were purchased from Becton Dickinson Biosciences (San Jose, California, USA).

The suspension is then washed with phosphate buffered saline and then the cells became ready for analysis. Flow cytometric analysis was done by FACSCalibur flow cytometry with Cell Quest software (Becton Dickinson Biosciences). Anti-human IgG was used as an isotype-matched negative control for each sample. CTCs defined as EpCAM + cytokeratin + CD45 − were detected (Fig. 1).

This was followed by analysis of both the percentages and absolute counts of positive samples. Patients were divided according to baseline CTC count into two prognostic groups: the first group included patients with low CTC count (<5 cells/7.5 ml blood) whereas the second group included patients with high CTC count (≥5 cells/7.5 ml blood).

Clinicians and patients were not informed of the results of the CTC analysis.

Statistical analysis

Data were analyzed using a computer software package IBM SPSS, version 21, (IBM, Chicago, Illinois, USA), as follows:

Numerical data were summarized as mean ± SD, whereas nominal and categorical data were described as frequencies and percentages. Median follow-up and the 95% confidence interval (CI) were calculated using the Kaplan–Meier method. Survival curves were estimated with the Kaplan–Meier method and compared using the log-rank test. DFS was defined as the time elapsed between the end of primary treatment and date of distant or local relapse.

Overall survival (OS) was defined as the time elapsed between randomization and death from any cause including tumor-induced death.

A multivariate Cox model was constructed. Multivariate Cox analysis included clinical variables whatever their univariate Cox *P* value.

Results

Patient's characteristics of 50 patients with primary breast cancer are shown in Table 1. At the time of presentation, CTC analysis was done for all patients after the complete resection of the primary tumor and before the start of systemic treatment, with 32 (64%) patients having CTCs less than 5/7.5 ml and 18 (36%) patients having CTCs more than or equal to 5/7.5 ml.

Table 1 Multivariate Cox proportional hazard regression analysis for disease-free survival and overall survival for different variables

Variables	HR	95% CI	*P*
OS			
Menopausal status (pre)	2.238	1.154-4.343	0.017
CTC in blood (≥5)	3.711	1.624-8.484	0.002
Hormone receptor status (negative)	2.695	1.342-5.414	0.005
Pathological LN (positive)	6.014	1.935-14.124	<0.004
DFS			
Menopausal status (pre)	7.678	1.628-36.219	0.010
CTC in blood (≥5)	3.136	1.335-7.367	0.009
Hormone receptor status (negative)	2.985	1.395-6.385	0.005
Pathological LN (positive)	5.521	1.781-14.052	<0.001

CI, confidence interval; CTC, circulating tumor cell; DFS, disease-free survival; HR, hazard ratio; LN, lymph node; OS, overall survival.

The median follow-up time was 35 months.

The relationship between circulating tumor cells at baseline and clinicopathological factors

At baseline, the menopausal status of the patient, the mean tumor size, receptor status, and the molecular subtype showed no statistically significant difference between patients with CTCs count less than 5/7.5 ml and patients with CTCs more than or equal to 5/7.5 ml.

Regarding nodal status, patients with baseline CTCs more than or equal to 5/7.5 ml showed positive lymph node for malignancy in 88.9% of patients and negative lymph nodes in 11.1%, whereas patients with CTCs less than 5/7.5 ml showed positive lymph nodes in 68.7% and negative lymph nodes in 31.3%, which was statistically significant between the two groups ($P = 0.049$).

Moreover, the mean numbers of lymph nodes in patients with CTCs less than 5 were 3.13 ± 1.6 and in patients with CTCs more than or equal to 5/7.5 ml were 8 ± 4.7, with a statistically significant difference between the study groups ($P = 0.02$).

Stage II was found among 22.2% of patients with CTCs more than or equal to 5/7.5 ml at baseline, whereas it was found in 37.5% of patients having CTCs less than 5/7.5 ml. On the contrary, stage III was found among 77.8% of patients with CTCs more than or equal to 5/7.5 ml at baseline, whereas it was found in 62.5% of patients having CTCs less than 5/7.5 ml, and there was a statistically significant difference between the two groups ($P = 0.007$).

Treatment outcome

Patterns of local and distant metastasis

At a median follow-up period of 35 months, 10 (20%) patients experienced distant and local metastasis, whereas 40 (80%) patients were free of metastasis until the end of the follow-up period.

Regarding the site of relapse, 8% of them were in the liver, 4% in the lung, 4% in the bone, and 4% local recurrence.

The relation between circulating tumor cells and metastasis

At baseline, 80% of patients who developed metastasis had CTCs more than or equal to 5/7.5 ml, whereas only 20% of the patients who developed metastasis had CTCs less than 5/7.5 ml, with a statistically significant difference ($P = 0.01$).

Mid-cyclic assessment of CTCs was done, and 60% of patients with metastasis had CTCs more than or equal to 5/7.5 ml, and 40% of the patients with metastasis had CTCs less than 5/7.5 ml, and this difference was not statistically significant.

On the contrary, after treatment, most patients (60%) with CTCs more than or equal to 5/7.5 ml developed metastasis, whereas most of the patients (90%) having CTCs less than 5/7.5 ml showed no metastasis, with a statistically significant difference ($P = 0.002$).

Circulating tumor cell changes over the study period

The relation between CTC at baseline and after completion of adjuvant chemotherapy (Table 2).

At baseline, 64% of the patients had CTCs less than 5/7.5 ml and 36% of the patients had CTCs more than or equal to 5/7.5 ml, whereas after six cycles of systemic chemotherapy, only 20% of the patients had more than or equal to 5 CTCs, with a significant difference ($P = 0.04$).

The relation between changes in CTC levels (Fig. 2).

There is a strong negative correlation (–0.89) between changes in the CTC levels from baseline (A) and after three cycles of systemic chemotherapy (B), that is, A–B, and from this point and after completion of chemotherapy (C), that is, B–C, with a significant difference ($P = 0.001$).

Table 2 Circulating tumor cell analysis at baseline and after completion of adjuvant chemotherapy among cases

	CTC after completion of adjuvant chemotherapy [*n* (%)]		Total [*n* (%)]	*P*
	<5	≥5		
CTC at baseline				
<5	24 (60)	8 (80.0)	32 (64)	
≥5	16 (40)	2 (20.0)	18 (36)	
	40 (80)	10 (20)	50 (100)	0.040*

CTC, circulating tumor cell. *Fisher-Exact test was used

Survival analysis

DFS

(1) The relation between baseline CTC and DFS (Fig. 3):
Patients with CTCs less than 5/7.5 ml have a median DFS of 47 months, whereas patients with CTCs more than or equal to 5cells/7.5 ml have a median DFS of 44 months, with a statistically significant difference ($P < 0.001$)

(2) The relation between posttreatment CTC and DFS (Fig. 4):
Patients with CTCs less than 5 cells/7.5 ml have a median DFS of 47 months, whereas patients with CTCs more than or equal to 5 cells/5 ml have a median DFS of 45 months, with a statistically significant difference ($P < 0.04$).

OS:

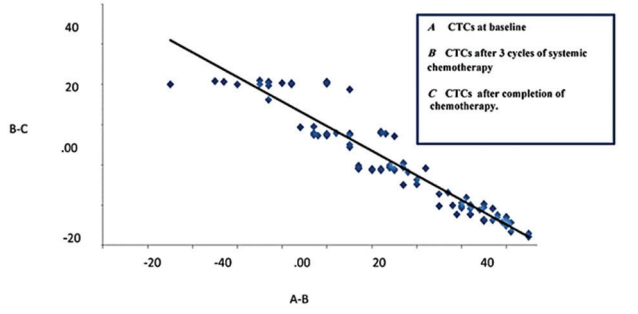
(1) The relation between baseline CTCs and OS (Fig. 5)
Patients with CTCs less than 5 cells/7.5 ml have a median OS of 47 months, whereas patients with CTCs more than or equal to 5 cells/5 ml have a median OS of 45 months, with a statistically significant difference ($P = 0.003$)

(2) The relation between posttreatment CTC and OS (Fig. 6)
Patients with CTCs less than 5 cells/7.5 ml have a median OS of 46 months, whereas patients with CTCs more than or equal to 5 cells/7.5 ml have a median OS of 44 months, with a statistically significant difference ($P < 0.05$).

Multivariate Cox regression analysis of survival

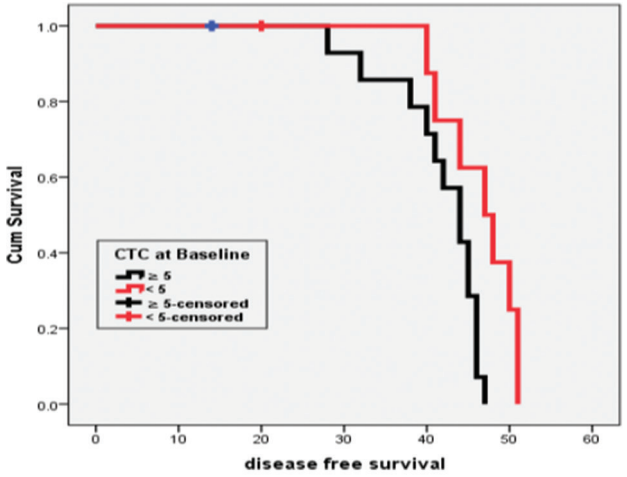
Table 3 shows the final Cox regression model, which contained four significant predictors: the menopausal status, CTCs more than or equal to 5, hormone receptor

Figure 2



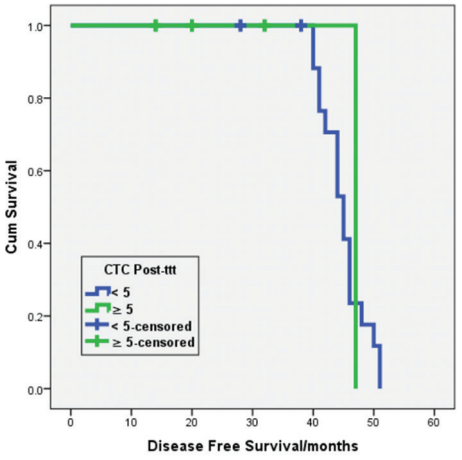
CTC level changes over the study period. CTC, circulating tumor cell.

Figure 3



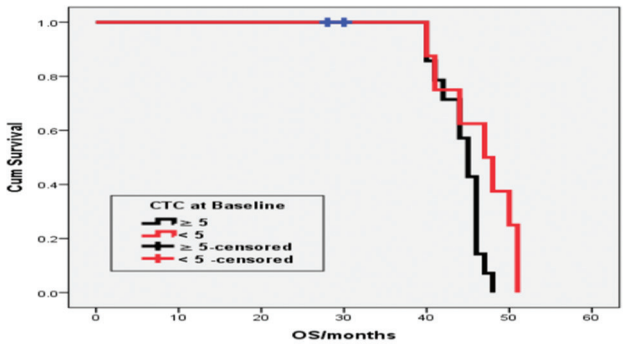
Effect of CTC at baseline on the DFS. CTC, circulating tumor cell; DFS, disease-free survival.

Figure 4



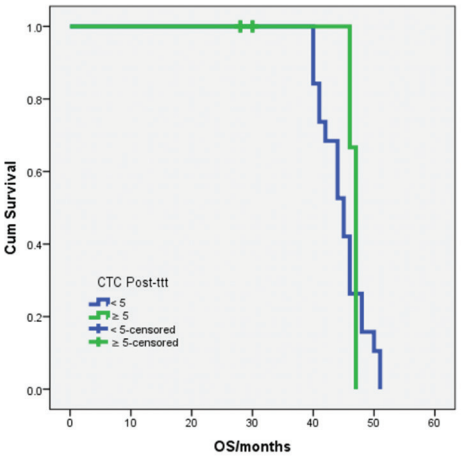
Effect of CTC after treatment on the DFS. CTC, circulating tumor cell; disease-free survival.

Figure 5



Effect of CTC at baseline on the overall survival (OS). CTC, circulating tumor cell.

Figure 6



Effect of CTC after treatment on the OS. CTC, circulating tumor cell; OS, overall survival.

status negative, and positive lymph node status. It was confirmed that CTCs more than or equal to 5/7.5 ml adjusted for other factors to be an independent prognostic factor for reduced DFS and OS with hazard ratio (HR) of 3.71 and 95% CI 1.624–8.484, in addition to premenopausal status, negative hormonal status, and pathological positive lymph node.

Discussion

A significant proportion of adequately treated patients with low tumor burden experience a relapse; in these patients, novel diagnostic tools to assess prognosis are necessary. CTC numbers correlate with clinical outcome in metastatic BC, and numerous trials have been launched to address this issue in early breast cancer [10].

In this study, baseline CTCs more than or equal to 5/7.5 ml was detected in 36% of cases postoperatively and before starting adjuvant chemotherapy. This result was comparable to that of Lang and colleagues, where 38% of patients had evidence of CTCs. In smaller cohorts, CTCs were reported in 31% of patients with T1 or T2 tumors [11,12]. On the contrary, the results of the German Success Group and REMAGUS 02 trial showed that CTCs were positive in 21.5 and 23%, respectively. However, they used a different cutoff point, which is the presence of at least one CTC in 7.5 ml of blood [10,13].

Although an increasing proportion of CTC-positive patients is seen at an advanced stage, a significant proportion of patients with early-stage disease shows the presence of CTCs. This implies that an advanced stage is not necessary for cancer cells to enter the circulation and spread. In this study, most patients with CTCs at baseline more than or equal to 5/7.5 ml were stage III. In another study done at MD Anderson by Karhade, the majority (65%) of patients in their study

Table 3 Patient's characteristics and the relationship between circulating tumor cells at baseline and other clinicopathological factors

	CTC at baseline		*P*
	<5 (*n*=32)	≥5 (*n*=18)	
Age (years)			
Mean±SD	49.3±9.1	51.3±7.8	0.265*
Median (range)	54 (35-65)	47 (35-65)	
Menopausal state [*n* (%)]			
Pre	16 (50)	14 (77.8)	0.176**
Post	16 (50)	4 (22.2)	
Tumor size (cm)			
Mean±SD	3.53±1.6	5.36±3.2	0.107*
Number of LNs			
Mean±SD	3.13±1.6	8.00±4.7	**0.028***
Nodal status [*n* (%)]			
Negative	10 (31.3)	2 (11.1)	**0.049*****
Positive	22 (68.7)	16 (88.9)	
Cancer stage [*n* (%)]			
II	12 (37.5)	4 (22.2)	**0.007****
III	20 (62.5)	14 (77.8)	
Receptor status [*n* (%)]			
ER +ve and PR +ve	12 (37.5)	8 (44.4)	1.000**
ER +ve and PR -ve	6 (18.8)	2 (11.1)	0.279**
ER -ve and PR +ve	4 (12.5)	2 (11.1)	0.544***
ER -ve and PR -ve	10 (31.3)	6 (33.3)	0.322**
HER-2 +ve	2 (6.25)	2 (11.11)	0.386***
Molecular subtype [*n* (%)]			
Luminal A	20 (62.5)	12 (66.7)	0.373**
Luminal B	2 (6.2)	0 (0)	
HER-2	0 (0)	2 (11.1)	
Triple negative	10 (31.3)	4 (22.2)	
Radiotherapy [*n* (%)]	30 (93.8)	16 (88.9)	0.528**
Hormonal treatment [*n* (%)]	22 (68.8)	12 (66.7)	0.561**

CTC, circulating tumor cell; ER, estrogen receptor; HER-2, human epidermal growth factor receptor 2; LN, lymph node; PR, progesterone receptor; *Mann-whitney test was used to compare the mean difference; **Fisher exact test was used to compare proportions; ***Significant

were early-stage (stages I and II) patients. Overall, 15% of stage I, 21% of stage II, and 32% (nine of 28) of stage III patients had CTCs [14].

In this study, the patients with positive lymph nodes were statistically significantly more often CTC-positive than node-negative patients. The same findings were present in the German Success Group, where N0 group was CTCs positive in 19.6%, and N1 group was CTCs positive in 22.4% ($P < 0.001$). On the contrary, the presence of CTC more than or equal to 5/7.5 ml was not statistically significantly associated with other clinicopathological characteristics like menopausal status, tumor size, hormonal receptor status, and HER-2 status. Moreover this result was comparable to that of Riethdorf *et al.* [15], Lucci *et al.* [16], and Rack *et al.* [10] who confirmed that the presence of one or more CTCs could not predict any of the standard tumor characteristics. However, another study by Lang *et al.* [11] found out that HER-2 status was the only factor that reliably predicted the presence of CTCs.

After completion of chemotherapy, CTCs more than or equal to 5/7.5 ml were detected in only 20% of the patients. This may be explained by that the number of CTCs was decreased on exposure to chemotherapeutic agents. However, assessment of CTCs after three cycles of systemic chemotherapy was not statistically significant. Another study was done by Daskalaki and colleagues in which paired samples of peripheral blood and bone marrow were obtained from 165 patients with stage I–III breast cancer before the initiation of adjuvant chemotherapy. In 84 patients, paired blood and bone marrow samples were also available after chemotherapy. The detection of CK-19 mRNA-positive CTCs and dissenated tumor cells (DTCs) was assessed by real-time PCR. CK-19 mRNA-positive CTCs and DTCs were detected in 55.2 and 57.6% of patients before chemotherapy, respectively. After chemotherapy, CTCs and DTCs were identified in 52.4% and 51.2% of the 84 patients, respectively. However, this was not statistically significant ($P = 0.169$) [17]. In the German Success Group, CTC analysis after completion of adjuvant chemotherapy was performed in a subgroup of 1492 patients. At this time point, CTCs were detected in 22.1% of the patients ($n = 330$ of 1493). There was no difference in CTC counts before and after chemotherapy [10].

In the current study, there was a significant relation between baseline and posttreatment CTCs count more than or equal to 5/7.5 ml and the occurrence of metastasis. This result was comparable to a study by Xenidis and colleagues where CTC-positive patients were significantly more at risk for developing a relapse than CTC-negative women. In another study by Rack and colleagues, the patients who developed local and distant metastasis were more frequently CTCs positive. Furthermore, a study by Zedan *et al.* [18] found that both mean baseline and mean posttreatment CTCs counts were significantly higher in relapsed patients than in nonrelapsed patients [10,19].

Our findings showed that the presence of more than or equal to 5/7.5 ml CTCs both at baseline and at the end of treatment predicts decreased DFS and OS in patients with primary nonmetastatic breast cancer receiving adjuvant chemotherapy. These results were also confirmed in the largest cohort trial (German Success), where CTCs were analyzed in 2026 patients before starting adjuvant chemotherapy and in 1492 patients after chemotherapy. The presence of CTCs was associated with poor DFS and OS. CTCs persistence after chemotherapy showed a negative influence on DFS and OS [10]. Another study by Lucci and colleagues, who evaluated CTCs at the time of definitive surgery from chemo-naive patients with

stages 1–3 breast cancer, found that detection of one or more CTCs predicted both decreased progression-free survival and OS. However, this study did not evaluate the posttreatment CTC count and its relation with survival [16].

Furthermore, we confirmed our results by doing multivariate Cox regression analysis and found that the presence of five or more CTCs showed great HRs for both relapse and death in patients with operable breast cancer. Higher numbers of CTCs carried HRs as prognostically powerful as lymph node metastasis. Other risk factors were estrogen receptor and progesterone receptor negative and premenopausal status.

Regarding this study, it was the first one done in our hospital to evaluate CTCs as a de novo prognostic marker in the early disease setting with regular monitoring of the CTC count throughout the treatment course; however, increased sample size is warranted for better understanding of the trends and conclusive multivariate analyses.

Conclusion

The current study postulated that identification of CTCs within the blood would independently predict shorter survival, irrespective of axillary lymph node status or standard tumor markers. If the presence of CTCs was to contribute independently to the currently available prognostic factors, this information might be useful in disease staging and in identifying patients who might benefit from additional adjuvant therapies. However, this will be answered in the upcoming years through future studies addressing this issue.

Acknowledgments

Funding was received from Faculty of Medicine, Assiut University.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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