RESEARCH ARTICLE

Editorial Process: Submission:03/16/2022 Acceptance:08/06/2022

Stromal Tumor Infiltrating Lymphocytes (sTILs) Were Associated with a Higher Grade and a Lower Stage of Indonesian Triple Negative Breast Cancers

Irianiwati Widodo[1], Ahmad Ghozali[1], Ibnu Purwanto[2], Paranita Ferronika[1]*

Abstract

Objective: This study aimed to investigate the association of sTILs with clinicopathological parameters and overall survival (OS) in patients with triple negative breast cancer (TNBC). **Methods:** One hundred and twenty-five paraffin embedded tissue of patients with TNBC, collected from Dr. Sardjito General Hospital Yogyakarta, Indonesia, between 2008-2017, were used in this study. Stromal TILs were examined from hematoxylin and eosin (H&E)-stained samples, and classified as either low or high score using 20% cut-off. Analysis of the association of sTILs with clinicopathological parameters, relative risk (RR) and OS used 95% confidence interval (CI) with significance set as $p<0.05$. **Results:** The higher proportion of TNBC patients in this study were ≥40 years old (83.3%), high tumor grade (68%), tumor stage >IIIa (56%), alive (50.4%), and with low sTILs (54.4%). The results showed significant association between sTILs and a higher grade or a lower stage of tumor (B = 0.259, 95%CI = 0.090-0.468, p = 0.004 and B = -0.255, 95%CI = -0.433 - -0.080, p = 0.005, respectively). Meanwhile sTILs were not associated with age at diagnosis (B = 0.027, 95%CI = -0.193 – 0.264 p = 0.758 nor 3-year OS of patients (HR = 0.342, 95%CI = 0.41 - 1.43 p = 0.402). **Conclusion:** The results indicate that sTILs may serve as an additional pathological parameter for TNBC.

Keywords: Lymphocytes- tumor infiltrating- neoplasm grading- neoplasm staging- triple negative breast neoplasms

Asian Pac J Cancer Prev, **23 (8)**, 2749-2754

Introduction

Triple negative breast cancer (TNBC) is an aggressive subtype of breast cancer with a poor prognosis. This cancer is characterized by the absent expression of hormone receptors and HER2. TNBC accounts for 15 to 20% of breast cancer cases worldwide (Foulkes et al., 2010; Hubalek et al., 2017). Our previous study found that Indonesian TNBC accounted for 29.3% of breast cancer cases, with an aggressive behavior including large size tumor, high grade and 70% with lymph node metastasis (Widodo et al., 2019). TNBC is unresponsive to endocrine therapy or therapy that targets Her2. Chemotherapy has become a standard treatment for TNBC. Although TNBC tends to respond well to initial chemotherapy, it also recurs more frequently than other breast cancer subtypes. New effective treatment strategies for TNBC are urgently needed (Alluri and Newman, 2014).

Recent studies have evaluated the important role of tumor-infiltrating lymphocytes (TILs) in influencing the disease course of TNBC. These TILs are widely recognized in intra-tumor and adjacent stromal tissues as a predictor of good prognosis in both adjuvant and neoadjuvant settings of TNBC (García-Teijido et al., 2016; Vikas et al., 2018). The presence of TILs may aid in selecting patients who may benefit from chemotherapy such as Carboplatin. A high number of TILs was also associated with an increased pathologic complete response (pCR), and as a predictive marker of immunotherapy response (Borcherding et al., 2018). New immune modulator agents, including immune checkpoint inhibitors, have shown promising effects in patients with TNBC (García-Teijido et al., 2016).

Previous studies found the association of TILs with some clinicopathological parameters including patient's prognosis. TNBC with a high level of TILs showed better short-term and long-term patient's prognosis (Gao et al., 2020), even in the patients without adjuvant chemotherapy (Park et al., 2019). A study in India found that high sTILs were significantly associated with tumor stage, but not with tumor grade and age of patients with TNBC (Goel et al., 2013). Another study found that TIL score was significantly increased with the increasing histologic grade of breast cancer (Cha et al., 2018). Higher tumor grade was also associated with higher sTILs. Distribution of TILs was also significantly associated with positive lymph node status and higher tumor grade of breast cancer.

*[1]Department of Anatomic Pathology, Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada, Indonesia. [2]Department of Internal Medicine, Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada, Indonesia. *For Correspondence: paranita.ferronika@ugm.ac.id*

Higher TIL proportions were associated with an aggressive biological phenotype, which tended to be more responsive to chemotherapy (Miyoshi et al., 2018). Up to now, there are limited studies of TILs among Indonesian TNBC patients. This study aimed to determine the association of sTILs with clinicopathological parameters and its role in predicting the overall survival (OS).

Materials and Methods

This retrospective study used 125 paraffin embedded tissue samples of patients with TNBC, collected from Dr. Sardjito General Hospital Yogyakarta Indonesia, between 2008- 2017. All of the patients underwent surgery with axillary lymph node dissection. None of patients had received preoperative therapy. Clinicopathological parameters including age of patient, tumor stage, age at diagnosis, and status of patient during follow-up period, were obtained from the patients' medical records along with the informed consent from the patients. The cut-off value of age at diagnosis used in our statistical analysis is 40 years, as suggested by others (Khanal et al., 2020; Liedtke et al., 2013; Verma et al., 2021). The tumor grade was classified into two categories; low grade and high grade. The tumor stage was classified into two categories; early stage (stage I-II) and late stage (stage IIIa). The use of human material and clinical data from patients in this study was approved by the Medical and Health Research Ethics Committee at the Faculty of Medicine, Universitas Gadjah Mada (UGM), Indonesia, as recognized by the Forum for Ethical Review Committee in Asia and the Western Pacific/FERCAP (KE/FK/0751/EC/2018).

Stromal TILs were evaluated from hematoxylin and eosin (H&E) stained slides and defined as percentage of lymphocytes over the analyzed stromal area. The evaluations were performed in five high power fields (400x magnification) and the counts were averaged. As suggested by others (Pruneri et al., 2016; Ruan et al., 2018),the percentage of sTILs between 1-20% was considered low, while the percentage of sTILs > 20% was considered high (Figures 1A and 1B). Evaluation of sTILs was conducted by two independent observers.

Statistical analysis was performed using SPSS statistical software: release 26 (IBM Corp., Armonk, NY). The association between sTILs, age at diagnosis, tumor grade, and tumor stage was examined by univariable analysis using linear regression model, which were done two-sided with p-value <0.05 considered statistically significant.

The association of each parameter with 3-year overall survival was tested. The survival time was measured from the time of first diagnosis to the time of death in months. Cox regression models were used to estimate hazards ratios (HR) as relative risks (RR) with 95% confidence intervals (CI). Analyses were done without adjustment for other parameters (first model) and a multivariable-adjusted model included the following confounders: sTILs, age at diagnosis, tumor grade, and tumor stage (second model). A two-sided p-value <0.05 was considered as statistically significant. Kaplan-Meier curves and Breslow tests were used to evaluate the OS with sTILs and tumor stage.

Results

Mean age of patients with TNBC studied was 50.67 ± 11.23 years old. Out of 125 patients with TNBC, the majority of the cases were ≥ 40years old (83.20%), with high tumor grade (68%) and high tumor stage (56%) (Figure 2). The number of patients alive at the end of the study was 50.40% and the number of low sTILs was 54% (Figure 2).

In the present study, we found a statistically significant association between sTILs and a higher tumor grade or a lower tumor stage of patients with TNBC. Meanwhile, sTILs were not associated with the age at diagnosis (Table 1).

In the Cox regression models, we observed no significant association between sTILs and 3-year OS (Table 2). Although not statistically significant, Kaplan Meier curves showed patients with high sTILs had higher survival rates than the patients with low sTILs (Figure 3a). Meanwhile, a significant association was found between tumor stage and 3-year OS in the first model and second model (Table 2). This association was also visualized by

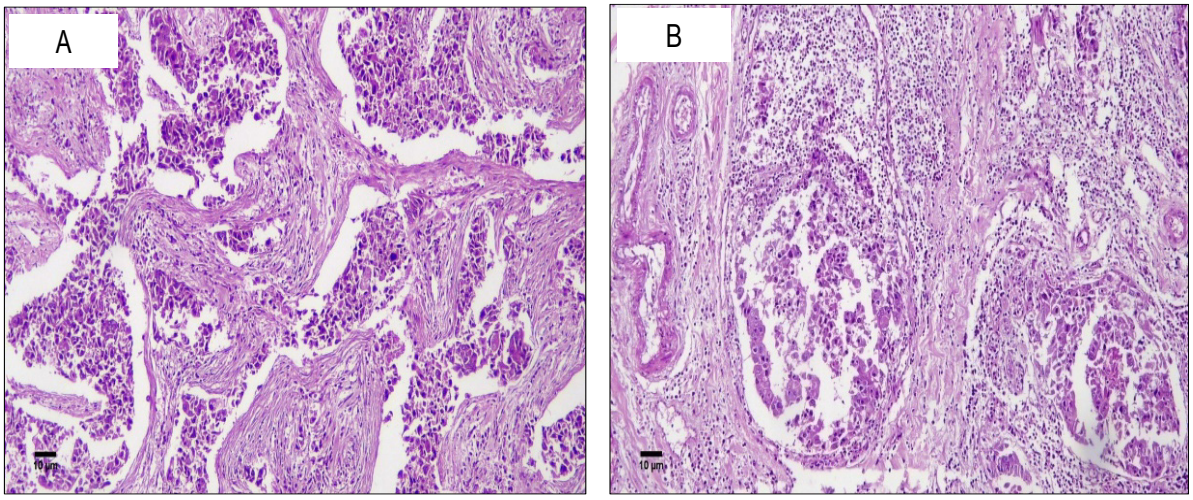

Figure 1. Histopathological Picture of TNBC Patients with Stromal Tumor-Infiltrating Lymphocytes (sTILs) Categorized as Low (A) and High Score (B) (H&E stain, ×100).

Table 1. Linear Regression Model on the association between Stromal Tumor-Infiltrating Lymphocytes (sTILs) and Clinicopathological Characteristics of Patients with Triple Negative Breast Cancer (TNBC). The cut-of for a statistically significant p-value is 0.05.

Characteristics		sTILs		p-value	B (coefficients)	(95% CI for B)
		High	Low			
Age						
	≥ 40 yo (104)	48	56	0.758	0.027	(-0.193 - 0.264)
	< 40 yo (21)	9	12			
Grade						
	High (86)	45	41	0.004	0,259	(0.090 - 0.468)
	Low (39)	12	27			
Stage						
	Late (70)	26	44	0.005	-0.255	(-0.433 - -0.080)
	Early (55)	31	24			

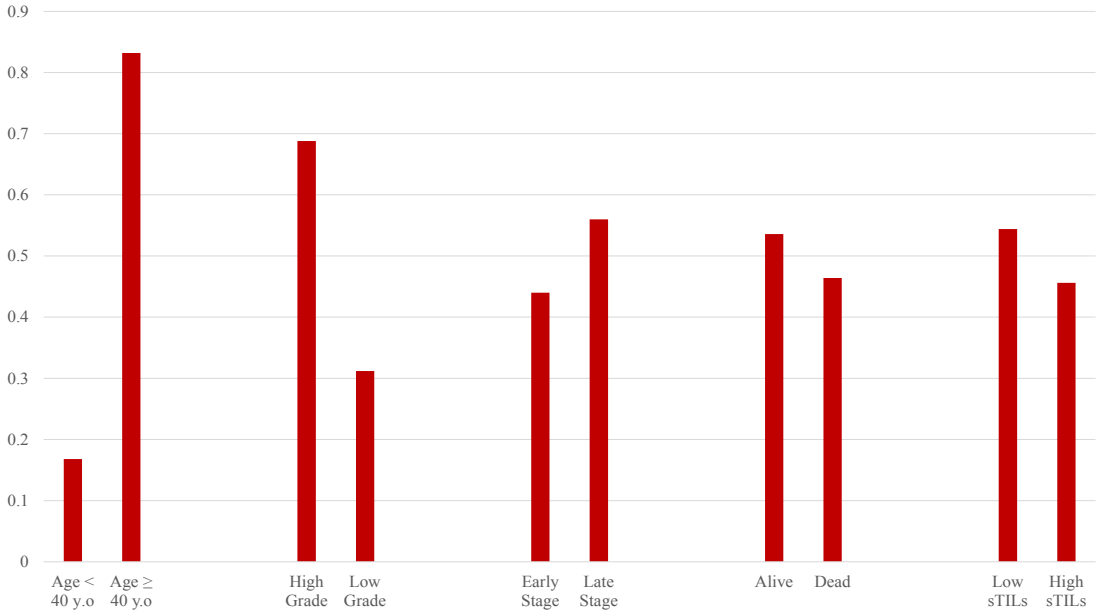

Figure 2. Characteristic of Local Triple Negative Breast Cancer (TNBC) Samples

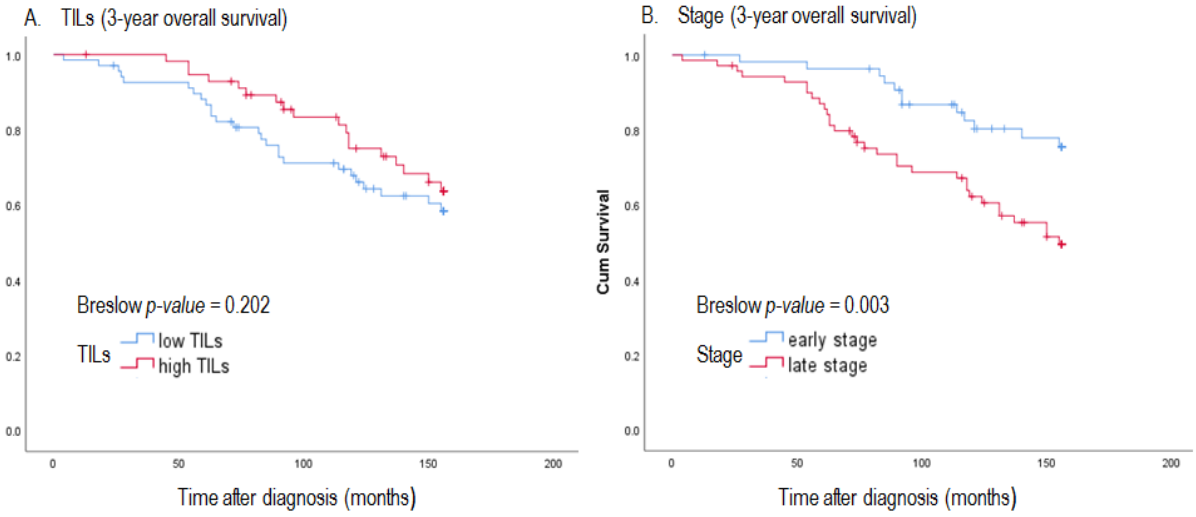

Figure 3. Kaplan Meier Curves for the Association of Tumor Infiltrating Lymphocytes / TILs (A) and stage (B) with 3-Year Overall Survival

Table 2. Hazard Ratios (HRs) for Overall Deaths Truncated at 3 Years of Follow-up According to Parameters of Age, TILs, Tumor Grade, and Tumor Stage. The cut-off for a statistically significant p-value is 0.05

Parameters	Total no. of cases	No. of deaths	Survival time, months	Model 1 [a]			Model 2 [b]		
				HR	(95% CI)	p-value	HR	(95% CI)	p- value
TILs									
High	57	18	126	1	Ref.		1	Ref.	
Low	68	26	116	0.75	(0.41-1.36)	0.342	0.77	(0.41-1.43)	0.402
Age									
≥40	104	25	126	1	Ref.		1	Ref.	
<40	21	9	118	0.75	(0.36-1.56)	0.433	0.6	(0.29-1.28)	0.186
Grade									
High	86	35	119	1	Ref.		1	Ref.	
Low	39	9	124	1.85	(0.89-3.84)	0.101	1.74	(0.81-3.76)	0.158
Stage									
Late	70	32	113	1	Ref.		1	Ref.	
Early	55	12	131	2.56	(1.32-4.98)	0.006	2.36	(1.18-4.71)	0.015

[a], Without adjustment for other parameters; [b], Adjusted for other parameters (TILS, age at diagnosis, tumor grade, and tumor stage)

Kaplan Meier curves (Figure 3B).

Discussion

Most of the patients with TNBC in our study were <40 years old and the mean age at diagnosis was 50.67 ± 11.23 years. Previous studies of TNBC patients in US showed higher mean age at diagnosis, for example, 59.87±15.67 years (Tariq et al., 2014) and 59 years (Yeh et al., 2017). Meanwhile, three studies in South Asia showed lower mean age of TNBC patient with 46.26 ± 12.22 years in Pakistan (Sajid et al., 2014), 49 ± 12 years in Nepal (Khanal et al., 2020), and 46 ± 11.23 years in India (Verma et al., 2021). Clinical characteristics of TNBC were found to be influenced by the age of patients. Patients ≤40 years have poorer survival despite aggressive systemic therapy (Liedtke et al., 2013). By categorizing patients with TNBC into younger (<40 years old) and older (>74 years old) groups, a Swedish study found significantly higher histopathological grade and ki67 index in the younger group. The same study also showed worse outcome including shorter survival times and disease-free survival in the older group (Tzikas et al., 2020).

Our study found that sTILs were not associated with 3-year OS of patients with TNBC (Table 1). This result is inconsistent with previous studies in which the presence of sTILs was associated with the positive outcome, such as longer survival (Denkert et al., 2010; García-Teijido et al., 2016; Park et al., 2019). In the present study, tumor stage as a previously established tumor prognostic parameter (Urru et al., 2018), was significantly associated with 3-year OS.

Many studies proved that tumor lymphocytic immune infiltrates (TILs) have an important role in TNBC. The presence of TILs predicts which patient will benefit from chemotherapy such as Carboplatin (Denkert et al., 2010). High levels of stromal TILs (sTILs) have been associated with improved disease-free survival and OS rates in TNBC patients with and without any treatment. The presence of sTILs in the stroma can also predict the responses of both neoadjuvant and adjuvant chemotherapy treatments. High numbers of sTILs correlate with an increased pathologic complete responses (pCR) in TNBC. Thus, the immune cells appeared to strongly influence the prognosis of patients with TNBC (García-Teijido et al., 2016; Park et al., 2019). New immune modulatory agents, including immune checkpoint inhibitors, have shown promising treatment in TNBC. Increased programmed cell death protein 1 ligand (PD-L1) expression on the surface of immune cells provides the rationale for implementing therapeutic strategies targeting the PD-1/PD-L1 axis in TNBC (Brockhoff et al., 2018).

The relationship between clinicopathologic parameters of TNBC and TILs needs to be further studied. Our study results demonstrated that sTILs was associated with a higher tumor grade and a lower tumor stage of TNBC. Similar results were found by other previous studies that showed higher scores of sTILs were related with higher histological grade and higher ki67 index (Cha et al., 2018; Krishnamurti et al., 2017; Ruan et al., 2018). Inconsistent with these findings, results from a study conducted by Goel et al. (2013) found sTILs were significantly associated with tumor stage, but not with tumor grade (Goel et al., 2013). The use of different cut-off value of TILs might contribute to the inconsistency in these results.

Up to now there is no standardized methodologies for TILs assessment, nor for establishing the cut-off value of TILs to predict therapeutic response. The International TILs Working Group recommendation suggested 50% as the cut-off for lymphocyte-predominant breast cancer (LPBC), however, the low proportion of the cases limits its power. Several researchers used different cut-offs for TILs assessment, such as 10% (Loi et al., 2014), 20% (Pruneri et al., 2016; Ruan et al., 2018), 25% (Goel et al., 2013) and 40% (Herrero-Vicent et al., 2017). The use of standardized cut-off value of TILs is very important, especially when in it is used to study two different subgroups of patients: LPBC which is suggested to have a high response to neoadjuvant chemotherapy and non-LPBC which is suggested to have a low response

(Herrero-Vicent et al., 2017), Further study is needed to evaluate the best cut-offs for TILs assessment.

In conclusion, in local TNBC patients, sTILs were not associated with age and survival, but associated with a higher tumor grade and a lower tumor stage. Up to now, there is no standardized methodology for TILs evaluation nor uniform cut-off value of TILs, which might contribute to the inconsistency among studies. Accordingly, a standardized cut-off of TILs is needed for integrating this parameter in standard histopathological practice and clinical settings.

Author Contribution Statement

IW contributed in conceptualization, investigation, formal analysis, visualization, and writing the original draft. PF contributed in conceptualization, formal analysis, visualization, and writing the original draft. AG and IP participated in the investigation, provision of sample resources, and editing the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank the staff of Klinik Bahasa, Universitas Gadjah Mada for editorial advice.

Ethical clearance

The use of human material and clinical data from patients in this study was approved by the Medical and Health Research Ethics Committee at the Faculty of Medicine, Universitas Gadjah Mada (UGM), Indonesia (KE/FK/0751/EC/2018).

Conflict of interest

The authors have no conflicts of interest to declare.

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