A high CD8 to FOXP3 Ratio in The Tumor Stroma is Associated with Improved Survival in Non-Metastatic Triple-Negative Breast Carcinoma.

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Abstract

Triple-Negative Mammary Carcinoma (TNBC) is an aggressive breast cancer subtype associated with dismal prognosis. The interaction between the immune system and the cancer cells plays a crucial role in tumor development and progression. However, it is still unclear how each diverse cell of the immune system contributes to the prognosis of patients with breast cancer. Purpose: In this study, we investigated how the cell composition of the immune cell infiltrated modifies the survival of patients with resected TNBC. Methods: Retrospectively, we collected data from 76 patients diagnosed with non-metastatic TNBC with available tissue blocks for Tissue Micro-Array (TMA) construction. The TMA was constructed using two cores from each tumor block. The expression of CD4, CD8, FOXP3, CD20, CD68, CD163, PD-1, PD-L1, PTEN and phosphoSTAT1 was determined by immunohistochemistry. Results: We observed that the inflammatory infiltrate in TNBC is enriched for M2 macrophages and T lymphocytes ($CD4^+$, $CD8^+$ and $FOXP3^+$). We found a correlation between TIL and PD-L1 expression in stroma cells (p=0.001) and in tumor cells (p=0.028). There was no association between PD-L1 expression and OS. The number of $FOXP3^+$ cells and the lack of expression of PTEN in tumor cells were associated with OS. Conclusion: We did not observe any association CD8 and CD4 cell counts in the tumor and survival, however a higher CD8/FOXP3 ratio was associated with improved survival. This was confirmed in the METABRIC TNBC cohort, where a high CD8A to FOXP3 gene expression ratio was also associated with longer survival.

Keywords: Triple-negative breast cancer, Immune infiltrate, CD8, FOXP3, Survival.

Accepted on 06 September, 2021

Introduction

Triple-Negative Breast Cancer (TNBC) refers to breast carcinomas that lack the expression of hormone receptors (estrogen and progesterone receptors), and that do not express the human epidermal growth factor receptor 2 (HER2) and do not have amplification of the corresponding gene (ERBB2). TNBC corresponds to 15%-20% of all newly diagnosed breast cancer cases. Despite the emergence of new drugs and targeted therapies for the treatment of other breast cancer subtypes, patients with TNBC continue to have a dismal prognosis. The interaction between the immune system cells and cancer cells plays an important role in tumor development and progression and involves a complex interaction of tumor cells with chemical mediators (cytokines and chemokines) and cells of the innate and adaptive immune system. Tumor-Infiltrating Lymphocytes (TIL) comprise a mixture of cytotoxic T cells, helper T cells, as well as B lymphocytes, macrophages, Natural Killer (NK) cells, and dendritic cells. Gene expression and cytometry analysis have shown that the percentage of TILs represents an indirect marker of a pre-existing anti-tumor activated T cell response. TILs are commonly observed at increased levels in TNBC and HER2-positive tumors as compared to Estrogen-Receptor (ER) positive, HER2-negative tumors. Recently, TILs have been reported to be associated with better prognosis and higher response rates to neoadjuvant therapy in early-stage breast cancer as well as improved response to chemotherapy and trastuzumab besides tumor infiltration by cells expressing CD3, CD8 and CD20 are a potential predictive biomarker of response to chemotherapy [1].

The cytotoxic $CD8^+$ T lymphocytes are crucial components of tumor-specific cellular adaptive immunity which can specifically recognize and kill tumor cells. $CD8^+$ T cells induce tumor cell cytostasis and kill them through cell cycle inhibition, induction of apoptosis, angiostasis, and induction of macrophage tumoricidal activity. In contrast, the FOXP3 positive regulatory T cells (Tregs) effectively suppress the proliferation and activation of CTLs in a contact-dependent manner or via the release of cytokines such as transforming growth factor-beta. STAT-1 gene is a pleiotropic protein with multiple transcriptional functions. STAT-1 is phosphorylated upon T cell activation by interferon-gamma. Activation of PD-1 by PD-L1 or PD-L2 decreases T cell activity, reduces cytokine production and induces antigen tolerance. Clinical studies with therapies involving the inhibition of these so called immune checkpoints, i.e. the inhibition of co-inhibitory molecules of the immune system (such as PD-1/PD-L1), has demonstrated high tumor response rates in several tumors, and PD-L1 expression is correlated with response to those treatments. The loss of PTEN correlates, specifically, with the development of hormone receptor-negative breast cancer, and, in addition to inducing the expression of PD-L1, its loss suppresses the proliferation and survival of T lymphocytes. In our study we evaluated the prognostic value of the different

immune cell subtypes and, as well as, the association between some immunophenotypic immunohistochemical biomarkers in the inflammatory infiltrate of early triple-negative breast cancer [2].

Materials and Methods

Population

The study population consisted of all patients diagnosed with non-metastatic (stages I, II and III) TNBC, defined by immunohistochemistry (following the CAP 2010 definition), that had available FFPE tissue blocks, treated at the A. C. Camargo Cancer Center, Brazil, from January 2002 to December 2014. Exclusion criteria were in situ ductal carcinoma and a previous invasive cancer in the last 5 years preceding study entry. The analysis was performed only on biopsies obtained prior to the initiation of any systemic treatment or radiotherapy. Demographics, clinical-pathological characteristics and treatment information were collected from the electronic medical records.

Analysis of the tumor infiltrating lymphocytes (TIL)

The intensity of the inflammatory infiltrate was evaluated in H and E stained slides using the criteria and recommendations described and standardized. All slides were evaluated by two pathologists (V.A.A. and L.G.) with experience in mammary pathology.

TMA construction

The original slides were revised to confirm histological classification and two representative areas of the tumor were selected to obtain two 1.0 mm cores of each sample, which were transferred from the original tumor block to a receptor block to build the TMA. Areas containing necrosis, hemorrhage, and artifacts were avoided.

Imumunohistochemical reactions

The expression of CD20 (B lymphocyte B), CD4 and CD8 (T lymphocyte), FOXP3 (regulatory T-lymphocyte-Treg), CD68 (macrophages), CD163 (M2 response macrophages), pSTAT1 and PD-1 was evaluated in inflammatory cells located in the tumor stroma. PD-L1 and PD-L2 expression was evaluated both in the tumor cells and in the inflammatory infiltrate in the tumor stroma. PTEN expression was evaluated in tumor cells (absence of labeling) only. Immunohistochemical reactions used an automated protocol and ready-to-use reagents and were performed in the Benchmark Ultra (Ventana Medical Systems, Inc.) equipment following standardized dilutions and reaction conditions for each antibody.

PD-L1 expression analysis

PD-L1 expression was evaluated with the SP263 clone and was analyzed by direct optical microscopy by a pathologist (M.M.P.) with specialized training. PD-L1 expression was counted as the percentage of cells with positive membrane staining and was evaluated separately in tumor cells and in the stroma. Percentages were registered as 0%, 1%, 5%, 10% and then in increments of 10%. For each sample, the average value obtained from the duplicates was used in the statistical analysis. When only one core was evaluable, the value obtained from this core was employed in the analysis.

Immune infiltrate analysis

Automated counting of the total number of positive cells in the tumor stroma from each core to each biomarker studied (except for PD-L1) was performed using the Aperio AT2 (Leica Biosystems) equipment and the manufacturer's suggested protocols for membrane (Aperio Membrane Algorithm) and cytoplasmic (Positive PixelCount v9) staining. The area to be analyzed was manually selected by a pathologist (V.P.A.), without knowledge of the clinical data. In each of the technical replicas in the TMA, an area was delimited, and the number of positive cells for each marker was calculated as cells/mm^2. The average of the total number of labeled cells obtained in each of the two cores analyzed for each patient was calculated (for the cases with only one evaluable core, that single core was used, and the cases with loss of the two cores in TMA were excluded from the analysis for that biomarker) [3].

Statistical analysis

Optimal cutoff points for the biomarkers associated with survival were determined by the maximization of the log-rank test method described. Association between specific cell population counts and clinical-pathological variables was evaluated by the Chi-squared test or Fisher’s exact test. Correlations were determined by the Spearman’s correlation test. Survival curves were calculated by the Kaplan-Meier method and compared by the log-rank test. All tests were deemed statistically significant when $p<0.05$. The calculation of the optimal gene expression cutoff for the determination of two groups was defined by the maximization with several approximations of the log-rank p-value using the maxstat R package described by Horthorn.

Results

Patient selection and characteristics

Initially, 379 patients identified as TNBC in the A. C. Camargo Cancer Center medical records were selected. Of these, 267 fulfilled all inclusion and exclusion criteria from whom clinical and demographic information was collected. Of this group, 166 patients with HE slides available in the Pathology Department archive file were selected for analysis of TIL, and among them, 76 patients also had paraffin blocks available which were used to construct the TMA for immunohistochemical analysis (Figure 1).

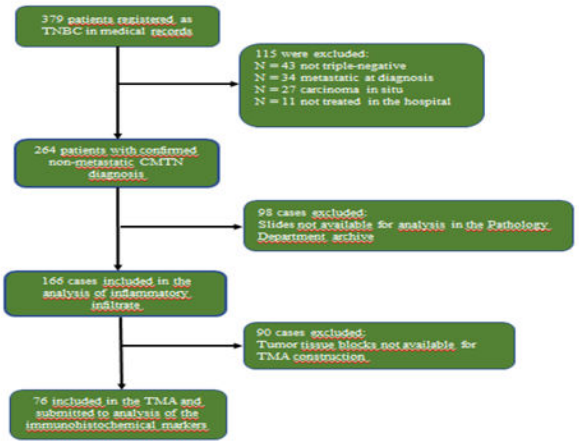

Figure 1: *Flowchart of the description of cases included and excluded in the study.*

Clinical-pathological features and outcomes of patients included in the TMA analysis

The mean age of the 76 women whose tumors were included in the TMA was 48.4 years old. Twenty-one (27.6%) patients were white and 7 (9.2%) were BRCA1 mutated. Regarding clinical staging, most patients were diagnosed in stage IIA, 29 (38.2%), or IIB, 14 (18.4%). Seventy-one tumors (93.4%) were of ductal histology and them the majority of were grade 3, (60.5%) 46 patients. Sixty-two (81.6%) received adjuvant treatment and 14 (18.4%) received neoadjuvant chemotherapy. Among the 14 patients who received neoadjuvant treatment, 2 (14.2%) had a complete pathologic response, 6 (42.8%) partial response and 3 (21.4%) had no response. Regarding the clinical outcomes, among the 76 patients analyzed, 18 (23.7%) relapsed, 11 (14.5%) died, 59 (77.6%) had no evidence of disease at the time of the last follow-up, 10 (13. 2%) presented disease progression and 7 (9.2%) had loss of follow-up (Table 1).

Table 1: Clinical-pathological features and outcomes of the 76 patients included

in the TMA analysis.

Variables	Descriptor	Frequency (N%)
Age	Mean	48.4
BRCA mutation	Wild-type	1 (1.3)
	Mutated	7 (9.2)
	Unknown	68 (89.5)
Clinical staging	IA	13 (17.1)
	IB	2 (2.6)
	IIA	29 (38.2)
	IIB	14 (18.4)
	IIIA	7 (9.2)
	IIIB	9 (11.8)
	IIIC	2 (2.7)
Histology	IDC	71 (93.4)
	ILC	1 (1.3)
	Metaplastic	4 (5.3)
Histological grade	G1	3 (3.9)
	G2	27 (35.6)
	G3	46 (60.5)

Analysis of the immune cell infiltrate composition

The average percentage of TIL was 25.78%, and the mean percentage of cells expressing PD-L1 in the tumor and stroma was 14.48% and 20.85%, respectively. We observed that the inflammatory infiltrate in TNBC is enriched for macrophages (cells expressing CD68 and CD163) and T lymphocytes (cells expressing CD8 and CD4), with few cells expressing FOXP3 and pSTAT1 (Table 2).

Variable	N*	Mean cells/mm2	Standard deviation	% (Mean)
CD8	76	1513.53	1353.5	
FOXP3	76	26.37	42.87	
CD20	76	530.54	1093.47	
CD4	76	1288.91	1354.23	
CD163	76	2001,41	1630.75	
CD68	76	2853.62	2247.43	
PTEN	76	119.58	48.47	
pSTAT1	76	119.76	197.67	
PD1	73	252.59	365.51	
PD-L1 Tumor	62			4.53
PD-L1 Estroma	48			12.17
Percentage of TIL	71			28.34

Association of immune cell subtypes with clinical-pathological characteristics and correlation between biomarkers

PD-L1 expression in the stroma was associated with the percentage of TILs (p=0.018). PD-L1 expression in the tumor was associated with the percentage of TIL (p=0.049) and with N staging (p=0.046). PD1 expression was associated with histological subtype (p=0.022). The other markers did not show association with any clinical-pathological variable. We found a positive and statistically significant, although weak, correlation between the percentage of TILs in the stroma and the expression of PD-L1 in stromal cells (p=0.02) and in tumor cells (p=0.027). The following markers also showed a correlation between them: CD4 with CD163, CD8, CD68 and FOXP3; CD163 with CD8, CD68, FOXP3, PD-L1 in the

stroma and absence of PTEN expression; CD8 with CD68 and FOXP3; FOXP3 with absence of PTEN expression; CD68 with FOXP3 and absence of PTEN expression; and stromal PDL-1 with tumor PD-L1 and absence of PTEN expression. The number of FOXP3 positive cells were also inversely correlated with the expression of PD-L1 in the tumor.

Impact of biomarker expression on overall survival

The only variables associated with overall survival were the lack of expression of PTEN in tumor cells and the number of cells positive for FOXP3 (Figure 2A and 2B).

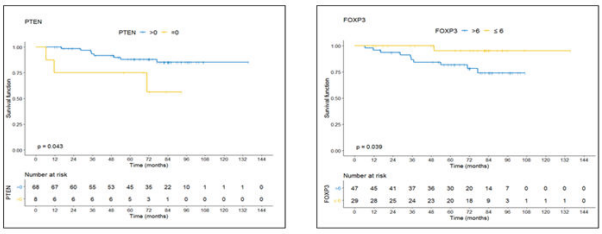

Figure 2:*Overall survival according to the number of tumor cells lacking PTEN expression and overall survival according to the number cells positive of FOXP3 positive in the stroma. Survival curves were calculated by the Kaplan-Meier method and compared by the log-rank test.*

Although the median survival was numerically different (77 and 120 months, respectively for PD-L1 expression in tumor >1% and ≤ 1%; p = 0.224) for patients whose tumors had high PD-L1 expression, this was not statistically significant (Figure 3). Similarly, PD-L1 expression in the stroma and the number of cells positive for CD8, CD4, CD20, pSTAT-1, CD68, CD163 or PD-1 in the stroma were not associated with overall survival (Supplementary Online Material). We also evaluated the impact of the ratio between CD8 and FOXP3, CD4 and FOXP3 and CD20 and CD4 on OS, and we observed that individuals with high a CD8/FOXP3 ratio (above the optimal cut-off point) in their tumors, as well as those with a high CD4/FOXP3 (Supplementary Online Material), had improved OS.

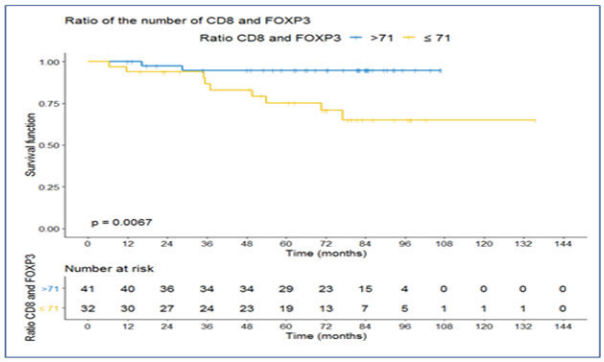

Figure 3: *Overall survival curves according to the ratio of the number of CD8 and FOXP3 positive cells. Survival curves were calculated by the Kaplan-Meier method and compared by the log-rank test.*

Impact of CD8A to FOXP3 ratio on the survival of TNBC in the METABRIC cohort

We calculated the overall survival of non-metastatic, triple-negative breast cancer patients included in the METABRIC according to the ratio between the expression of CD8A and FOXP3 genes (CD8A/FOXP3 ratio). Similarly to what was observed in our cohort, patients whose tumors had a high CD8A to FOXP3 ratio had improved overall survival (Figure 4).

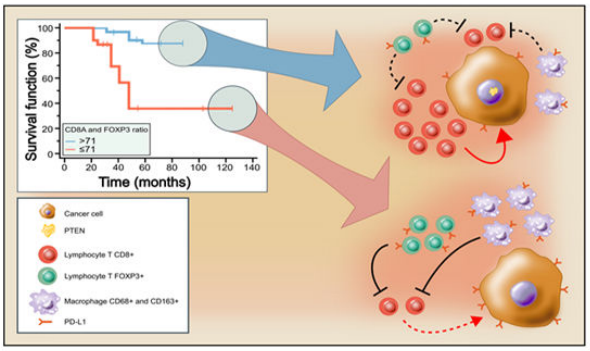

Figure 4:The Immune microenvironment in CMTN is associated with overall survival. A more exuberant mononuclear infiltrate is associated with increased overall survival, as well as with increased PD-L1 expression in both the stroma and tumor cells. As that characteristic (PD-L1 expression) was also associated with infiltration by CD8 expressing cells, we believe that those tumors (upper group in the figure) represent a more immunogenic subgroup which actively recruits the adaptive immune system, however, at the same time, as a mechanism of adaptive resistance, it triggers the expression of PD-L1 (exhaustion). Effective anti-tumor activity depends on the ratio between cells expressing CD8 (cytotoxic T lymphocytes) and FOXP3 (regulatory lymphocytes). Besides, loss of PTEN expression was also associated with poorer survival and infiltration by macrophages (cells expressing CD68 and CD163) and regulatory lymphocytes. The red arrows represent the likely final effect on the tumor. Barred arrows indicate inhibitory effect. Tipped arrows indicate anti-tumor activity.

Discussion

In our TNBC cohort, the immune/inflammatory infiltrate observed in tumor samples was enriched with macrophages (mainly with M2 phenotype) and T lymphocytes. Although we observed a high number of $CD8^+$ and $CD4^+$ cells in the stroma of TNBC, these cells were directly correlated with increased numbers of $CD68^+$ and $CD163^+$ cells, as well as $FOXP3^+$ cells. Besides, the number of $CD8^+$ cells also correlated with a higher number of PD-L1+ cells in the stroma. These data suggest that, although TN breast tumors may effectively attract T lymphocytes at some point in their progression, these lymphocytes probably have their effector activity suppressed by co-recruitment of immunosuppressive cells (M2 macrophages and regulatory lymphocytes) and acquisition of an exhausted phenotype due to the PD-L1 expression. The

description of the frequency of these cells in the intratumoral or stromal compartments is very heterogeneous in the literature, as well as the determination of its clinical significance. The evaluation of the intratumoral cytokine profile and a more detailed evaluation of the immunophenotype of the cells in the tumor immune infiltrate by flow or mass cytometry would be very informative [4].

We also found a positive and statistically significant correlation between the percentage of cells expressing PD-L1 in the stroma (p=0.02) and PD-L1 in the tumor (p=0.027), reinforcing some data from the literature suggesting that the higher the inflammatory tumor infiltrate the higher the expression levels of PD-L1. Nevertheless, PD-L1 expression, both in the stroma and in the tumor was not associated with OS. Lymphocytes infiltrated into the tumor are an important immune component of the cancer response. The density of CD4, CD8 and CD68 leukocytes in tumor tissues obtained at the time of primary surgery from 179 patients with breast cancer who were treatment naive. They observed that a high density of CD4+ T cells and low density of $CD8^+$ T cells correlated with reduced OS, while CD68+ cell density was not associated with OS. However, there was an inverse correlation between stromal infiltration by $CD68^+$ macrophages and $CD8^+$ T lymphocytes. According to the number of $CD8^+$ T cells is a predictor of better clinical outcome. Intratumoral infiltration by $CD8^+$ T cells was associated with a higher cancer-specific survival (HR 0.55, 95% CI, 0.39-0.78, p=0.001). In our cohort, we observed a trend for improved survival associated with a higher $CD8^+$ (p=0.054) and $CD4^+$ (p=0.082) cell counts, but the results were not statistically significant.

The only variables associated with overall survival were the lack of PTEN expression in tumor cells and the number of $FOXP3^+$ cells in the stroma. Since the optimal cut-points for OS evaluation of these variables were not associated with any clinical variable, we believe that these can be independent markers of OS, but, given the small sample size, we could not perform a multivariate analysis to check this hypothesis. However, as we analyzed the association of OS with the ratio between the number of $CD8^+$ and $FOXP3^+$ cells (p=0.007) and of $CD4^+$ and $FOXP3^+$ cells (p=0.034), we found that a higher relative proportion of $CD8^+$ or $CD4^+$ lymphocytes in relation to $FOXP3^+$ lymphocytes was associated with improved OS. Together, these data suggest that the T lymphocytes in the tumor microenvironment may exhibit antitumor activity and promote growth control, as opposed to events favoring immunosuppression (regulatory lymphocyte infiltration). This data is replicated in the TNBC cohort of METABRIC for those patients whose tumors have a high CD8A/FOXP3 ratio. The association between T cells (Treg) and $CD8^+$ Cytotoxic T Lymphocytes (CTLs) with patient survival, histopathologic features, and molecular subtypes in 1,270 cases of invasive breast carcinoma [5]. They showed that an increased infiltration by Tregs and CTLs inside the tumors was associated with unfavorable characteristics, like high histologic grade and negative ER and PR status. In addition, high Treg infiltration was also associated with decreased Overall Survival (OS) and Progression-Free Survival (PFS). On the other hand, a high CTL/Treg ratio in the tissue surrounding the tumor was significantly associated with improved OS and PFS. The expression of CD8, FOXP3, and CD3 in 177 patients with primary, invasive, unilateral early-stage breast cancer of all molecular subtypes and observed that T-cell infiltration was associated with hormone receptor negativity, high proliferation rate, high histological grade, and with large tumors. Basal-like tumors had the highest number of $FOXP3^+$ T-cells, with an unfavorable ratio to cytotoxic $CD8^+$.

Our data suggest that the presence of exuberant inflammatory infiltrate in TNBC is associated with PD-L1 expression both in the tumor itself and in the tumor stroma, as well as with enrichment for T lymphocytes ($CD8^+$ and $CD4^+$), which may make these tumors important targets for treatment with PD-1 or PD-L1 inhibitors. Furthermore, macrophages and regulatory T lymphocytes probably represent important counter-regulatory mechanisms that the tumor recruits to allow its escape of the adaptive immune response and a high CD8 to FOXP3 ratio in the tumor stroma is a predictor of improved survival in non-metastatic TNBC (Figure 4). In the future, it would be interesting to evaluate the role of strategies that deplete $FOXP3^+$ cells in the tumor microenvironment.

Conflict of Interest

All authors declare not to have any conflicts of interest.

Ethical Approval

The study was conducted in accordance with 1964 Declaration of Helsinki and was approved by the local institutional Ethics Committee under the number 1914/14.

Informed Consent

Due to the retrospective and non-interventional nature of the study a waiver for the application of a free informed consent was allowed by the institutional Ethics Committee.

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Citation: *Tavares MC, Sampaio CDAT, de Lima GE, et al.. A high CD8 to FOXP3 Ratio in The Tumor Stroma is Associated with Improved Survival in Non-Metastatic Triple-Negative Breast Carcinoma. J Clin Oncol Cancer Res 2021;4(4):1-6.*

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