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The role of Circulating Tumor Cells (CTCs) in Predicting the Response of Primary (Neoadjuvant) Chemotherapy and its Impact as a Prognostic Factor in Early Breast Cancer

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Abstract

Background: The enumeration of circulating tumor cells (CTCs) has long been regarded as an attractive diagnostic tool of malignancy, as these cells are thought to reflect aggressiveness of the tumor and may assist in therapeutic decisions in patients with solid malignancies. Primary or neoadjuvant chemotherapy (NACT) which was a standard of care in patients with inoperable locally advanced or inflammatory breast cancer, is now may be considered for patients with early breast cancer. Even though at early stages tumors are clinically restricted to loco-regional tissue, there is often early dissemination of viable tumor cells. One of the purposes of systemic NACT is to attack these circulating tumor cells. This fact has potentiated the interest in the use of NACT

Aim of the study: to detect and measure the count of CTCs in the blood of patients before starting "baseline CTCs count" and after finishing neoadjuvant chemotherapy "post-treatment CTCs count" for early (non-metastatic) breast cancer patients. In addition to determine the correlation between baseline CTCs count with relapse rate, other prognostic factors, disease free survival (DFS) and overall survival (OS).

Patients and methods: Forty patients with confirmed early non-MBC at South Egypt Cancer Institute were recruited to participate in this study with treatment protocols. All patients received three to four cycles of NACT either with AC (adriamycin and cyclophosphamide); FAC (fluorouracil, doxorubicin and cyclophosphamide) or FEC (fluorouracil, epirubicin and cyclophosphamide). CTCs count was measured using flowcytometry in all patients before starting treatment and in only 25 patients after the end of therapy. The study approved by the local ethics committee.

Results: There was statistically significant difference between baseline and post-treatment CTCs counts ($P<0.003$). Also, there was statistically significant difference between primary tumor size before and after NACT ($p=0.001$). Pathological complete response (pCR) rate was 55%. The mean baseline and post-treatment CTCs counts were significantly higher in patients who did not achieve pCR than in patients who achieved pCR ($P=0.001$ and 0.003 respectively). Patients were divided according to baseline CTCs count into 2 prognostic groups: the first group included patients with low CTCs count (<5 cells/ 5 ml blood) while the second group included patients with high CTCs count (≥5cells/ 5 ml blood). There was statistically significant higher relapse rate in the high CTCs count group compared to low CTCs count group ($P<0.001$). Also, DFS and OS were significantly shorter in the high CTCs count group compared to low CTCs count group ($P=0.001$ and 0.008 respectively). There was no significant correlation between baseline CTCs count and other prognostic factors.

Conclusion: CTCs count in breast cancer patients before starting neoadjuvant chemotherapy could predict response to neoadjuvant chemotherapy. High CTCs count is associated with an increased risk of disease recurrence or relapse and shortened DFS and OS. We should consider detection on a large scale and more standardization of the methodology.

Background

The outcome of breast cancer largely depends on the development of metastases in the course of the disease. Given this vital importance of metastases, the detection and monitoring of their existence are continuously sought for. The detection of circulating tumor cells (CTCs) is one field of research focusing on a new method to detect metastatic disease earlier, less invasive and more reliably than currently available conventional methods, such as clinical presentation, radiographic evaluation and serum tumor markers do [1].

Historically, most cancer research has been focused on studying the biology of either primary tumors or metastases. However, the intermediate steps of the process, including events such as cell departure from the tumor mass, intravasation, lymphatic and circulatory dissemination and extravasation, have been less studied. There has been an increasing interest in understanding thoroughly all processes involved in the metastatic cascade, including the transit journey of tumor cells in the circulatory and lymphatic systems [1].The systemic nature of breast cancer is characterized by the migration of tumor cells even at early stages of the disease when the primary tumor shows a relatively small size [2]. CTCs are defined as tumor cells circulating in the peripheral blood of patients, shed from either the primary tumor or its metastases. Thus, CTCs in peripheral blood could be regarded as the pre-stadium of clinically manifest distant metastases [3, 4]. Moreover, it is acknowledged that a thorough understanding of the biology of CTCs may open new paths for the future development of potential anticancer strategies [5, 6].A considerable number of studies have been accomplished on the determination of CTCs as a prognostic and/or predictive biomarker for different types of cancers [7].

Primary or neoadjuvant chemotherapy (NACT), initially used only in non-resectable breast cancer to reduce the tumor's size [8], is now also an option in resectable tumors [9, 10]. For some patients affected with primary breast cancer, the standard of care is systemic neoadjuvant therapy, followed by surgical resection of the malignant tissue. NACT may result in local tumor regression or even in a complete tumor response, which may directly influence the surgical procedure of choice, going from radical mastectomy to some type of breast-conserving surgery – without risking patient survival [11]**.** Even though at early stages tumors are clinically restricted to loco-regional tissue, there is often early dissemination of viable tumor cells. One of the purposes of systemic NACT is to attack these circulating tumor cells. This fact has potentiated the interest in the use of NACT [12]. In early non-metastatic breast cancer, however, it remains necessary to construct more studies to correlate the level of CTCs with overall survival (OS) and with disease-free disease (DFS).

We designed our study to assess the potential role of CTCs in prediction of response to NACT and risk of relapse and death in a cohort of patients with early breast cancer.

Patients and Methods

Patient population: Forty patients with confirmed early non-metastatic breast cancer treated at the South Egypt Cancer Institute were recruited to participate in this study after approval of the local ethics committee and patient consent. Patients were diagnosed between 2011 and 2014.

Inclusion criteria: female patients aged ≥18 years with histologically proven invasive non-metastatic breast cancer (stages II and III; T2-T4, N0-N3, M0) according to the American Joint Committee on Cancer and the International Union for Cancer Control (AJCC-UICC) TNM breast cancer staging system. ECOG performance state 1 or 2, adequate hematological, renal, cardiac and hepatic functions.

Exclusion criteria: included prior treatment with any anti-cancer agent, women who were pregnant, lactating or refuse effective contraception, secondary malignancy, history of another primary malignant disease, active infection, any other concomitant severe clinical condition making implementation of the treatment difficult. Administration of other cytotoxic, hormonal agents or radiation therapy was not permitted during the study, with the exception of contraceptives, corticosteroids given as antiemetic treatment or growth factors for neutropenic patients.

Work-up: The routine diagnostic work-up included clinical examination, breast ultrasonography to detect tumor size before and after NACT, needle tumor biopsy, chest x-rays, abdominal ultrasound, bone scan, blood sampling for complete blood count, renal and hepatic functions. Estrogen and progesterone status was determined and the cut-off used to define hormone receptor positivity was 1% of stained cells.

Treatment Schedule: All patients received three to four cycles of standard anthracycline-based neoadjuvant chemotherapy regimens including AC (doxorubicin 50 mg/m^2 and cyclophosphamide 500 mg/m^2) (q 21days); FAC (fluorouracil 500 mg/m^2, adriamycin 50 mg/m^2 and cyclophosphamide 500 mg/m^2) (q 21days) or FEC (fluorouracil 500 mg/m^2, epirubicin 100 mg/m^2, and cyclophosphamide 500 mg/m^2) (q 21days).

After NACT, all patients underwent definitive surgery either modified radical mastectomy or breast conservation as indicated. Surgical breast and axillary node resection specimens were evaluated for pathologic tumor response. Patients who had no remaining invasive cancer in the breast and who were lymph node negative were considered to have pathological complete response (pCR).

Surgery was followed by another two to three cycles of the same regimen used in the neoadjuvant setting to complete full course of six cycles of chemotherapy, after which post-operative radiotherapy was given followed by adjuvant hormonal therapy (tamoxifen or aromatase inhibitors) in case of hormonal positive tumors.

CTCs detection: CTCs were detected by modification of the method of Hristozova et al, 2011[13]. CTC identification and counting were done by flowcytometry (figure 1). After discarding the first 1ml of blood to avoid potential contamination with skin epithelial cells, peripheral blood samples (5ml). After lysis of erythrocytes of the 5ml blood, the cell suspension was incubated for 20 minutes in dark with fluorescein isothiocyanate (FITC) labeled pan-cytokeratin, phycoerythrin (PE) ladeled CD66 and peridinium-chlorophyll-protein (Per-CP) labeled CD45. All monoclonal antibodies were purchased from Becton Dickinson (BD) Biosciences, San Jose, USA. After wash with phosphate buffered saline (PBS), the cells were ready for analysis. Flowcytometric analysis was done by FACSCalibur flowcytometry with Cell Quest software (BD Biosciences). Anti-human IgG was used as an isotype-matched negative control for each sample. The absolute numbers of CTCs per 5 ml blood were deremined by recording all events in the whole suspension.

CTCs count was determined before starting treatment and three to four weeks after the end of NACT (before surgery). Neither the patients nor the clinicians were informed of the results of CTCs analysis. Patients were divided according to baseline CTCs count into 2 prognostic groups: the first group included patients with low CTC count (<5 cells/ 5 ml blood) while the second group included patients with high CTC count (≥5cells/ 5 ml blood).

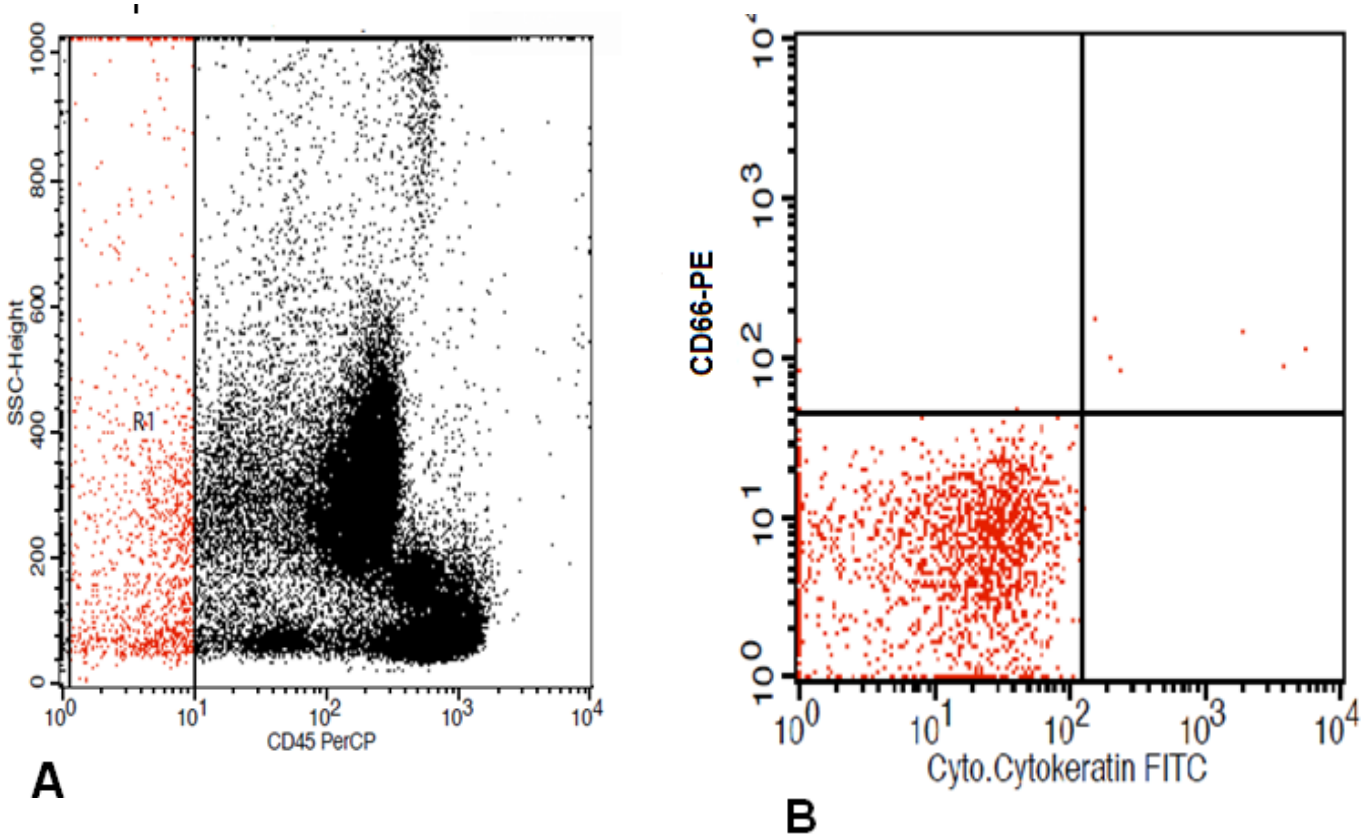

Figure (1): Flowcytometric detection of circulating tumor cells (CTCs)
A: CD45 and side scatter histogram was used to select the CD45- cells (R1).
B: The expression of CD66 and cytokeratin in CD45− cells (R1) was detected.
CTCs defined as CD66+cytokeratin+CD45

Statistical Analysis

Data obtained from all enrolled patients using the statistical package for social sciences (SPSS) Software version 18 (Chicago. USA). DFS was calculated from the date of surgery to the date of relapse or last follow up. OS was calculated from the date of surgery to the date of death from any cause or last follow up. Survival curve was estimated with the Kaplan-Meier method and compared using the log-rank test. A multivariate cox model was constructed. Multivariate-cox analysis included all relevant clinical variables whatever their univariate cox p-values, namely: age, menopausal status, hormonal receptors, tumor size, lymph node status and grade. Probability (p-) values equal or less than 0.05 were considered statistically significant.

Results

Patients' characteristics:

The median age of the patients at the time of diagnosis of breast cancer was 50 years (24-75) and 60% of the patients were postmenopausal. The

histopathologic subtype of breast cancer for all patients was invasive duct carcinoma (IDC). 57.5 % were estrogen receptor positive and 40% were progesterone receptor positive. 70% of cases were of grade 2, 25% were grade 3 while grade 1 was found in the remaining 5%. 82.5% of study patients were lymph node positive (table 1).

Table (1): Patients' and Tumors' characteristics of the whole group of patients included in this study

Item	No (%)
Menopausal	
pre	16 (40.0%)
post	24(60.0 %)
Estrogen receptors	17(42.5 %)
Negative	23(57.5%)
positive	
Progestron receptors	24(60.0%)
Negative	16(40.0%)
Positive	
Tumor size	
T1	---
T2	18 (40.0%)
T3	11(27.5%)
T4	11(27.5%)
Grade	
G1	2(5.0%)
G2	28(70.0%)
G3	10(25.0%)
Lymph Node	
negative	9(22.5%)
positive	31(77.5%)

Circulating tumor cells detection and counting:

Baseline CTCs count was measured in all the 40 patients with a mean level of (4.068±2.4086)cells/5ml (rang 0 – 10). Post-treatment CTCs count was measured only in 25 patients with a mean level of (2.896±2.0479)cells/5ml (rang 0 – 8) with statistically significant difference between CTC count before and after NACT (P<0.003). Regarding baseline CTCs count, a cut off value of 5 circulating tumor cells per 5 ml of blood was chosen to classify patients into low CTCs count group (<5 cells/ 5 ml blood) (no=28) (70%) and high CTCs count group (≥5 cells/ 5 ml blood) (no=12) (30%).

Response to NACT

Pre-treatment mean tumor size (in cm) was 8.1800 ±4.48739, which was statistically significantly higher than a mean post-treatment size of 1.9560 ±2.12232 (P=0.001). Also, we found negative correlation between the decrease in primary tumor size and the baseline CTCs counts before NACT (r = - 0.4, P=0.05) (figure 2).

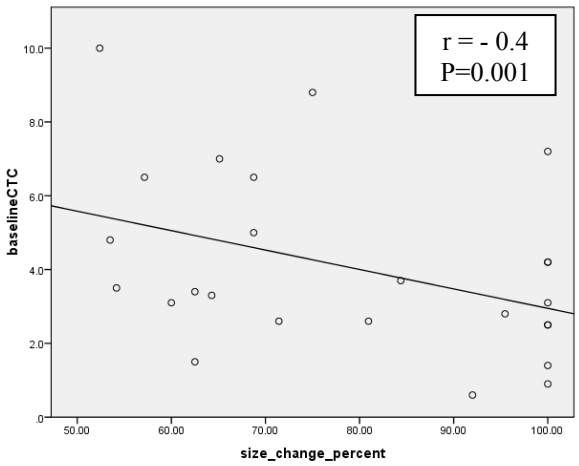

Figure (2): Correlation between baseline CTCs count and tumor size change percent

Twenty-two of the 40 patients (55%) showed pCR with a mean baseline CTCs count of 2.714±1.8491, while 18/40 with no pCR had a mean baseline CTCs count of 5.172±2.4757, and the difference between the mean CTCs counts between the two patients groups was statistically significant (P=0.001). Also, the mean post-treatment CTCs count in patients achieving pCR was 1.500±.8692, which was significantly lower than that in patients not achieving pCR (3.827±2.0937) (P=0.003).

Table (2): Patterns of disease progression or relapse in patients of study group

Item	No (%)
• Good	26(65.0%)
• Relapse	14(35.0%)
• Death	7(17.5%)
Site of relapse	
• Bone	5(12.5%)
• Liver	4(10%)
• Lung	6(15%)
• Brain	2(5%)
• L. N	1(2.5%)
• Local recurrence	3(7.5%)

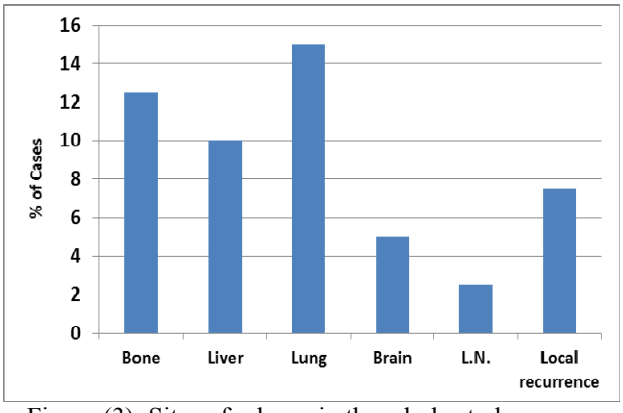

Figure (3): Sites of relapse in the whole study group

Relapse rate and its relation to prognostic factors:

The median follow up period for all patients was 20 months during which follow up of patients was done clinically using breast examination and imaging.

Fourteen patients (35%) relapsed, of them seven patients (17.5%) died. Sites of relapse included lung (15%), bone (12.5%), liver (10%), local recurrence (7.5%), brain (5%) and lymph node (2.5%) (figure 3 and table 2). There was no statistically significant effect of any of known prognostic factors on relapse rate except menopausal status and progesterone receptor status (*P*= 0.034 and 0.0149 respectively) (table 3). Both mean baseline and mean post-treatment CTCs counts were significantly higher in relapsed patients than in non-relapsed patients (*P*<0.001 for both) (table 4).

Table (3): Clinico-pathological characteristics and their relation to relapse rate

Item	No relapse "n=26" No (%)	Relapse "n=14" No (%)	P value
Menopausal			
Pre	7(26.92%)	9(64.29%)	0.034*
Post	19(73.08%)	5(35.71%)	
ER			
Negative	10(38.46%)	7(50.00%)	0.205
Positive	16(61.54%)	7(50.00%)	n.s
PgR			
Negative	12(46.15%)	12(85.71%)	0.0149*
Positive	14 (53.85%)	2(14.29%)	
Tumor Size			
T2	11(42.31%)	7(50.00%)	
T3	9(34.62%)	2(14.29%)	0.473
T4	6(23.07%)	5(35.71%)	n.s
Grade			
G1	2(7.70%)	---	
G2	17(65.38%)	1(78.57%)	0.432
G3	7(26.92%)	3(21.43%)	n.s
Lymph Node			
Negative	7(26.92%)	2(14.29%)	0.406
Positive	19(73.08%)	12(85.71%)	n.s

* Statistically significant, n.s not significant

Table (4) Correlation between mean baseline and post-treatment CTCs count with relapse

CTCs	No relapse (n=26) Mean ± SD	Relapse (n=14) Mean ± SD	P-value
Baseline CTC	2.32±1.52	5.84±1.99	P<0.001
Post-treatment CTC	1.52±0.77	4.64±1.8	P<0.001

The study included 28 patients with low baseline CTCs count and 12 patients with high baseline CTCs count. There was statistically significant higher relapse rate in the high CTCs count group (9/12, 75%) compared to low CTCs count group (5/28, 18%) (*P* <0.001). 5/9 (55%) of the relapsed patients in high CTCs count group died compared to 2/5 (40%) patients in the low CTCs count group.

Survival analysis:

The mean DFS and OS for all patients were 25 and 28.5 months respectively (figure 4, 5). DFS and OS for patients with high baseline CTCs count were significantly shorter than patients with low CTC count (*P*=0.001 and 0.008 respectively) (figure 6, 7). Among clinicopathologic factors, only menopausal status and progesterone receptor status significantly affected DFS (*P*=0.023 and 0.026 respectively) (figure 8, 9), while no factor affected OS.

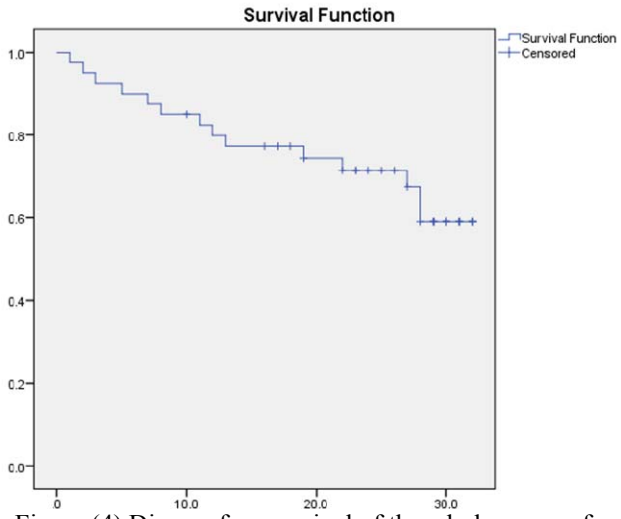

Figure (4) Disease free survival of the whole group of patients

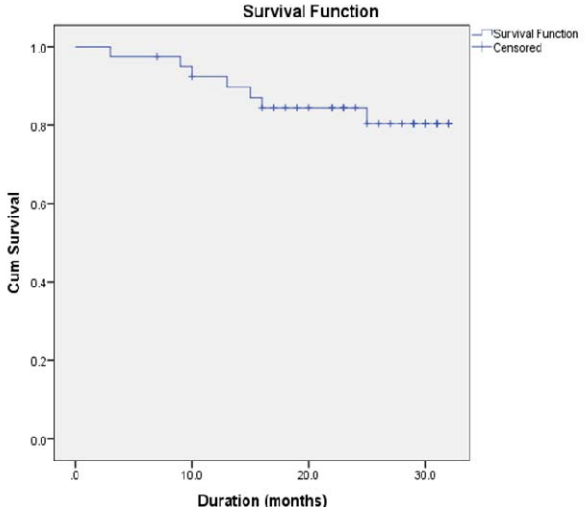

Figure (5) overall survival of the whole group of patients

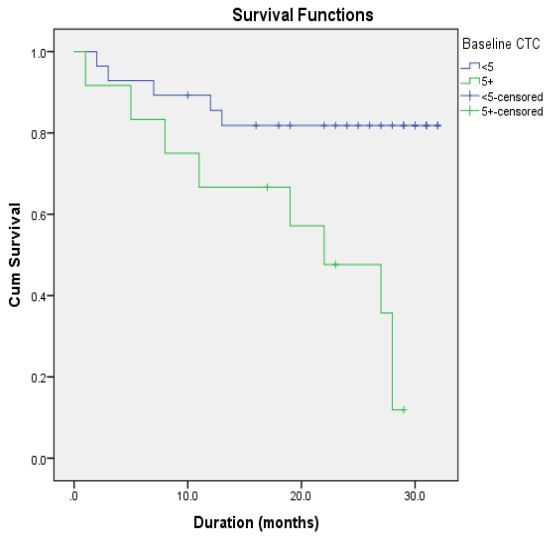

Figure (6) Correlation between baseline CTCs count and DFS

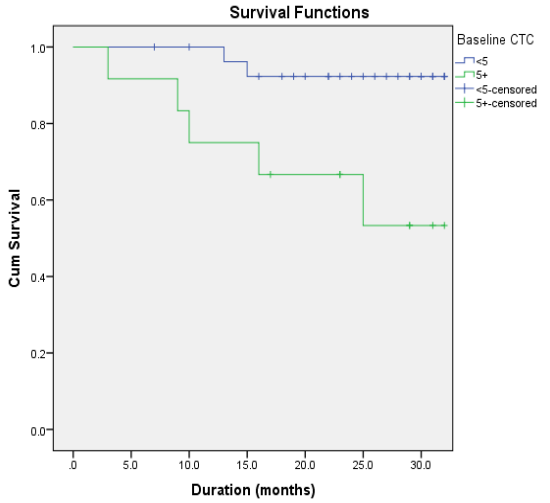

Figure (7) Correlation between baseline CTCs count and OS

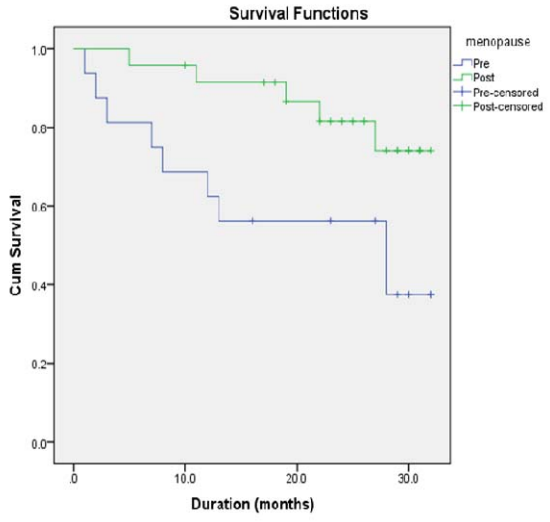

Figure (8): Correlation between menopausal status and DFS

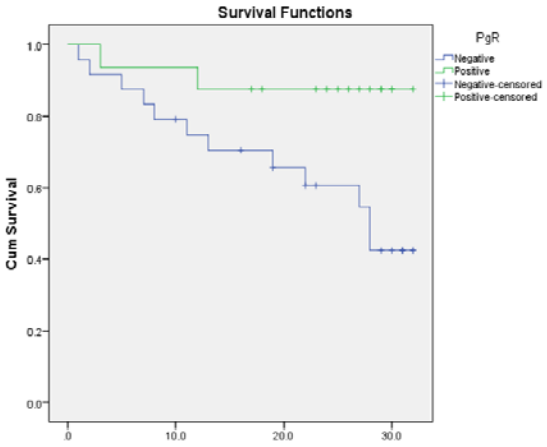

Figure (9): Correlation between progesterone receptor status and DFS

Discussion

CTCs can be detected in many patients with solid tumors but rarely in healthy subjects [14]. CTCs are generally defined as nucleated cells lacking CD45 and expressing cytokeratin [15]. In the current study, 12/40 (30%) presented with base-line high CTCs count (≥5 cells/5ml blood) compared to only 5/25 patients (20%) after the end of NACT. ***Riethdorf et al, 2010*** [16] in the GepartQuatro neoadjuvant study, found that only 5% of pre-operative (post-NACT) samples carried ≥5 CTCs/sample. ***Rack et al, 2007*** [17] evaluated 1767 patients for the presence of CTCs before and after treatment. 10% of patients had >1 CTCs before treatment, while 7% patients had >1 CTC after treatment.

We found statistically significant reduction of mean post-therapy CTCs count when compared to mean baseline count (P<0.003). Pre-and post-treatment tumor sizes were measured using breast ultrasonography and there was statistically significant reduction of mean tumor size after NACT when compared to pretreatment mean size. As the origin of CTCs is the primary tumor so it is logic to find that the decrease in CTC count is correlated to the primary tumor response to chemotherapy. We found negative correlation between the decrease in primary tumor size and the baseline CTCs counts before NACT. ***Pachman et al, 2008*** [18] showed the existence of a strong correlation between the presence of CTCs and a decrease in tumor size after neoadjuvant chemotherapy. They theorized that the reduction in the tumor size during treatment could be a consequence of the release of CTCs from the primary tumor mass.

Regarding pCR rate, there was statistically significantly higher mean base-line and post-NACT CTCs counts in patients who do not achieved pCR than in those who pCR after NACT. However, in another study conducted by ***Cristofanilli et al, 2005*** [19], no correlation was found between CTCs and tumor response to neoadjuvant therapy. Also, ***Bidard et al, 2010***[20], in a phase II trial (REMAGUS02) found that

CTCs was not correlated with the primary tumor response to chemotherapy.

Mounting evidence during recent years suggests that the presence of CTCs correlates with disease progression in patients with breast cancer [21]. In the present study, we demonstrated a significant relation between CTCs count and disease progression or relapse. Relapse rate was higher in patients with high baseline CTC count. In our study, we observed no significant relation between CTC count and other clinicopathologic factors which may affect prognosis such as age, menopausal status, hormonal receptors, tumor size, grade and lymph node status; also we showed in multivariate analysis the count of CTCs before NACT was a strong independent prognostic factor. Similar to our findings, in the GepartQuatro study, ***Riethdorf et al***, 2010 [16] observed no significant correlation between CTC detection and primary tumor characteristics, such as tumor stage, histologic type, lymphnode stage or homone receptor status. ***Pierga et al***, 2008 [22] in a smaller cohort of patients in the REMAGUS02 trial, also found no significant correlation between CTC detection and most characteristics presented in the primary tumor. Our results in the current study also agree with ***Krishnamurthy et al***, 2010[23] who conducted a study to evaluate the occurrence of CTCs in peripheral blood and to find the correlation between their detection and the standard prognostic factors like tumor size, tumor histologic grade, ER status, progesterone receptor status, HER2 status and axillary lymph node status. There was no correlation between occurrence of CTCs and the standard prognostic factors. ***Lucci et al***, 2012[24] also concluded that there was no correlation between primary tumor characteristics and detection of CTCs

As regard survival in our study, menopausal status, PgR status and baseline CTCs count were the prognostic factors affected DFS, while baseline CTCs count was the only factor which affected OS. Patients with high baseline counts clearly showed a shorter DFS and OS. So, the count of CTCs in the pre-neoadjuvant context can be used as a independent prognostic factor. These results were similar to that conducted by ***Bidard et al, 2010***[20] in the REMAGUS02 neoadjuvant trial as they concluded that detection of CTCs in non-MBC patients was correlated with metastasis-free and overall survival when neoadjuvant chemotherapy was chosen as a treatment modality. Also the study conducted by ***Lucci et al, 2012***[24] evaluated the prognostic value of CTCs in early stage breast cancer and concluded that presence of CTC was associated with significantly shorter relapse free survival (RFS).

Conclusion

The count of CTCs in breast cancer patients before starting neoadjuvant chemotherapy could predict response to neoadjuvant chemotherapy and it is associated with an increased risk of disease recurrence or relapse and shortened DFS and OS. We should consider detection on a large scale and more standardization of the methodology.

Conflict of Interests

The authors declare that they have no conflict of interests.

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