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 more often 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
eyear 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/m² and cyclophosphamide 500 mg/m²) (q 21days);
FAC (fluorouracil 500 mg/m², adriamycin 50 mg/m²
and cyclophosphamide 500 mg/m²) (q 21days) or FEC
(fluorouracil 500 mg/m², epirubicin 100 mg/m², and
cyclophosphamide 500 mg/m²) (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) labeled 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 determined 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 (≥5 cells/ 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

<table>
<thead>
<tr>
<th>Item</th>
<th>No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Menopausal</strong></td>
<td></td>
</tr>
<tr>
<td>pre</td>
<td>16 (40.0%)</td>
</tr>
<tr>
<td>post</td>
<td>24 (60.0%)</td>
</tr>
<tr>
<td><strong>Estrogen receptors</strong></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>17 (42.5%)</td>
</tr>
<tr>
<td>positive</td>
<td>23 (57.5%)</td>
</tr>
<tr>
<td><strong>Progesterone receptors</strong></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>24 (60.0%)</td>
</tr>
<tr>
<td>positive</td>
<td>16 (40.0%)</td>
</tr>
<tr>
<td><strong>Tumor size</strong></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>18 (40.0%)</td>
</tr>
<tr>
<td>T2</td>
<td>11 (27.5%)</td>
</tr>
<tr>
<td>T3</td>
<td>11 (27.5%)</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>2 (5.0%)</td>
</tr>
<tr>
<td>G2</td>
<td>28 (70.0%)</td>
</tr>
<tr>
<td>G3</td>
<td>10 (25.0%)</td>
</tr>
<tr>
<td><strong>Lymph Node</strong></td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>9 (22.5%)</td>
</tr>
<tr>
<td>positive</td>
<td>31 (77.5%)</td>
</tr>
</tbody>
</table>

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).

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

<table>
<thead>
<tr>
<th>Item</th>
<th>No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Good</td>
<td>26 (65.0%)</td>
</tr>
<tr>
<td>Relapse</td>
<td>14 (35.0%)</td>
</tr>
<tr>
<td>Death</td>
<td>7 (17.5%)</td>
</tr>
</tbody>
</table>

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

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 \( (P=0.034 \text{ and } 0.0149 \text{ respectively}) \) (table 3). Both mean baseline and mean post-treatment CTCs counts were significantly higher in relapsed patients than in non-relapsed patients \( (P<0.001 \text{ for both}) \) (table 4).

![Survival Function](image)

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

![Survival Function](image)

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

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 \( (P=0.001 \text{ and } 0.008 \text{ respectively}) \) (figure 6, 7). Among clinicopathologic factors, only menopausal status and progesterone receptor status significantly affected DFS \( (P=0.023 \text{ and } 0.026 \text{ respectively}) \) (figure 8, 9), while no factor affected OS.

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

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

* Statistically significant, n.s not significant

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

<table>
<thead>
<tr>
<th>CTCs</th>
<th>No relapse ( (n=26) ) Mean ± SD</th>
<th>Relapse ( (n=14) ) Mean ± SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline CTC</td>
<td>2.32±1.52</td>
<td>5.84±1.99</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Post-treatment CTC</td>
<td>1.52±0.77</td>
<td>4.64±1.8</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>
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 baseline 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 GeparQuatro 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 hormone 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 an 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).

**Conflict of Interests**

The authors declare that they have no conflict of interests.

**References**


