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ORIGINAL ARTICLE

Tumor Infiltrating Lymphocytes in Breast Cancer Patients with Progressive Disease during Neoadjuvant Chemotherapy

Jacques Raphael[1,2] · S. Nofech-Mozes[3] · T. Paramsothy[1] · N. Li[4] · S. Gandhi[1]

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Abstract

A minority of breast cancer (BC) patients progress during neoadjuvant chemotherapy (NCT). The aim of this study was to assess the value of Tumor infiltrating lymphocytes (TILs) in such a high-risk population where valid biomarkers are eagerly needed. A retrospective review identified BC patients who either progressed during NCT or achieved a pathologic complete response (pCR). An experienced BC pathologist semi-quantified stromal TILs in pre-treatment core biopsies using hematoxylin and eosin stained slides. The primary outcome was to compare the levels of TILs between the 2 groups as a continuous and categorical variable using the t-test and X^2 test as appropriate. The secondary outcome was to compare survival outcomes between patients with high versus low TILs level using the log-rank test. Fifty patients were successfully identified and assessed for TILs: 21 progressed during NCT and 29 had a pCR. Patients with progressive disease were older with more advanced disease ($p = 0.03$, $p = 0.0001$ respectively). A significantly lower mean level of TILs was found in patients with progressive disease compared to patients with pCR: 14.3% (Standard Deviation (SD): 16.9) versus 32.8% (SD: 31), $p = 0.01$). The level of TILs was neither associated with baseline characteristics nor with survival outcomes. BC patients progressing during NCT have low TILs levels compared to patients with pCR. Prospective studies are needed to establish the utility of TILs as early biomarkers of tumor response, particularly in patients with disease progression who need novel treatment approaches.

Keywords Breast cancer · Disease progression · Neoadjuvant chemotherapy · Tumor infiltrating lymphocytes

Introduction

Patients with locally advanced breast cancer (LABC) are usually treated with upfront chemotherapy followed by surgery. At the time of surgery, achieving a pathologic complete response (pCR) is associated with improved disease free and overall survival (DFS, OS) [1–4]. However, around 20% of non-metastatic BC patients recur and eventually die from their disease [5]. In order to identify these patients, several predictors of tumor response and resistance to neoadjuvant chemotherapy (NCT) were assessed and described in the literature [6–12].

A subset of patients progress during their NCT and need salvage therapies subsequently. Few studies assessed this population and identified putative markers predictive of tumor progression during NCT [13, 14]. Nevertheless, most of these markers namely high tumor grade and low or negative Estrogen Receptor (ER) status are also predictors of tumor response, rendering their use in the clinical practice very limited to identify patients with higher risk of progression during NCT.

Tumor-infiltrating lymphocytes (TILs) are emerging as promising biomarkers for establishing response rates to NCT. They were recently identified as predictive markers for response to NCT and prognostic markers after adjuvant chemotherapy [15–20]. Their presence in the stroma or inside the tumor (intratumoral) was shown to be associated with better survival outcomes and tumor responses mainly in triple negative (TN) and HER2 positive BC subtypes [15, 19, 20]. Yet the role of TILs is still unclear in patients with progressive disease during NCT.

✉ Jacques Raphael
raphaeljack13@hotmail.com

[1] Division of Medical Oncology, Department of Medicine, Sunnybrook Health Sciences Centre, 2075 Bayview Avenue, Toronto, ON M4N3M5, Canada

[2] Division of Medical Oncology, Department of Oncology, Western University, London, ON, Canada

[3] Department of Pathology, Sunnybrook Health Sciences Centre, Toronto, ON, Canada

[4] Sunnybrook Research Institute, Toronto, ON, Canada

We aimed to assess and quantify the presence of TILs in such a population at our centre and compare their levels to the level of TILs in patients with excellent tumor responses after NCT.

Methods

Study Design and Data Source

We conducted a retrospective cohort study using data from the REB-approved Sunnybrook Cancer Research Biomatrix database. Biomatrix is a secure, privacy protected data warehouse, developed under support of The Canada Foundation for Innovation. It integrates a wide spectrum of information pertaining to a patient's journey through our cancer program, including detection, pathologic diagnosis and treatment of disease with links to images, tumor and tissue, sociodemographic data and outcomes onto a searchable web-based platform to facilitate multidisciplinary cancer research. Baseline patients, tumor and treatment characteristics and their outcomes were captured and this included: age, tumor stage (TNM stage as per the American Joint Committee on Cancer staging criteria (AJCC) [21]), Estrogen and Progesterone receptor status (ER, PR), HER2 status, presence of lymphovascular invasion (LVI), histologic type, tumor grade, type of surgery (mastectomy versus breast conserving surgery, sentinel lymph node biopsy versus axillary dissection) and dates of recurrence, death and last follow up.

Patients

All BC patients treated with NCT at Sunnybrook who consented to prospective data collection in Biomatrix were identified. Patients with clinical and/or radiologic progression during their therapy requiring change in regimen and salvage treatment were eligible for our analysis. For comparison, a similar number of LABC patients treated during the same time period, who achieved a pCR, were randomly identified. However this cohort was not matched for the other baseline characteristics because of the small number of patients identified. Patients treated with neoadjuvant endocrine or radiation therapy were excluded.

Of 413 LABC patients in the database, 30 patients (7.3%) with progression and 30 patients with pCR were identified.

A written consent was obtained from all patients to capture their data into biomatrix and our institutional review board approved the study protocol.

Pathology Assessment

A pathologist with BC expertise reviewed all core biopsies to select the optimal slide for TILs assessment. An optimal slide referred to a slide with preserved morphology (minimal artifactual changes) that best represents the variability in tumor morphology. At the time of assessment, the pathologist was blinded to the clinical outcomes of all patients.

Clinical and pathologic staging system was based on the seventh edition of the AJCC staging criteria [21]. Tumor grading was based on the Nottingham score. ER and PR status were determined by immunohistochemistry (IHC; 1% cut-off for positivity, using the ASCO/CAP guidelines [22]), and HER2 positivity by IHC (3+) and/or gene amplification on fluorescence in situ hybridization based on the 2013 guidelines [23]. Pathologic complete response (pCR) was defined as the absence of residual invasive cancer on hematoxylin and eosin (HE) evaluation of the complete resected breast specimen and all sampled regional lymph nodes following the completion of NCT [24] (ie, ypT0/Tis ypN0 in the current AJCC staging system); non-invasive breast residuals (ductal carcinoma in situ) were allowed.

TILs Analysis After using the self-training tutorial supplementing the recently published guideline recommendations by the international TILs working Group 2014 [25], an experienced BC pathologist semiquantified stromal TILs using 4–5 μm thick HE-stained slides. Stromal TILs are mononuclear inflammatory cells that are seen in the stroma between nests of tumor cells. The term intratumoral TILs referred to mononuclear inflammatory cells that may be found in areas of solid nests of tumor where neoplastic cells are touching with no intervening stroma. Evidence suggests that the extent of lymphocytic infiltration in tumor tissue can be assessed as a major parameter using standardized visual assessment by evaluation of HE-stained tumor sections. According to these recommendations the proportion of surface area occupied by any mononuclear inflammatory cell infiltrate including lymphocytes, and plasma cells (granulocytes and other polymorphonuclear leukocytes are excluded) in designated areas of stroma was recorded. Available data suggest that evaluation of stromal TILs is more reproducible than intratumoral TILs evaluation [26]. TILs were assessed as the percentage of tumor stroma containing infiltrating lymphocytes and plasma cells as a continuous scale. Cases with 50% TILs or more were categorized as lymphocyte-predominant BC (LPBC) [25].

Study Outcomes and Hypothesis

The primary outcome of this observational study was to assess and quantify the presence of TILs in BC patients with progressive disease during NCT and to compare their level to patients with excellent tumor response (pCR). As for the secondary outcome and in an exploratory analysis we aimed to study the association between the level of TILs (LPBC versus low TILs) and the baseline characteristics as well as the survival outcomes.

Table 1 Baseline characteristics by group (patients with pathologic Complete Response (pCR) versus patients with progressive disease)

Baseline characteristics	Patients with pCR $N = 29$	Patients with progressive disease $N = 21$	p-value
Mean age (SD[a])	50.2 (0.2)	57.5 (12.9)	0.03*
Node + (%)	15 (71)	21 (75)	0.8
Tumor grade			
G2[b] (%)	9 (36)	4 (19)	0.2
G3[b] (%)	16 (64)	17 (81)	
TNM staging			
Stage 2 (%)	20 (71)	5 (24)	0.001*
Stage 3 (%)	8 (29)	16 (76)	
ER[c] status			
ER+ (%)	8 (28)	4 (19)	0.5
PR[d] status			
PR+ (%)	6 (21)	2 (9.5)	0.4
HER2 status			
HER2+ (%)	18 (62)	5 (24)	0.007*
TNBC[e] (%)	8 (26)	12 (57)	0.035*
Chemotherapy regimens[f] N (%)			
dd AC-P[g]	4 (19)	15 (52)	
FEC-D[h]	6 (28.5)	8 (28)	
AC-D[i]	0	1 (3)	
TC[j]	5 (24)	2 (7)	
ET[k]	0	1 (3)	
Other	6 (28.5)	2 (7)	

[a] SD: Standard deviation

[b] Grade2/Grade3

[c] Estrogen Receptor

[d] Progesterone Receptor

[e] Triple negative breast cancer

[f] Chemotherapy regimens with/without Trastuzumab

[g] dose dense Adriamycin Cyclophosphamide Paclitaxel

[h] 5 FU Epirubicin Docetaxel

[i] Adriamycin Cyclophosphamide Docetaxel

[j] Taxotere Cyclophosphamide

[k] Epirubicin Taxotere;

[*] statistically significant

The study hypothesis was that patients with progressive disease have lower levels of TILs and worse outcomes.

Statistical Methods

All the treatment, patient and tumor's characteristics deemed to be clinically relevant for our study hypothesis were captured at diagnosis and surgery.

All continuous variables were reported as means and medians with standard deviation and interquartile ranges as appropriate. Normality was assessed by the Shapiro-Wilk test of normality and histograms. All categorical variables were reported as frequency counts and proportions.

Median follow up time was estimated using the reverse Kaplan Meier estimator [27].

Primary Outcome In order to compare the level of TILs as a categorical and continuous variable between patients with progressive disease and the ones with pCR, a t test, Wilcoxon test and Fisher's exact test were used as appropriate.

Exploratory Outcome A Fisher's exact test was used to assess the association between the baseline characteristics (age, ER, PR and HER2 status, tumor stage, grade and LVI) and TILs (LPBC) separately. T0 was defined as the time at diagnosis. DFS was defined as the time from T0 till the date of first recurrence or the date of last follow-up if no recurrence

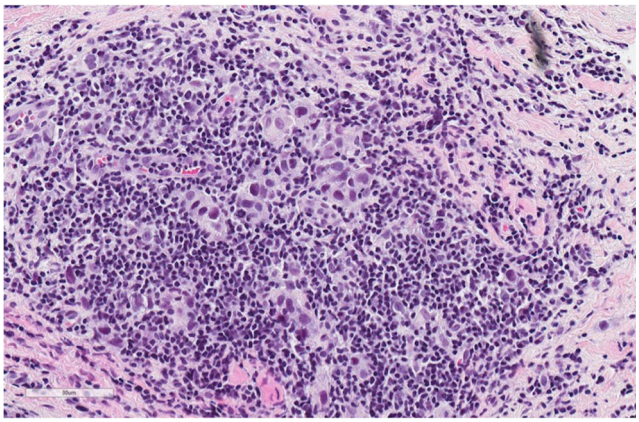

Fig. 1 Breast cancer patient with a pathologic complete response and a high level of tumor infiltrating lymphocytes (> = 50%) (Magnification 400×)

occurred. OS was defined as the time from T0 till the date of death or the date of last follow-up if no death occurred. Patients with no recurrence or death at last follow-up were censored. A Kaplan Meier method was used to estimate the survival outcomes and a log-rank test to compare the survival outcomes between 2 groups [28]: patients with high (LPBC, > = 50%) versus low TILs (<50%).

We did not conduct multivariable regression models for the primary and secondary outcomes as the number of events and patients were too low to fit any model. SAS University Edition was used for the analysis. A two-tailed *p*-value of <=0.05 was considered statistically significant for our analyses.

Results

Patients' Characteristics

Out of 413 patients with early and locally advanced BC treated with NCT, 30 patients (7.2%) had disease progression during their therapy. These patients were identified along with 30 other patients who achieved pCR. Out of the 60 patients evaluated, 50 were successfully assessed for TILs analyses: 21 with disease progression and 29 with pCR.

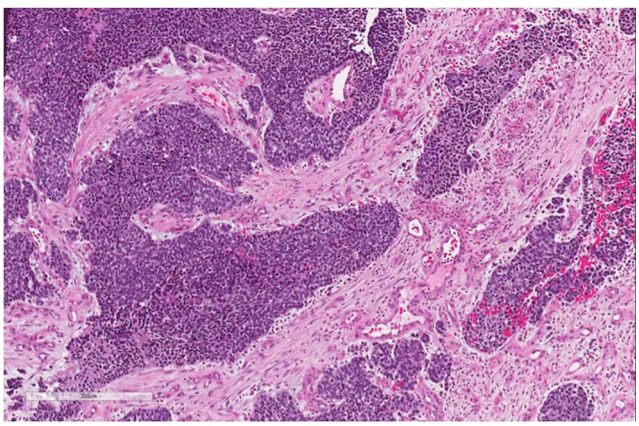

Fig. 2 Breast cancer patient with disease progression during neoadjuvant chemotherapy and low levels of tumor infiltrating lymphocytes (<50%) (Magnification 400×)

Table 2 Association between baseline characteristics and Tumor Infiltrating Lymphocytes (TILs) (Lymphocyte Predominant Breast Cancer (LPBC) versus low TILs)

Association between baseline characteristics and TILs	p-value
Nodal status	0.24
Tumor grade	0.29
ER[a] status	0.25
PR[b] status	0.66
HER2 status	0.3
TN[c] phenotype	0.78
T stage	0.12
TNM stage	0.6
LVI[d]	1.0

[a] Estrogen Receptor
[b] Progesterone Receptor
[c] Triple negative
[d] Lymphovascular invasion

The median follow up period for the study population was 35.52 months (24.73–46.97). Patients with progression during NCT were older with more advanced disease (stage III) than patients with pCR ($p = 0.03$ and $p = 0.001$ respectively). Furthermore, more patients with TN disease progressed during NCT and more HER2+ patients achieved pCR ($p = 0.03$, $p = 0.007$ respectively). Less than 20% of the patients in each group had a hormone receptor positive, HER2 (−) phenotype (luminal A and B). The 2 most common chemotherapy regimens used were FEC-D (5FU, Epirubicin, Cyclophosphamide, Docetaxel) (6/21) and TC (Taxotere, Cyclophosphamide) (5/21) for the patients who progressed during therapy and dose dense AC-P (Adriamycin, Cyclophosphamide, Paclitaxel) (15/29) and FEC-D (8/29) for the patients who achieved a pCR. The baseline characteristics are presented in Table 1.

TILs Assessment by Group (Patients with Progression Versus Patients with pCR)

The mean level of TILs was significantly lower in patients with progression compared to the patients with pCR. Patients with progression had a mean level of TILs of 14.3 (Standard Deviation (SD): 16.9) compared to 32.8 (SD: 31) ($p = 0.01$). When TILs were assessed as a categorical variable (LPBC) there was only a trend toward significance in the same direction (2 patients with progression and 9 patients with pCR were LPBC respectively (9.5% versus 31%, $p = 0.06$)).

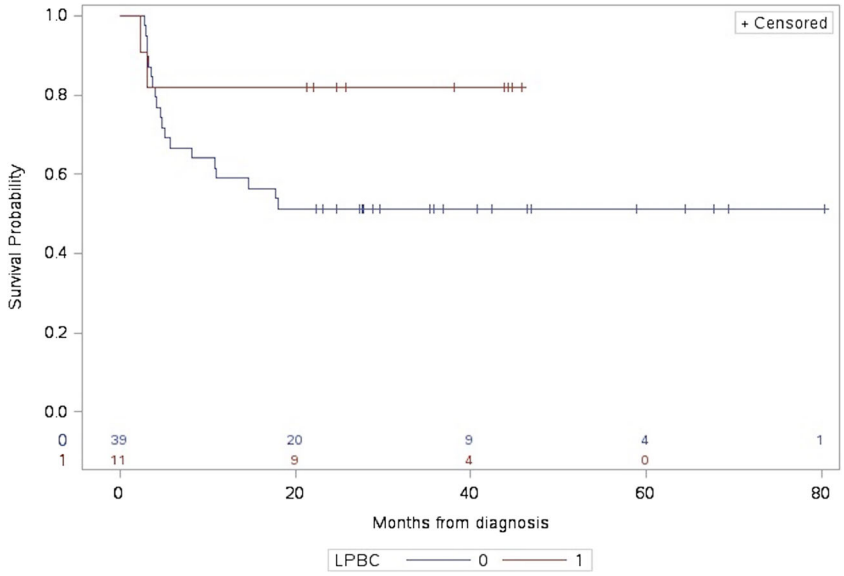

Fig. 3 Association between tumor infiltrating lymphocytes (TILs) (Lymphocyte predominant breast cancer (LPBC) versus low TILs) and Disease Free Survival (DFS). *Log-rank test, p = 0.12*

To illustrate the results, Figs. 1 and 2 represent a patient with pCR and high level of TILs and a patient with disease progression during NCT and low level of TILs respectively.

Association between Baseline Characteristics and TILs (LPBC)

None of the baseline characteristics were associated with TILs when each characteristic was assessed as a categorical variable separately. Furthermore in a univariate analysis, the type of chemotherapy regimen used in the neoadjuvant setting was neither associated with the TILs level nor with the pCR rates at surgery ($p > 0.05$). We were only able to conduct a univariate analysis because of the small number of patients limiting the possibility to include potential confounders in the analysis. The results are presented in Table 2.

Association between TILs (LPBC) and Survival Outcomes (DFS and OS)

TILs were not statistically associated with survival outcomes. However numerically, patients with high TILs (LPBC) seemed to have better survival outcomes. At 3 years from diagnosis, 51.2% versus 82% of the patients with low versus high TILs (LPBC) were recurrence-free respectively (log-rank test, $p = 0.12$) (Fig. 3) and 80% versus 86% of the patients with low

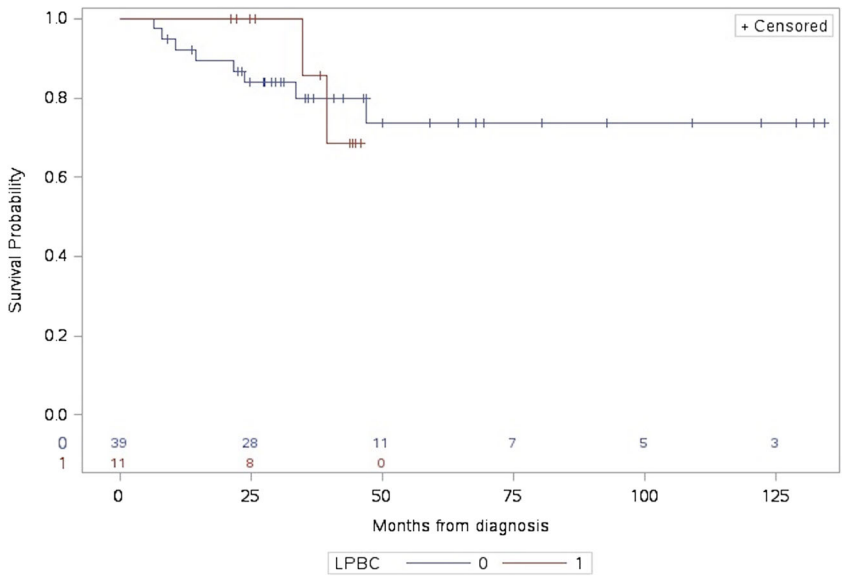

Fig. 4 Association between tumor infiltrating lymphocytes (TILs) (Lymphocyte predominant breast cancer (LPBC) versus low TILs) and Overall Survival (OS). Log-rank test, $p = 0.9$

versus high TILs (LPBC) were still alive (log-rank test, $p = 0.9$) (Fig. 4). Of note, the type of chemotherapy regimen used in the neoadjuvant setting was not associated with the survival outcome ($p = 0.95$).

Discussion

A minority of patients with BC progress during NCT [13, 14] and only few studies assessed this population and identified markers of progression in order to optimize tumor response and survival [13, 14]. Nevertheless, the markers identified (high tumor grade, low or negative ER status) could also serve as markers of tumor response [6–8], rendering their use very limited in the clinical setting.

Consequently, other valid biomarkers are needed to identify these patients early during their therapy in order to implement different treatment strategies to increase tumor response rates and ultimately survival outcomes. Among those, TILs might play an interesting role in that setting as we already know that patients with high levels of TILs known as LPBC were shown to have better pCR rates and survival outcomes mainly in TN and HER2+ BC phenotypes [15, 19, 20].

To our knowledge, this is the first study quantifying TILs in BC patients who progress during NCT. Our findings are in line with our hypothesis and the literature as patients with progression, who are considered poor responders, had significantly lower levels of TILs compared to patients with pCR despite the small number of patients included.

On another hand, neither the baseline characteristics nor the survival outcomes differed between patients with low versus high TILs even if numerically patients with high TILs seemed to have better survival outcomes. The lack of association between TILs and baseline characteristics is in line with a recent meta-analysis, where TILs were not associated with BC clinical or pathologic features [29] and only predicted better pCR rates and outcomes as discussed previously.

We acknowledge that this study has several limitations mainly related to its observational retrospective nature. In addition, the low number of patients identified and included could have rendered the study underpowered to detect any significant association between TILs and the baseline characteristics as well as the survival outcomes and prevented us from conducting multivariable analyses to control for any potential confounder. However only few BC patients progress during NCT (≈5%) [13, 14] and have a poor outcome. Hence the need to have multi-institutional work to expand the cohort size to achieve practical and statistical significance and to evaluate at an early stage this high-risk population encountered in the clinic. Furthermore, although estimation of TIL levels in breast tumors has appeared in recent pathology guidelines, 2 pathologists should have independently scored the TIL levels with the concordance reported and a consensus or average result derived for each specimen. However, the pathologist involved in this project is an experienced senior BC pathologist who previously scored several TILs projects and was blinded to the patients' clinical outcomes; thus the risk of measurement bias was reduced significantly.

Our study results are exploratory in nature and we cannot have any strong conclusions regarding the role of TILs in BC patients progressing during NCT. However these findings are hypothesis generating in that TILs can play an important role in identifying BC patients who will progress during NCT at an early stage before clinical and/or radiographic progression. In that setting, TILs might help us select patients who will benefit the most from a neoadjuvant chemotherapy approach (patients with high levels of TILs) and implement different and/or new treatment strategies [e.g. immunomodulators (checkpoint inhibitors) with or without radiation therapy] for patients with low TILs to increase their expression and improve tumor responses rates and patients' outcomes subsequently. This hypothesis should be tested in a well-designed prospective study to validate the predictive value of TILs in this specific population and ultimately improve patients' prognoses.

As the American Society of Clinical Oncology has outlined recently that the use of TILs as prognostic and predictive biomarkers in early BC may represent a new dawn, but it is not yet ready for prime time [30].

Conclusion

Patients with non-metastatic BC who progress during NCT have lower levels of TILs compared with patients achieving pCR. However in this study, TILs were not associated with baseline characteristics and survival outcomes. Further prospective work is needed to study the utility of TILs as valid biomarkers in identifying this high-risk population at an early stage and implement new treatment strategies to improve tumor response rates and survival.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Informed Consent Obtained from all patients.

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