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Role of Ultrasonographic and Clinicopathological Characteristics in Assessing Stromal Tumor-Infiltrating Lymphocyte Density in Triple-Negative Breast Cancer

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Background: A high density of stromal tumor-infiltrating lymphocytes (sTILs) is positively correlated with the pathological complete response rate and favorable survival in patients with triple-negative breast cancer (TNBC). Heterogeneity in stromal lymphocyte distribution and limited tumor sampling may affect the accuracy of sTIL quantification in biopsy samples from patients receiving neoadjuvant therapy. Thus, identifying additional biomarkers to complement sTIL evaluation is essential.

Aims: To identify biomarkers that could be used to complement sTILs evaluation.

Study Design: Retrospective cohort study.

Methods: A total of 162 patients with invasive TNBC were enrolled in the study. The following data were gathered: sTIL density, Ki67, nuclear grades of cancer cells, lymphovascular invasion status, the American Joint Committee on Cancer stage, axillary lymph node metastasis status, and ultrasonographic parameters of the tumor (size, shape, orientation, margin, internal echo pattern, posterior feature, and vascularity). The relationship between sTIL density and ultrasonographic or clinicopathological characteristics was investigated using both continuous and categorical analyses.

Results: Posterior features of the primary tumors was associated with sTIL density ($p = 0.038$). Additionally, the Ki67 levels and nuclear grades were also associated with sTIL density ($p < 0.001$ and $p = 0.024$, respectively). When stratified according to a 20% cut-off of sTIL density, the posterior features of primary tumors, Ki67 levels, and nuclear grades significantly differed between the high and low sTIL density tumors. Tumors with high Ki67 levels were more likely to exhibit high sTIL density than low sTIL density [odds ratio (OR): 2.75, $p = 0.021$]. Furthermore, nuclear grade III tumors demonstrated significantly higher sTIL density than nuclear grade I-II tumors (OR: 2.49, $p = 0.014$). Additionally, tumors with posterior enhancement or no posterior features were more likely to exhibit high sTIL density than tumors with acoustic shadows (OR: 2.91, $p = 0.028$; OR: 2.74, $p = 0.022$, respectively).

Conclusion: Low sTIL density is frequently observed in tumors exhibiting acoustic shadows on ultrasound. However, high sTIL density is more common in tumors with posterior enhancement or no posterior features. Furthermore, high Ki67 levels (> 40%) and high nuclear grades are positively correlated with high sTIL density. Our study findings highlight the need for closer surveillance of these biomarkers to complement sTIL evaluation in TNBC.

INTRODUCTION

The immune system influences the prognosis of breast cancer.[1,2] Stromal tumor-infiltrating lymphocytes (sTILs) are frequently observed in highly proliferative tumors. Furthermore, there is a positive correlation between sTILs and both the pathological complete response and survival rate in patients with triple-negative breast cancer (TNBC).[3-5] TNBC is the most immunogenic of the breast cancer subtypes, displaying a prominent lymphocytic infiltration in the tumor stroma. Thus, the evaluation of sTILs may provide clinically significant prognostic data in TNBC.[7-10]

The estimation of sTILs is based on the visual assessment of lymphocytes and plasma cells on routine hematoxylin and eosin (H&E)-stained sections. In patients receiving neoadjuvant therapy, the baseline sTIL density in primary tumors is determined in pre-

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treatment specimens obtained using core needle biopsies. However, heterogeneity in stromal lymphocyte distribution and limited tumor sampling affect the accuracy of sTIL quantification in the biopsy samples.[11,12] Thus, additional biomarkers should be identified to complement sTILs evaluation in patients receiving neoadjuvant therapy.

Obtaining ultrasound parameters from routine clinical procedures is relatively simple. Ultrasound allows for the evaluation of the morphology and internal characteristics of breast tumors, which may provide insights into the density of sTILs. Furthermore, a few studies have postulated a potential correlation between the ultrasonographic characteristics of breast tumors and sTIL density.[13-15] However, the disparate outcomes of studies highlight the necessity for further investigation. Thus, in this study, we aimed to identify potential biomarkers that could be used to complement the evaluation of sTILs.

MATERIALS AND METHODS

Patient population

This study was approved by the Medical Ethics Committee of Nanfang Hospital, Southern Medical University (approval number: NFEC-2023-295; date: 06.07.2023). Patients with stage I-III invasive TNBC, who had been admitted to Nanfang Hospital, Southern Medical University between January 2012 and December 2018, were included in the study. Patients who had been administered neoadjuvant therapy and patients with insufficient pathological tissue for sTILs assessment, multifocal tumors, or missing pre-treatment ultrasonographic images of the breast were excluded. Ultimately, 162 patients were included in the study.

Data processing

Ultrasound was performed before the biopsy and treatment using several ultrasound machines, including the Aplio 500 (Toshiba, Japan), IU22 (Philips, Holland), and Logic E9 (GE Healthcare, USA), with high-frequency linear array transducers. The following tumor characteristics were documented by retrospectively reviewing the ultrasonographic images: size, shape, orientation, margin, internal echo pattern, posterior features, and vascularity (color Doppler). Two radiologists with > 6 years of experience, who were blinded to the imaging reports and pathological information, analyzed all the ultrasonographic images separately. The tumor features were recorded according to the Breast Imaging Reporting and Data System terminology.[16] Disagreements between the two radiologists were settled via a discussion until a consensus was reached.

The sTIL density was evaluated according to recommendations proposed by the International sTILs Working Group on Breast Cancer.[17] sTIL density was defined as the percentage of immune cells in the tumor stroma that exhibited a mononuclear immunological infiltrate. Two trained pathologists assessed the sTIL density by reviewing H&E-stained surgical specimens, using full-face tissue slices instead of biopsy sections. The entire slide was assessed, and the mean of the sTIL percentages in six regions was recorded. Thirty randomly selected patients, corresponding to 20% of the enrolled population, were separately annotated by the two pathologists to assess the inter-observer consistency of the readings. The estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and Ki67 indexes were examined by immunohistochemistry (IHC). Specimens with a HER2 index of 3+ on IHC or HER2 positivity on fluorescence in situ hybridization (FISH) were defined as HER2 positivity. However, a HER2 index of 0 or 1+ on IHC or HER2 negativity on FISH were classified as HER2 negativity. Breast cancer with an ER index < 10%, PR index < 10%, and HER2 negativity was classified as TNBC.[18] A sTIL density of < 20% and ≥ 20% was classified as low and high, respectively, on the basis of our prior study's optimal prognostic cut-off value for disease-free survival in TNBC (Li et al. 2024, manuscript submission under review) as well as another study conducted by Ruan et al.[19] We determined the cut-off value (maximum Youden index) of Ki67 levels using receiver operating characteristic analysis. Ki67 levels of ≤ 40% and > 40% were categorized as low and high, respectively. Axillary lymph node metastasis (ALNM), lymphovascular invasion (LVI), and nuclear grades of the cancer cells were confirmed by reviewing pathological data. The 8th edition of the American Joint Committee on Cancer's (AJCC) tumor, nodes and metastases staging system was used for the tumor staging.

Statistical analysis

Categorical variables have been reported as numbers, and continuous variables have been presented as medians and interquartile range (IQR). To investigate the relationship between sTIL density and ultrasonographic or clinicopathological characteristics in a comprehensive manner, we initially employed the Mann-Whitney U and Kruskal-Wallis tests with the sTIL density as a continuous variable. Subsequently, the cohort was classified into two categories according to the sTIL density (high and low). Thereafter, univariate and multivariate logistic regression analyses were utilized to examine the relationship between high or low sTIL density and the ultrasonographic or clinicopathological characteristics. Variables that were statistically significant in the univariate logistic regression were included in the multivariate regression. All analyses were carried out using SPSS Statistics (version 29.0.2.0; IBM). A $p \leq 0.05$ was considered statistically significant between groups.

RESULTS

Analyses using sTIL density as a continuous variable

The mean age of the recruited patients was 48 years (range: 27-76 years). The median sTIL density of the study patients was 12.5% (IQR, 5.8-29.7%). The ultrasonographic and clinicopathological characteristics of the study patients and their correlation with sTIL density are summarized in Table 1. Only the posterior features of the primary tumor demonstrated a correlation with sTIL density ($p = 0.038$, Table 1, Figure 1).

We examined the ALNM status, nuclear grades of cancer cells, AJCC stage, LVI status, and Ki67 levels of the tumors and their association with sTIL density. We identified a correlation between Ki67 levels and nuclear grades and the sTIL density (Table 1). Tumors with high

TABLE 1. Ultrasonographic and Clinicopathological Characteristics of Tumors and Their Association with sTIL Density

Variables	Number	sTIL density	p
Shape			0.113
Regular	30	20.8 (6.7, 46)	
Irregular	132	11.7 (5.8, 26.7)	
Orientation			0.813
Parallel	40	14.6 (5.3, 29.7)	
Vertical	122	12.3 (6.2, 29.5)	
Margin			0.558
Circumscribed	26	9.2 (5.8, 28.9)	
Indistinct	24	9.8 (5.3, 21)	
Microlobulated	33	15.8 (10, 35)	
Angular	55	11.7 (4.9, 29.2)	
Spiculated	24	16.1 (8.3, 29.6)	
Internal echo pattern			0.577
Hypoechoic	152	13.9 (5.8, 30)	
Isoechoic	1	11.7	
Complex cystic and solid	9	7.5 (6.5, 12.8)	
Hyperechoic	0	-	
Heterogeneous	0	-	
Posterior features			0.038
Shadowing	57	10 (4.8, 19.2)	
Enhancement	39	16.7 (8.2, 35.8)	
No posterior features	66	15 (5.9, 31.5)	
Vascularity			0.138
Absent	42	10 (5, 21.7)	
Internal or peripheral	120	14.2 (6.17, 31.2)	
Tumor size			0.085
< 2cm	57	9.2 (4.5, 26.7)	
≥ 2cm	105	14.2 (6.5, 30.8)	
ALNM			0.76
No	113	12.5 (5.7, 30)	
Yes	49	12 (6.2, 28.3)	
AJCC stage			0.114
I	47	8.9 (4.3, 24.2)	
II	77	18.3 (6.2, 31.1)	
III	38	11.7 (6.9, 22.3)	
Nuclear grade			0.024
I-II	76	10.9 (5, 22.3)	
III	86	14.3 (6.7, 35.8)	
Ki67 level			<0.001
≤ 40%	47	7.5 (4, 16.9)	
> 40%	115	15 (6.7, 33.6)	
LVI			0.332
No	143	13.6 (6.2, 30)	
Yes	19	11.7 (5, 23.8)	
Sum	162	12.5 (5.8, 29.7)	

*Data has been presented as median (interquartile range). sTIL, stromal tumor-infiltrating lymphocyte; ALNM, axillary lymph node metastasis; AJCC, American Joint Committee on Cancer; LVI, lymphovascular invasion.

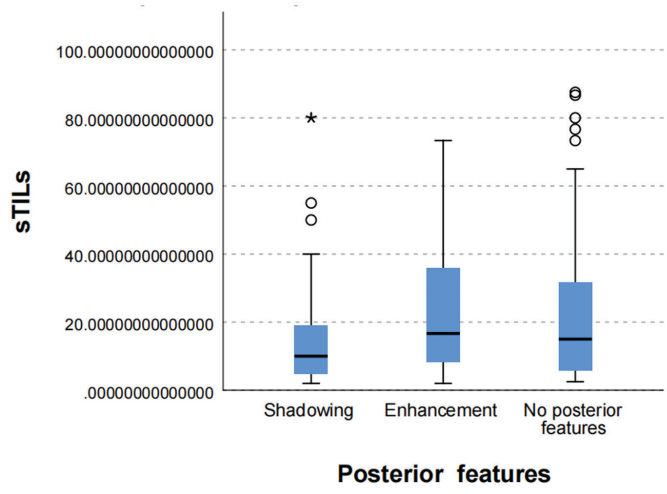

FIG. 1. Tumors with acoustic shadows on ultrasonography exhibited a lower median sTIL density than tumors with posterior enhancement or no posterior features.
sTIL, stromal tumor-infiltrating lymphocyte.

Ki67 levels exhibited a higher median sTIL density than those with low Ki67 levels (15% vs. 7.5%, $p < 0.001$, Table 1, Figure 2a). Tumors classified as nuclear grade III demonstrated a higher median sTIL density than those classified as nuclear grade I-II (14.3% vs. 10.9%, $p = 0.024$, Table 2, Figure 2b). No discernible difference in sTIL density was observed across groups with varying LVI status, AJCC stages, or ALNM status ($p = 0.760$, $p = 0.114$, and $p = 0.332$, respectively; Table 1).

Analyses using sTIL density as a categorical variable

After classifying tumors on the basis of a cut-off sTIL density of 20%, 103 (63.6%) tumors were included in the low sTIL density group, while 59 (36.4%) were included in the high sTIL density group. The association between ultrasonographic or clinicopathological characteristics and high or low sTIL density is summarized in Table 2.

The posterior features of the primary tumors, Ki67 levels, and nuclear grades were significantly different between tumors with high or low sTIL density. Tumors with high Ki67 levels were more likely to exhibit high sTIL density than low sTIL density [odds ratio (OR): 2.75, 95% confidence interval (CI): 1.17-6.46, $p = 0.021$, Table 2]. Only 9 (19.1%) of the 47 tumors with low Ki67 levels exhibited high sTIL density. Tumors classified as nuclear grade III exhibited

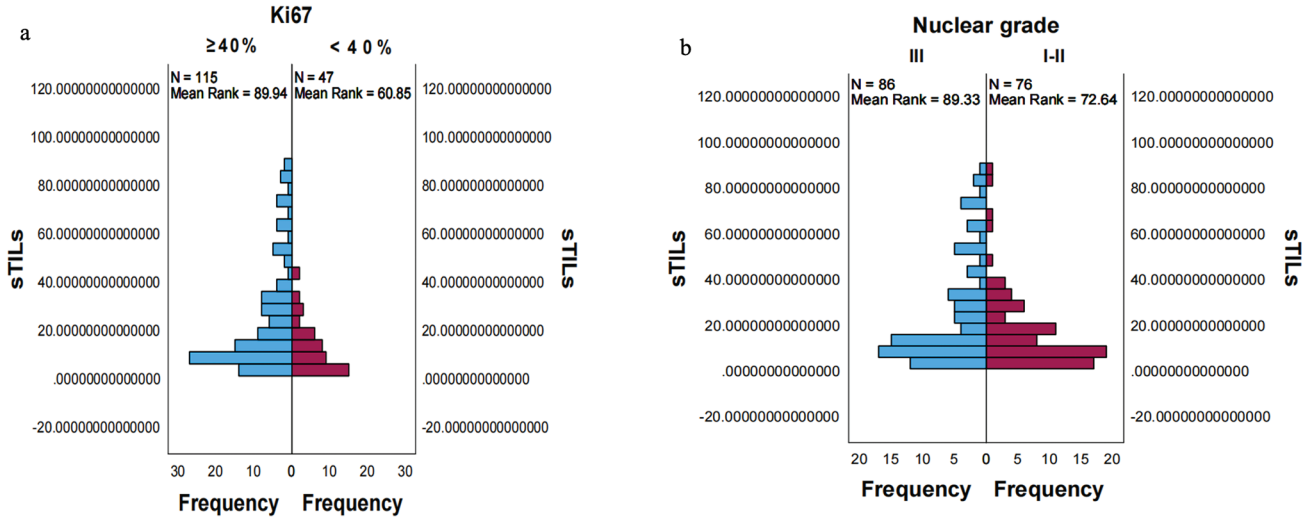

FIG. 2. (a) Tumors with high Ki67 levels exhibited a higher sTIL density than those with low Ki67 levels. (b) Tumors classified as nuclear grade III demonstrated a higher sTIL density than those classified as nuclear grade I-II.

sTIL, stromal tumor-infiltrating lymphocyte

a significantly higher sTIL density than those classified as nuclear grade I-II (OR: 2.49, 95% CI: 1.20-5.17, $p = 0.014$, Table 2). Tumors with posterior enhancement or no posterior features were more likely to exhibit high sTIL density than tumors with acoustic shadows (OR: 2.91, 95% CI: 1.12-7.52, $p = 0.028$; OR: 2.74, 95% CI: 1.16-6.47, $p = 0.022$, respectively; Table 2). A majority (77.2%) of the tumor s with acoustic shadows had a low sTIL density.

A representative example of the posterior features on ultrasound and sTIL density in specific patients are shown in Figure 3. In one 47-year-old female patient with a right-sided TNBC, the ultrasound revealed a hypoechoic tumor with posterior enhancement, and the H&E-stained specimen revealed dense lymphocytic infiltration in the stroma (high sTIL density of 70%) (Figure 3a, b). In another 51-year-old woman with right-sided TNBC, the ultrasound revealed a hypoechoic tumor with an acoustic shadow, and the H&E-stained specimen revealed sparse lymphocytic infiltration in the stroma (low sTIL density of 5%) (Figure 3c, d).

DISCUSSION

To guarantee the reliability of the sTIL evaluation, the two pathologists underwent comprehensive training, and the inter-observer consistency was evaluated. The results demonstrated that the median sTIL density among the enrolled patients with invasive TNBC was 12.5%. Furthermore, 63.6% of the TNBC tumors exhibited a low sTIL density, while 36.4% demonstrated a high sTIL density. This finding is consistent with those of previous studies.[7,20]

The percentage of sTILs was a continuous factor, ranging from 0 to nearly 100%. From a statistical perspective, the continuous analysis of sTIL density may provide a more precise description of tumor biology. However, categorizing patients into different groups makes stratification in clinical trials easier. Thus, classification of sTILs into low- and high-density groups may be useful for future clinical applications. In our study, both the continuous and categorical analyses of sTIL demonstrated comparable results of a correlation between sTIL density and the tumor posterior features on ultrasound, Ki67 levels, and nuclear grades.

Till date, only a few studies have assessed the relationship between sTIL density and the ultrasonographic features of breast tumors.[13-15] However, the results are inconclusive. In the current study, we identified a significant association between sTIL density and the posterior features of the tumor. Tumors with acoustic shadows were more likely to exhibit low sTIL density than high sTIL density. This finding is consistent with that of Fukui et al.[13] and Candelaria et al.[15] However, Çelebi et al.[14] did not identify a significant difference in sTIL density between the groups of posterior features. Nonetheless, in their study, tumors with high sTIL density exhibited circumscribed margins, a round shape, heterogeneous echogenicity, and a larger size on ultrasound imaging. Conversely, Candelaria et al.[15] discovered that TNBC tumors with high sTIL density were more likely to have a complex cystic and solid echogenicity than a heterogeneous echogenicity. This discrepancy may be attributed to the fact that Candelaria et al.[15] investigated all subtypes of breast cancer.[14] sTILs are a promising prognostic biomarker in TNBC.[21-24] Thus, we focused on TNBC. One advantage of our approach was we assessed sTILs using full-face tissue sections of the entire tumor, which differs from the use of pre-treatment core biopsy specimens used Candelaria et al.[15] These tissue sections are preferred over biopsies.

Despite the evaluation of ultrasound images by two seasoned radiologists, the low reproducibility of visual identification may have exerted a significant influence. Furthermore, the interpretation of ultrasonographic features was significantly influenced by the experience of the operator, and it lacked quantitative assessment. Ultrasonographic evaluation provides information regarding the entire tumor region. Thus, it may be a useful complementary evaluation to sTIL density estimation. However, ultrasonographic

TABLE 2. Ultrasonographic and Clinicopathological Characteristics of the Tumors, Stratified According to Low or High sTIL Density

	Univariable					Multivariable		
Variables	**n**	**Event (n)**	**OR**	**95% CI**	**p**	**OR**	**95% CI**	**p**
Shape								
Regular	30	16	-	-		-	-	-
Irregular	132	43	0.42	0.19, 0.94	0.036	0.60	0.25,1.41	0.240
Orientation								
Parallel	122	44	-	-				
Vertical	40	15	1.06	0.51, 2.23	0.870			
Margin								
Circumscribed	26	10	-	-				
Indistinct	24	6	0.53	0.16, 1.80	0.311			
Microlobulated	33	13	1.04	0.36, 2.99	0.942			
Angular	55	21	0.99	0.38, 2.58	0.981			
Spiculated	24	9	0.96	0.31, 3.01	0.944			
Internal echo pattern								
Hypoechoic	152	58	-	-				
Isoechoic or complex cystic and solid	10	1	0.18	0.02, 1.46	0.108			
Hyperechoic	-	-						
Heterogeneous	-	-						
Posterior features								
Shadowing	57	13	-	-		-	-	-
Enhancement	39	18	2.90	1.20, 7.01	0.018	2.91	1.12, 7.52	0.028
No posterior features	66	28	2.49	1.13, 5.48	0.023	2.74	1.16, 6.47	0.022
Vascularity								
Absent	42	13	-	-				
Internal or peripheral	120	46	1.39	0.65, 2.94	0.393			
Tumor size								
< 2cm	57	17	-	-				
≥ 2 cm	105	42	1.57	0.79, 3.12	0.200			
ALNM								
No	113	42	-	-				
Yes	49	17	0.90	0.45, 1.81	0.764			
AJCC stage								
I	47	12	-	-				
II	77	36	2.56	1.16, 5.67	0.020			
III	38	11	1.19	0.46,3.10	0.725			
Nuclear grade								
I-II	76	20	-	-		-	-	
III	86	39	2.32	1.20, 4.51	0.013	2.49	1.20, 5.17	0.014
Ki67								
≤ 40%	47	9	-	-		-	-	
> 40%	115	50	3.25	1.44, 7.34	0.005	2.75	1.17,6.46	0.021
LVI								
No	143	54	-	-				
Yes	19	5	0.59	0.20, 1.73	0.334			

sTIL, stromal tumor-infiltrating lymphocyte; OR, odds ratio; CI, confidence interval; ALNM, axillary lymph node metastasis; AJCC, American Joint Committee on Cancer; LVI, lymphovascular invasion.

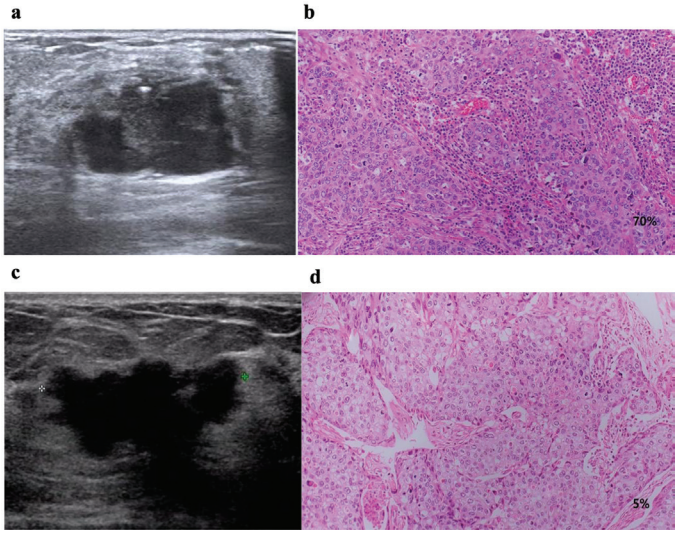

FIG. 3. In a 47-year-old female with a TNBC that exhibited a high sTIL density (70%), (a) the ultrasound revealed a hypoechoic tumor with posterior enhancement, and (b) the H&E-stained (x200 magnification) specimen revealed dense lymphocytic infiltration in the stroma. In a 51-year-old female with a TNBC that exhibited a low sTIL density (5%), (c) the ultrasound revealed a hypoechoic tumor with an acoustic shadow, and (d) the H&E-stained (x200) specimen demonstrated sparse lymphocytic infiltration in the stroma.

TNBC, triple-negative breast cancer; sTIL, stromal tumor-infiltrating lymphocytes; H&E, hematoxylin and eosin.

findings alone may be insufficient for providing accurate predictive information regarding sTIL density. A machine-learning approach to image analysis could offer a solution to this limitation.

The Ki67 index is a reliable marker of tumor cell proliferation activity. Furthermore, there is a correlation between Ki67 levels and sTIL density.[25,26] Our data suggests that Ki67 level of > 40% are more closely associated with high sTIL density than low sTIL density. Previous studies have demonstrated that breast tumors with high TIL levels are enriched with cell proliferation-related genes and exhibit increased expression of Ki67.[27] sTILs, similar to Ki67, have been proposed as a continuous parameter of tumor-immune cell interaction rather than a marker of a specific immune-activated tumor subtype.[26] This might aid in the elucidation of a favorable correlation between Ki67 and sTIL density. The nuclear grade is a morphological assessment of cancer cell proliferation. An increase in nuclear grade is indicative of a greater propensity for proliferative activity among the cancer cells. We found a positive correlation between nuclear grade and sTIL density in our study, which is similar to the finding of a previous study.[28]

Given the correlation between sTILs and high response rates to neoadjuvant chemotherapy and favorable prognosis,[29,30] precise estimation of this biomarker can aid in identifying breast tumors with good curative effect. Additionally, sTIL density could convey the prognosis of the approach to tumor management. Limited sampling and intrinsic tumor heterogeneity make it challenging to reliably evaluate sTILs using core biopsy samples from patients who have been administered neoadjuvant therapy. This can be overcome by evaluating biomarkers that correlate with sTIL density.

Our study has some limitations. Our study included a relatively small sample size and was a retrospective study conducted at a single institution. Thus, future studies should include a larger study population. Second, observer variability in ultrasonography, a user-dependent assessment tool, may have contributed to a discrepancy in determining the tumor characteristics, which may have influenced the results. Third, we only analyzed the conventional ultrasonographic features. Thus, further studies are required to evaluate the role of advanced sonographic characteristics in predicting sTIL density.

In conclusion, our study findings support the hypothesis that low sTIL density is frequently observed in tumors with acoustic shadows on ultrasonography. However, high sTIL density is more common in tumors with posterior enhancement or no posterior features. Furthermore, high Ki67 levels of > 40% and high nuclear grades are positively correlated with high sTIL density. Our study findings highlight the need for closer surveillance of these biomarkers to complement sTIL evaluation in patients with TNBC.

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Ethics Committee Approval: This study was approved by the Medical Ethics Committee of Nanfang Hospital, Southern Medical University (approval number: NFEC-2023-295; date: 06.07.2023).

Informed Consent: Retrospective study.

Data Sharing Statement: The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Authorship Contributions: Concept- L.L.; Design- L.L.; Supervision- Y.L.;Materials- L.L.; Data Collection or Processing- L.L.; Analysis or Interpretation- L.L.; Literature Search- L.L., Y.L.; Writing- L.L.; Critical Review- Y.L.

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