Effect of neoadjuvant chemotherapy on circulating immune cells in breast cancer patients: A pilot study

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Background: Innate and adaptive immunity affect tumor growth and progression. Likewise, immune cell subsets may have a promising approach in prognosis and treatment strategies. Aim: The aim of this pilot study was to investigate the neoadjuvant paclitaxel’s effect on the relative and absolute numbers of lymphocytes, monocytes, granulocytes and CD11b and its maturation markers and their role in breast cancer patients’ clinical response. Patients and Methods: Breast cancer patients (n=10) and healthy donors (n=4) were recruited from Tanta Cancer Center, Egypt. Peripheral blood samples (2ml) were collected in EDTA tubes from breast cancer patients during neoadjuvant chemotherapy (prior to each cycle of paclitaxel) as well as healthy volunteers. The relative and absolute numbers of lymphocytes, monocytes and granulocytes were obtained using gating strategy after acquisition with Flow-cytometer. The absolute numbers of CD11b⁺ and its maturation markers (HLADR⁺ and CD83⁺) were also evaluated by flow-cytometry after staining with specific antibodies. The absolute number was calculated using total peripheral blood leucocytes of each subject. Results: Comparing between the absolute number of monocytes prior to each cycle, significant difference was found between the 1st cycle vs the 3rd cycle and the 4th cycle. Significant difference was also found in CD11b expressing cells and its maturation markers (HLADR) between responder and non-responder patients. Conclusion: Our study concluded the importance of CD11b and its maturation marker in clinical response in neoadjuvant BC patients as a prognostic tool. Immunological parameters should be considered during application of treatment strategies as a prognostic tool and promising approach in immunotherapy. Keywords: neoadjuvant chemotherapy, breast cancer, paclitaxel, immune cells, CD11b

INTRODUCTION

Breast cancer (BC) is the most frequently diagnosed and the leading cause of cancer related death for women worldwide. It is a heterogeneous disease with clinical features and different outcomes. It is classified into three relevant biological types (estrogens/progesterone receptor positive (ER+/PR⁺), human epidermal growth factor receptor 2 (HER2) amplified, and triple negative) and multiple molecular subtypes (e.g., Luminal A/B, HER2, basal like, normal like) (Jemal et al., 2011). Non-metastatic breast cancer (BC) is considered as a systemic disease rather than a local disease, which requires early systemic neoadjuvant therapies to prevent cancer metastasis. However, neoadjuvant chemotherapy (NACT) was used for the treatment of locally advanced and non-operable tumors to decrease tumor size, NACT has become nowadays a standard treatment in primary BC to obtain high pathologic complete response and better prognosis (Corso et al., 2017). It is important to note that immune cells have significant role in tumor outcome. Tumor progression and metastasis can be affected by both innate and adaptive immunity (Cattin et
Monocytes, granulocytes as well as lymphocytes, mostly immature forms, contribute to cancer progression by promoting immunosuppression, angiogenesis, cancer cell survival, growth, invasion, and metastasis (Coffelt and de Visser 2016). Furthermore, tumor associated macrophages (TAM) are the generated from peripheral monocytes (Franklin et al., 2014). TAM are correlated with poor clinical response in BC (Campbell et al., 2011). It has been shown that peripheral blood monocytes are affected by tumor immune changes (Wang et al., 2020). Previous studies showed correlation between lower lymphocytes count and poor survival in many tumor models (Mandó et al., 2018). Lymphopenia has been found to be associated with increased risk of febrile neutropenia. It has also been found as a prognostic tool in overall survival such as in soft tissue sarcomas and hormone resistant metastatic breast cancer patients (Ray-Coquard et al., 2009). Furthermore, correlation was found between lower monocytes count and clinical response in BC patients after neoadjuvant chemotherapy (Talamantes et al., 2020). Similarly, preclinical studies with GB1275, a salt form of leukadherin-1, showed that activation of CD11b improves the antitumor immune response and enhances the response to immunotherapy in mouse models of pancreatic adenocarcinoma, breast cancer and lung cancer (DeNardo et al., 2021).

For this purpose, we aimed in this pilot study to investigate the effects of NACT on the relative and absolute number of lymphocytes, monocytes, granulocytes and CD11b expressing cells and their role in BC patients’ clinical response.

PATIENTS AND METHODS

Study subjects and Ethical approval

BC patients were recruited from Tanta Cancer Center(n=10) and healthy donors (n=4) from December 2019 to May 2020. Patients were receiving NACT paclitaxel after four cycles of Adriamycin/ Cyclophosphamide. Peripheral blood samples were withdrawn prior to each cycle of Paclitaxel as shown in figure 1 after obtaining informed consent. This study was approved by ethical committee of Ministry of Health and Population in Egypt according to declaration of Helsinki and World Health Organization guidelines and after submission of the Ethics committee in the Central Directorate of Research & Health Development in December 2018, Com.No/Dec.No:25-2018/8. The inclusion criteria of patients consist of age≥20 upon informed consent, diagnosed as malignant breast cancer and adequate baseline parameters of organ functions. The exclusion criteria included severe complications and metastatic breast cancer.

Data collection

Complete blood count (CBC) of patients were collected from their medical sheet records at Tanta Cancer Center. Their clinical pathologic features were also collected from their medical records as shown in Table 1. The absolute number of cells were calculated using patients’ total white blood cells/µl.

Table 1. Clinical pathologic features of patients.

<table>
<thead>
<tr>
<th>Breast cancer patients (n=10)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age range</td>
<td>49.4± 14.97. 28-74</td>
</tr>
<tr>
<td>ER: positive</td>
<td>7</td>
</tr>
<tr>
<td>ER: negative</td>
<td>3</td>
</tr>
<tr>
<td>PR: positive</td>
<td>6</td>
</tr>
<tr>
<td>PR: negative</td>
<td>4</td>
</tr>
<tr>
<td>HER-2: Positive</td>
<td>2</td>
</tr>
<tr>
<td>HER-2: negative</td>
<td>8</td>
</tr>
<tr>
<td>T: Tx</td>
<td>1</td>
</tr>
<tr>
<td>T1</td>
<td>3</td>
</tr>
<tr>
<td>T2</td>
<td>4</td>
</tr>
<tr>
<td>T3</td>
<td>2</td>
</tr>
<tr>
<td>N: N0</td>
<td>1</td>
</tr>
<tr>
<td>N1</td>
<td>4</td>
</tr>
<tr>
<td>N2</td>
<td>3</td>
</tr>
<tr>
<td>N3</td>
<td>2</td>
</tr>
<tr>
<td>M0:</td>
<td>10</td>
</tr>
<tr>
<td>Paclitaxel weekly:</td>
<td>5</td>
</tr>
<tr>
<td>Paclitaxel/ 3 weeks:</td>
<td>5</td>
</tr>
</tbody>
</table>

ER: estrogen receptor, PR: progesterone receptor, HER-2: human epidermal growth factor receptor, T: tumor stage, N: lymph nodes involvement, M: metastasis

Sample collection and flowcytometry analysis

Reagents

Red blood cells (RBC) lysis Buffer (10X) was used. Before using, 10X RBC lysis buffer was diluted to 1X by adding 1 ml lysing solution with 9 ml distilled water at room temperature.
Monoclonal antibodies

The monoclonal antibodies used for surface cell staining in this study were purchased from BD Pharmingen™, BD Biosciences (USA). These antibodies included HLADR-PE (catalog number: 555561, clone number: TU36), CD83-APC (catalog number: 551073, clone number: HB15e) and CD11b-FITC (catalog number: 562793, clone number: ICRF44).

Handling of blood samples for surface staining

Peripheral blood samples were collected in heparinized EDTA tubes. Samples of 100µl of whole blood were mixed with aliquots monoclonal antibodies (CD11b, HLADR, CD83) for surface staining of immune cells of peripheral blood. The samples contained whole blood and antibodies, were incubated at dark conditions for 30min then the samples were incubated for 15min at dark conditions after addition 2ml lysing solution (1X) to lyse (RBCs) then spin down the samples at 1500rpm in the centrifuge for 5min. The supernatant discarded, and the pellets were washed with 2ml phosphate buffer saline (PBS). The supernatant discarded, and the pellets were suspended in PBS. The cells were washed twice, then suspended in 500ml PBS, and then acquired by flow cytometer (BD FACS Canto II).

Gating strategy

The data were analyzed using Flowlogic software, Milteny Biotec. Each sample was analyzed using Forward and side scatter (FSC vs SSC) and dead cells were excluded. Gates around lymphocytes, monocytes and granulocytes were used to calculate relative numbers for each. To calculate relative number of CD11b⁻ HLADR and CD11b⁺HLADR expressing cells, we used four quadrant gating on monocytes and histogram of CD83 to calculate percentage from CD11b⁺HLADR⁺

Statistics

Statistical analysis was performed using GraphPad Prism software 8.4. The data were not normally distributed using Kolmogorov Smirnov normality test so we used Kruskal-Wallis test followed by Dunn’s multiple comparison test to show the difference between responders, non-responder BC patients and control group. P values of <0.05 were considered statistically significant.

RESULTS

The relative numbers of lymphocytes, monocytes and granulocytes prior to each cycle of paclitaxel were obtained using the gating strategy as shown in Figure 2. The absolute numbers of lymphocytes, monocytes and granulocytes were calculated using patients’ CBC prior to each cycle are shown in figure 3. Significant difference in absolute number of monocytes was found between 1st cycle vs 3rd cycle and 1st cycle vs 4th cycle of paclitaxel (P=0.0291,0.0194 respectively). Mean and standard deviation of immune cell subset from BC patients’ CBC and healthy donors are shown in Table 2. Significant difference was found in hemoglobin values in the second and third cycle of paclitaxel as compared to control group (p=0.02,0.037 respectively). Significant difference was also found in lymphocytes and monocytes count prior to each cycle as compared to control (p=0.005,0.0114 respectively). These all changes may be as a result of chemotherapy myelosuppression.

No significant difference was found for relative and absolute numbers of lymphocytes, monocytes and granulocytes between responders and non-responder BC patients as shown in Figure 4. Otherwise, absolute numbers of lymphocytes increased prior to third and fourth cycle with no significant difference. The median of relative and absolute numbers of lymphocytes and monocytes is higher in responder BC patients than non-responders with no significant difference. To obtain relative and absolute numbers of CD11b expressing cells and its maturation markers (HLADR & CD83), we used the gating strategy as shown in Figure 5. The percentage of CD83 from CD11b⁺HLADR⁺ was near zero (Figure 5).

No significant difference was found for relative and absolute numbers of CD11b⁺HLADR⁻ & CD11b⁺HLADR⁺ expressing cells between control group and BC patients prior to each cycle of paclitaxel (Figure 6). Significant difference was found for relative and absolute numbers of CD11b⁺HLADR⁻ & CD11b⁺HLADR⁺ between responder and non-responder BC patients as shown in Figure 7.
Table 2. A representative table showing mean ± SD of CBC of healthy donors and BC patients prior to each cycle of paclitaxel. P value for each cycle as compared to control group.

<table>
<thead>
<tr>
<th>Patient</th>
<th>1st cycle</th>
<th>2nd cycle</th>
<th>3rd cycle</th>
<th>4th cycle</th>
<th>Control</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hgb</td>
<td>11.05±0.766304</td>
<td>10.79±1.036501</td>
<td>11.11±0.641959</td>
<td>10.8222±0.319287</td>
<td>11.975±0.618466</td>
<td>control vs. 1st:0.1&lt;br&gt; control vs. 2nd:0.02 (<em>)&lt;br&gt; control vs. 3rd:0.13&lt;br&gt; control vs. 4th:0.037 (</em>)</td>
</tr>
<tr>
<td>TLC</td>
<td>6.39±1.378768</td>
<td>5.82±2.030216</td>
<td>6.15±1.286036</td>
<td>6.7444±2.712522</td>
<td>6.675±0.7932</td>
<td>0.8339</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1.657±0.412958</td>
<td>1.699±0.445033</td>
<td>1.89±0.633667</td>
<td>2.04±0.740169</td>
<td>2.9325±0.335398</td>
<td>0.0050 (*)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.478±0.18486</td>
<td>0.34±0.147874</td>
<td>0.364±0.157424</td>
<td>0.2875±0.099103</td>
<td>0.5725±0.038622</td>
<td>0.0114 (*)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>4.175±1.136087</td>
<td>3.698±1.529479</td>
<td>3.937±1.005331</td>
<td>4.3344±2.061808</td>
<td>2.9875±0.945282</td>
<td>0.5665</td>
</tr>
</tbody>
</table>

Figure 1. Schematic representative figure showing time of sample withdrawal. AC: Adriamycin/ Cyclophosphamide

Figure 2. A: A representative flow-cytometry analysis showing gating strategy for lymphocytes, monocytes and granulocytes. B: Box and Whiskerplot showing relative number of monocytes, lymphocytes and granulocytes.
Figure 3. Box and Whisker plot showing absolute numbers of lymphocytes, monocytes and granulocytes. A: from flow-cytometry analysis, B: from patients’ CBC. CBC: complete blood count.

Figure 4. Box and whisker plot showing Kruskal-Wallis test for: A: relative numbers of lymphocytes, monocytes and granulocytes, B: Absolute numbers of lymphocytes, monocytes and granulocytes.
Figure 5. A representative flow-cytometry analysis showing gating strategy of: A: FSC-A vs SSC-A gating on monocytes of healthy donors and breast cancer patients prior to each cycle of Paclitaxel, B: CD11b vs HLADR, C: Percentage of CD83 from CD11b+ HLADR+

Figure 6. Box and Whisker plot showing, A: relative numbers of CD11b+HLADR- & CD11b+ HLADR+ B: absolute numbers of CD11b+HLADR- & CD11b+ HLADR+
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**DISCUSSION**

The median of absolute and relative numbers of CD11b+HLADR & CD11b+HLADR+ expressing cells is higher in responder BC patients than non-responders as shown in figure 7. Significant difference was found in the relative number of CD11b+HLADR expressing cells in responder BC patients as compared to control and non-responders and control group (p=0.006). Significant difference was also found in the absolute number CD11b+HLADR+ and CD11b+HLADR expressing cells in responder BC patients as compared to non-responders and control group (p=0.034, 0.0053 respectively).

Clinical response and prognosis in cancer patients are known to be associated with immune parameters (Gouttefangeas et al., 2020). Comparing between the four cycles of paclitaxel, we found initial decrease in the absolute numbers of lymphocytes and monocytes followed by the increase prior to the 3rd and the 4th cycles. We also found significant difference in the absolute numbers of monocytes between the 1st cycle vs the 3rd cycle and the 4th cycle of paclitaxel was found (P=0.0291, 0.019 respectively).

Figure 7. Box and Whisker plot showing significant difference between responder and non-responder patients. A: relative numbers of CD11b+ HLADR- &CD11b+HLADR+ B: absolute numbers of CD11b+ HLADR- &CD11b+HLADR+
Timing of immunotherapy such as vaccination strategies and adoptive transfer of expanded tumor infiltrating (T-cell receptor engineered or re-educated) lymphocytes is important to control tumor growth (van der Burg et al., 2016). In preclinical mouse models which simulate advanced or end stage cancer patients, vaccination therapy failed when the time between tumor engraftment and vaccination increased. This was explained by accumulation of T regulatory (T-reg) and other immune suppressive cells such as myeloid derived suppressor cells (MDSC) (Berraondo et al., 2007). Better clinical outcomes are expected if in case of early stage or minimal residual disease.

In regards to granulocytes, they have no significant role in anti-tumor immunity and our study showed no significant difference in their relative and absolute numbers between responder and non-responder BC patients. Our findings concerning lymphocytes and monocytes are in conflict with another study which showed that lower monocytes and lymphocytes count that was correlated with poor prognosis (Cattin et al., 2021). We also showed significant difference of the absolute numbers of CD11b expressing cells between responders and non-responder BC patients. In line with Park et al., CD11b have a significant role in immunotherapy as preclinical studies suggested that hypothesis (Park et al., 2021).

CD11b expressing cells could be monocytes, monocytic-MDSC (m-MDSC) or myeloid dendritic cells (m-DCs). If they are HLADR+, they could be m-MDSC. In line with previous studies, m-MDSC decrease during Paclitaxel course (Diaz-Montero et al., 2009). A significant correlation was also found between tumor stage and number of m-MDSC in both human and murine models as they affect dendritic cell function in antigen presentation and T-cell response (Almand et al., 2001). CD11b expressing cells which are HLADR+, m-MDSC are excluded and they may be m-DCs or matured monocytes. M-DCs could be matured and express CD83 (Li et al., 2019) but our findings showed that percentage of CD83 was very low. Our study results showed significant difference between responder and non-responder BC patients. This support the significant role of monocytes differentiation and prognosis (Kitamura et al., 2018). Similarly-DCs have critical role in T-cell activation and anti-tumor response (Kuhn et al., 2015).

There are previous data suggested that immunotherapy may enhance efficacy of chemotheraphy. In HER-2/neu mice models, poly-regimen chemotherapy was administered. It consisted of cyclophosphamide or paclitaxel 1 day before and doxorubicin 7 days after vaccination with GM-CSF-secreting tumor cells. Enhanced anti-tumor effect was observed (Machiels et al., 2001). However, immunotherapy strategies such as immune checkpoint inhibitors, targeted antibodies and vaccines, there are many cancer patients resistant to chemotherapy. Hence, there is a high demand to improve immunotherapy mechanisms such as immunosuppressive pathways to result in better outcomes (Arina et al., 2016).

Finally, our study concluded the importance of CD11b and its maturation marker in clinical response in neoadjuvant BC patients. This immunological parameter could be considered during application of treatment strategies and may be used as prognostic tool. They also could be a promising approach in immunotherapy.

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All authors have made significant contributions to this work.

CONFLICT OF INTEREST

All authors have approved this article and declare no conflicts of interest.

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References


