ORIGINAL ARTICLE

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Programmed cell death protein 1 is a marker for neoadjuvant chemotherapy response in triple-negative breast cancer

Maria de Fátima Dias Gaui[1*], Luis Claudio Amendola[2], Danielle Carvalho Quintella[3], Nathalie Canedo[3], Adriana Bonomo[4]

SUMMARY

OBJECTIVE: Tumor-infiltrating lymphocytes are detectable in up to 75% of triple-negative breast cancer. The composition of these infiltrates may influence prognosis and is not known regarding regulatory or effector lymphocytes. The objectives of this study were to describe and quantify the composition of the tumor-infiltrating lymphocytes before and after chemotherapy (neoadjuvant chemotherapy) and to evaluate their association with complete pathological response and overall survival.

METHODS: This was a retrospective observational study. Clinical and pathological data from 38 triple-negative breast cancer patients treated with neoadjuvant chemotherapy at the University Hospital (HUCFF/UFRJ), between November 2004 and November 2018, were analyzed. The Stromal tumor-infiltrating lymphocytes (Stromal tumor-infiltrating lymphocytes) have been identified on hematoxylin and eosin-stained sections according to the guidelines of the "International tumor-infiltrating lymphocytes Working Group." Immunohistochemistry studies were performed to identify T-cell subsets (i.e., CD3, CD4, CD8, and FOXP3) and T-cell exhaustion (i.e., programmed cell death protein 1).

RESULTS: Statistically significant changes in stromal tumor-infiltrating lymphocyte categories were observed before and post-neoadjuvant chemotherapy, with 32% of intermediate cases becoming high. The correlation between pre-neoadjuvant chemotherapy stromal tumor-infiltrating lymphocytes and pathological response, pre-neoadjuvant chemotherapy and post-neoadjuvant chemotherapy, and stromal tumor-infiltrating lymphocytes and overall survival was not statistically significant. However, we noticed an increase of cells that favor the antitumor activity (i.e., CD3, CD8, and CD8/FOXP3 ratio) and decreased levels of cells inhibiting tumor activities (i.e., FOXP3 and programmed cell death protein 1) post-neoadjuvant chemotherapy. Importantly, programmed cell death protein 1 expression pre-neoadjuvant chemotherapy showed an association with pathological response.

CONCLUSION: In this study, we observed that chemotherapy significantly increases stromal tumor-infiltrating lymphocytes, CD8 T cells, as well as CD8/FoxP3 ratio. Most importantly, programmed cell death protein 1 expression before neoadjuvant chemotherapy positively correlates with pathological response suggesting the use of programmed cell death protein 1 as a prognostic marker before neoadjuvant chemotherapy.

KEYWORDS: Triple-negative breast cancer. Tumor-infiltrating lymphocytes. PD 1 protein.

INTRODUCTION

Breast cancer is the most frequent neoplasm and the leading cause of mortality among women worldwide[1]. Recent studies have demonstrated the importance of the tumor microenvironment (TME) and its prognostic implication regarding the behavior of various tumors, including breast cancer. Although breast cancer is not typically an immunogenic disease, tumor-infiltrating lymphocytes (TILs) are detectable in up to 75% of tumors, and approximately 20% of these tumors present particularly dense infiltrate[2].

There is growing evidence regarding the prognostic values of TILs correlating with survival, especially in triple-negative breast cancer (TNBC) cases and amplified HER2 cases[3]. Randomized studies comparing neoadjuvant treatment protocols in HER2+ and TN tumor cases have demonstrated that there is a significant correlation between TIL intensity in biopsies and better response to chemotherapy, as measured by the number of cases with complete pathological response (pCR)[4,5]. Thus, TILs have been shown to be biomarkers for the response to chemotherapy treatment and, consequently, survival[6].

A recently published meta-analysis that included individual patients from nine large studies confirmed the prognostic role of TILs in TN cases. Thus, it was suggested that TILs could be considered biomarkers for clinical use[7]. This recommendation

[1]Universidade Federal do Rio de Janeiro, Faculdade de Medicina, Departamento de Clínica Médica – Rio de Janeiro (RJ), Brazil.
[2]Instituto Nacional de Saúde da Mulher, Criança e do Adolescente Fernandes Figueira – Rio de Janeiro (RJ), Brazil.
[3]Universidade Federal do Rio de Janeiro, Faculdade de Medicina, Departamento de Anatomia Patológica – Rio de Janeiro (RJ), Brazil.
[4]Pesquisadora do Instituto Oswaldo Cruz/Fiocruz – Rio de Janeiro (RJ), Brazil.
*Corresponding author: mfgaui@hotmail.com
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Rua Almirante Guilhem 198/301 Leblon, Rio de Janeiro. CEP 22440-000 Tel: 021 999657032

was endorsed by the 16th International Breast Cancer Conference in St. Gallen, and it was proposed that TIL analysis in TN cases should be incorporated into the 8th edition of the American Joint Committee on Cancer staging system[8].

The most abundant cell population in TILs is T lymphocytes (75%). However, depending on the composition of these lymphocytes, i.e., whether they are effectors or regulators, the prognosis of breast cancer may vary. Studies have suggested that the best characterization of the immune infiltrate is obtained through immunohistochemistry (IHC), in terms of the levels of CD3 (total T lymphocytes), CD8 cytotoxic cells, and FOXP3 expressing Treg. The CD8/FOXP3 ratio in TILs correlates with a better response to neoadjuvant chemotherapy (NEOCT) and a greater chance of achieving a pCR[9]. Programmed cell death protein 1 (PD-1) can be overexpressed on the TILs[10]. The PD-1/PD-L1 pathway is crucial for the development of immune tolerance. In fact, blockage of the PD-1/PD-L1 interaction releases T-cell activity, and this is clear in many cancers where anti-PD-1 treatment with monoclonal antibodies allows a good clinical response mediated by T cells[11].

The predictive and prognostic function of immune biomarkers in TNBC remains unclear. This study aimed to quantify and identify the TILS components, along with their relationship with NEOCT.

METHODS

Patients and study design

This was an observational retrospective cohort study. We evaluated 133 patients who were treated at HUCFF/UFRJ with a diagnosis of initial or locally advanced breast cancer. These individuals underwent NEOCT followed by surgery between November 2004 and November 2018. From these, 40 patients with TN breast cancer defined through IHC, who were hormone receptor-negative and HER2-negative (0, 1+, or 2+ and FISH-negative) in accordance with the ASCO/CAP criteria, were selected.

The NEOCT regimen used was based on anthracycline and docetaxel, usually consisting of the FEC 3 docetaxel regimen (PACS protocol 01)[12]. At the end of chemotherapy, the patients were referred for breast surgery (conservative or radical) and axillary surgery (sentinel lymph node biopsy or axillary lymphadenectomy), at the surgeon's discretion. Out of the 40 TN patients, 38 were eligible for this study because sufficient histopathological material from before and after chemotherapy was available. This study was approved by the ethics committee of the UFRJ (CAAE:2800.3420.1.0000.5257).

Quantification and identification of tumor-infiltrating lymphocytes

TILs were identified in the biopsy material and surgical specimens by pathologists, using sections stained with hematoxylin and eosin at magnifications of 200-400×(10×ocular lens with 20-40×objective lens). Stromal TILs (sTILs) within the edge of the tumor scar were analyzed, after the exclusion of areas of ductal carcinoma in situ and tumor zones with necrosis and artifacts. The mean percentage of the stromal area occupied by mononuclear cells was scored, using the guidelines of the "International TILs Working Group," for the evaluation of TILs within the pre-treatment and post-chemotherapy scenarios. The quantity of sTILs was analyzed as a continuous measurement, using three predefined categories: low sTILs (0–10%), intermediate sTILs (10–40%), and high sTILs (40–90%)[13]. The sTILs were quantified blindly by two experienced pathologists at UFRJ.

The composition of the sTILs was identified by means of IHC. Counting of immunostained cells was performed in 3 fields of the stromal area (200–400× magnification). To evaluate CD3, CD4, and CD8 expressions, the following antibodies were used: Dako CD3 antibody (A0452) at a dilution of 1:800, CD8 SP clone (M3162) at a dilution of 1:100, and Bioscience FOXP3 (14-4777-82) at a dilution of 1:100.

Statistical analysis

The statistical assessment of the data was performed using R version 4.1.3 (R Development Core Team: http://www.R-project.com). For comparisons between strata (categories of variables), Student's t-test was used. p-value<0.05 were considered statistically significant.

The Kaplan-Meier statistical method was used for survival analysis. The start date for counting the length of survival was the time when the diagnosis was recorded. The observations began on the date when the first case included was diagnosed.

RESULTS

Cohort description

Out of the 40 TN breast cancer patients, 38 were eligible for inclusion because histopathological material from before and after chemotherapy was available.

The clinical and pathological features of the patients are described in Table 1.

Regarding the overall survival (OS) of the TN patients studied, 50% of the patients were still alive at 60 months. A pCR

Table 1. Clinical and pathological features of 38 patients.

All patients n=38		
Age	Mean (range)	54 (33-81)
Staging	IIA	1 (2.6%)
	IIB	10 (26%)
	IIIA	19 (50%)
	IIIB	3 (7.8%)
	NI	5 (13%)
Ki67	<20%	2 (5%)
	>20%	24 (63%)
	NI	12 (32%)
Histological type	Medullary	2 (5%)
	Metaplastic	1 (2.6%)
	Micropapillary	1 (2.6%)
	Infiltrating ductal carcinoma SOE	34 (89%)
Breast surgery	Mastectomy patey's	31 (82%)
	Conservative surgery	7 (18%)
Axillary surgery	Lymphadenectomy	30 (79%)
	Sentinel lymph node biopsy	8 (21%)
PCR		5 (13%)

Clinical staging based on the TNM of the International Union Against Cancer (UICC) 7th edition. Ki 67 considered low ≤20% and high >20%. N/I: without information. IDC NOS: infiltrating ductal carcinoma not otherwise specified.

was obtained in five patients (13%). In accordance with the literature, patients who achieved a pCR after NEOCT had better survival (p=0.030).

Quantification of tumor-infiltrating lymphocytes pre- and post-neoadjuvant chemotherapy and association with outcome

A total of 100% of the initial biopsy samples studied presented sTILs. Statistically significant changes in sTIL categories from before to after chemotherapy were observed in initially intermediate TILs patients only (p=0.016). At biopsy, sTILs were low in 10 (26%) cases, moderate in 22 cases (58%), and severe in 6 cases (16%). In the post-chemotherapy surgical specimen, we observed that 70% of low TIL cases remained low, 66% of high cases remained high, while 32% of intermediate cases became high and low in 12 cases (32%).

There was no statistically significant association between the intensity of sTILs in pre-chemotherapy biopsies and pCR (p=0.673). In addition, there was no statistically significant association between the intensity of sTILs in pre-chemotherapy biopsies and OS (p=0.98) or between post-chemotherapy sTILs and OS (p=0.24).

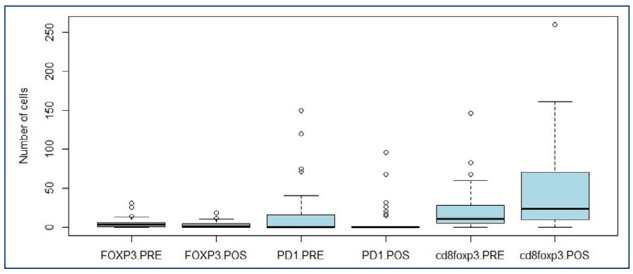

Figure 1. A box plot comparing immunophenotypes in pre- and post-chemotherapy biopsy samples: FOXP3, programmed cell death protein 1, and CD8/FOXP3, which shows significant increase in the pre- and post-CD8/FOXP3 ratio (p=0.001) and significant decrease in regulatory markers: FOXP3 (p=0.027) and programmed cell death protein 1 (p=0.01).

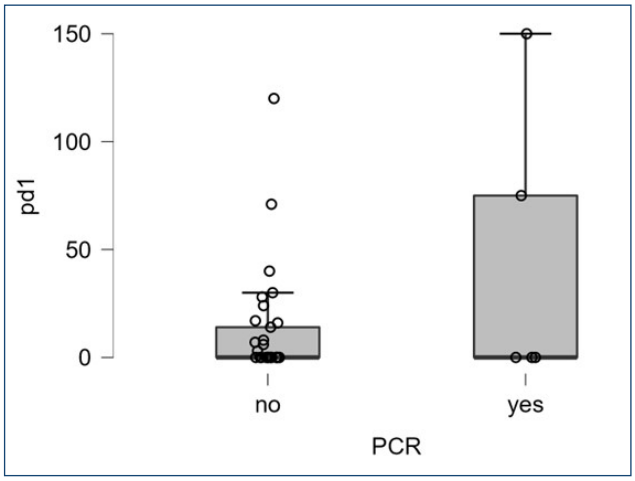

Figure 2. A box plot comparing programmed cell death protein 1 quantification (programmed cell death protein 1+cells) and pathological response. Programmed cell death protein 1 showed a significant correlation with complete pathological response (p=0.039).

Immunophenotypic analysis on stromal tumor-infiltrating lymphocytes

Immunophenotype analyses comparing pre-chemotherapy biopsies with post-chemotherapy surgical samples were performed. We observed non-significant increases in total T cells (CD3: 75.4-88) and in CD8+ T cells (CD8: 58.3-71.4). We also observed a significant decrease in FOXP3+cell levels (p=0.027) and PD-1+cells: 16-7.2 (p=0.011) leading to a significant increase in CD8/FOXP3 (p=0.001) (Figure 1).

However, when patients were separated according to their pathological response, and PD-1 expression quantified, high PD-1 expression was clearly correlated with complete response (p=0.039), while low pre-NEOCT expression was present in non-responding patients (Figure 2).

DISCUSSION

In this retrospective study, we evaluated 38 patients who had been diagnosed with TN breast cancer and were treated with NEOCT, in a single institution. Approximately 84% of them had tumors larger than 5 cm or positive lymph nodes. These findings were probably due to delayed diagnosis and late start of treatment. Only five patients achieved a pCR (13%), a result much lower than that has been reported internationally[14] and in Brazil[15]. The large tumor volumes may explain the poorer response to chemotherapy. However, even with the small number of pCRs, we were able to demonstrate, in accordance with the literature, that patients with a pCR had higher survival rates.

The association between pre-chemotherapy sTILs and pCR was not statistically significant (0.673). In addition, the correlations between pre-chemotherapy sTILs and OS (p=0.98) and between post-chemotherapy sTILs and OS (p=0.24) were not statistically significant, which were different from the literature that shows a correlation between TILs and OS[7].

There were statistically significant changes in the categories of sTILs from before to after chemotherapy. About 70% of low TILs remained low, 66% of high cases remained high, and 32% of intermediate cases became high, making us believe that this group of tumors is the one that best benefits from the immunogenic activation of NEOCT and subsequent immunotherapy. Only the intermediate group turned "cold" neoplasms into "warm" ones with chemotherapy induction. We believe that preexisting antitumor immunity is activated or enhanced during the initial cycle of chemotherapy, but only if infiltrating T cells were initially present at a certain level.

When we immunophenotyped the TILS, an increase in the profile of cells favoring immunity and antitumor activity and a significant decrease in the numbers of cells inhibiting tumor activities (FOXP3 and PD-1) were observed, and consequently, an increase in the CD8/FOXP3 ratio (Figure 1) was observed after NEOCT. This finding is compatible with the literature, in which chemotherapy is described as stimulating the immune response[16].

From all the markers used for immunophenotyping, only PD-1 in pre-chemotherapy samples showed a significant correlation with pCR. PD-1 receptor can be expressed in T cells, whereas PD-L1 is expressed in activated T and B cells, tumor-infiltrating macrophages or fibroblasts, and tumor cells. In the literature, the correlation of PD-1/PD-L1 with immunotherapy (ICB) has been widely explored, especially regarding the treatment of metastatic breast cancer, where PD-1/PD-L1 was used as a predictive biomarker to ICB therapy. The combination of an anti-PD-1 monoclonal antibody with NEOCT significantly increased the pCR rate and event-free survival[17,18] independently of the PDL1 level. These conflicting findings can be justified by the complex interaction between PD-1/PDL1, TILs, TME, and other immune checkpoints such as cytotoxic T-lymphocyte-associated antigen-4 and PD-L2, which are less studied targets in breast cancer[11]. More robust studies are needed to validate this finding. Based on this result, prospective randomized studies could test the addition of adjuvant immunotherapy only in PD-1+ patients without pCR or residual tumor[19]. A better understanding of this complex network can help in the use of new therapeutic targets.

The retrospective design, the small number of patients included, and the small number of pCRs obtained were the limitations of this study and may have influenced the finding of a correlation between the variables and outcomes. The strengths of this study were the use of a homogeneous population, and patients with locally advanced TNBC who underwent NEOCT in a single institution with quantification and immunophenotypic identification of TILs, along with their relationship with the treatment. Over the period covered by this study, NEOCT protocols for TN breast cancer did not undergo major changes.

CONCLUSION

Our study suggests that PD-1 levels in sTILs could be a candidate as a prognostic marker in response to NEOCT, independently of checkpoint inhibitor immunotherapy.

AUTHORS' CONTRIBUTIONS

MFDG: Conceptualization, Data curation, Investigation, Methodology, Project administration, Formal Analysis,Validation, Writing – original draft. **LCA:** Data curation , Investigation, Software, Writing – review & editing . **DCA:** Investigation, Methodology, Writing – review & editing. **NC:** Project administration, Resources, Supervision, Writing – review & editing. **AB:** Visualization , Resources, Supervision, Writing – review & editing.

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