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The correlation of tertiary lymphoid structures with tumor spread through air spaces and prognosis in lung adenocarcinoma: focusing on pathological spatial features

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Abstract

Lung adenocarcinoma (LADC) exhibits high spatial heterogeneity, with distinct spatial variations in pathological features. The distribution of tertiary lymphoid structures (TLS) in LADC is uneven, and different TLS characteristics play unique roles. To investigate the correlation between TLS features and other pathological characteristics, particularly tumor spread through air spaces (STAS), we analyzed TLS and other pathological features on whole-slide images stained with HE and CD20/CD23. Additionally, the 14-Gene assay was used to assess prognostic risk. Among 388 enrolled LADC patients, 226 (58.2%) were TLS-positive. TLS showed a negative correlation with various adverse pathological features, with boundary-area TLS demonstrating the strongest correlation with STAS quantity (r= -0.324, $P<0.001$). Multivariate Cox analysis identified boundary-area TLS as an independent prognostic factor for recurrence-free survival (HR = 0.856, 95% CI = 0.759–0.966, P = 0.026), while mature TLS was an independent factor for overall survival (HR = 0.841, 95% CI = 0.717–0.988, P = 0.035). High-density TLS at the tumor boundary was associated with low-risk stratification by the 14-Gene assay (P = 0.013). This study highlights the negative correlation between TLS and STAS, especially in boundary areas, and emphasizes the impact of tumor microenvironment spatial characteristics on clinical outcomes. Assessment of spatial heterogeneity in LADC facilitates precise risk stratification for patients.

Keywords Tertiary lymphoid structures, Tumor spread through air spaces, Spatial characteristics, Lung adenocarcinoma

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Background

Lung cancer is one of the leading causes of cancer-related deaths, with non-small cell lung cancer (NSCLC) accounting for the vast majority of cases [1]. Among NSCLC, lung adenocarcinoma (LUAD) is the most common histological subtype, exhibiting high heterogeneity. In the research of LUAD, there is an increasing emphasis on tumor spatial heterogeneity, which refers to the distinct differences in pathological, cellular, and molecular features across different regions within the tumor [2]. Many pathological features of LUAD have spatial characteristics. Among them, tumor spread through air spaces (STAS) is particularly typical. STAS refers to tumor cells spreading from the primary lesion of lung cancer to the normal alveolar spaces outside the lesion, involving the spatial distribution of tumor cells from inside the tumor to outside the tumor [3]. The dissemination of tumors is influenced by the tumor microenvironment (TME) at the tumor boundary, and the unique TME composition in the invasive margin plays a non-negligible role in tumor progression. Additionally, tertiary lymphoid structures (TLS) are also a pathological feature with spatial characteristics. TLS are ectopic lymphoid organs formed within non-lymphoid tissues, containing a plethora of diverse immune cells [4]. TLS are more commonly found at the invasive margins of solid tumor infiltration rather than the tumor core [5], suggesting that TLS at the boundary of tumors may influence the dissemination of cancer cells at the invasive margins of LUAD.

Currently, the presence of TLS is considered to be associated with a relatively favorable prognosis in various types of tumors overall [6]. However, different TLS also exhibit heterogeneity, with variations in cellular composition, maturity, spatial distribution, and structure. These differences may result in distinct roles of TLS within the TME [7]. Studying the diverse characteristics of TLS in LUAD may lead to more comprehensive understanding of the role of TLS in the TME, providing a theoretical basis for inducing TLS as a therapeutic approach for treating LUAD. In previous studies of lung cancer, the spatial characteristics of TLS were often overlooked, particularly in relation to their spatial distribution and various pathological features of LUAD. Due to the absence of a capsule, TLS are directly exposed to the TME, allowing the immune cells within TLS to directly contact nearby tumor antigens, thereby exerting immunological functions [8]. In LUAD, TLS in different locations may have varying effects on the local TME, thus impacting tumor development and patient prognosis. Consequently, we speculate that TLS at the boundary of tumor may influence the development of STAS, thereby affecting the prognosis. However, there is currently a scarcity of studies focusing on the mutual influence of their spatial distribution.

Therefore, a multicenter retrospective study was performed to assess the characteristics of TLS, including quantity, maturity, and spatial distribution, and their correlation with pathological features and patient prognosis in LUAD, with particular attention paid to the relationship between TLS characteristics and STAS.

Materials and methods

Subjects and clinical data

Study population: We retrospectively reviewed the clinical data of patients who underwent surgical treatment for lung cancer at Tianjin Chest Hospital, Tianjin Jinnan Hospital, and Fujian Provincial Hospital between January 2017 and December 2018 by accessing hospital databases. The inclusion criteria were as follows: (1) R0 resection for lung cancer; and (2) postoperative pathological diagnosis of invasive LUAD. Exclusion criteria were: (1) neoadjuvant therapy; (2) multiple primary lung cancers; (3) no tumor margin in paraffin section; and (4) loss to follow-up.

Clinical and pathological data: The clinical characteristics of enrolled patients included gender, age, and smoking history. Pathological characteristics included pathological staging, lymph node metastasis, maximum tumour diameter, pleural invasion, vascular invasion, pathological subtypes, and STAS. Micropapillary, solid, and complex gland components were considered as high-grade components or "high risk" patterns [9].

Tissue preparation

Two adjacent slices were used for hematoxylin and eosin (HE) staining and double CD20/CD23 immunohistochemical (IHC) staining. The double CD20/CD23 IHC was performed using a primary antibody mixture of mouse anti-CD20 (Kit-0001, Maixin) and rabbit anti-CD23 (18642-1-AP, Proteintech), along with a double IHC staining kit (DS-0004, Zhongshan Golden Bridge), and the procedures were performed according to the instructions. CD20 + cells were stained in pink on the cell membrane, while CD23 + cells exhibited brown staining on the cell membrane.

Evaluation of TLS

In this study, our method for evaluating TLS was based on previous literature [5]. TLS were defined as organized lymphoid node-like structures containing CD20 + B cells, made up of at least 50 immune cells. When at least one TLS was observed within the tumor, it was defined as TLS-positive; otherwise, it was defined as TLS-negative. Additionally, it is necessary to distinguish TLS from lymphoid aggregates, which were defined as discrete collections of lymphocytes without the architectural organization or cell types of TLS [10]. Mature TLS (mTLS) were defined as TLS containing a visible

germinal center (GC) or more than one CD23 + follicular dendritic cells [5, 11]. The number of TLS and mTLS within the tumor were recorded. Further, the tumors were divided into the boundary area (≤ 1 mm inside from the tumor boundary) and the core area (> 1 mm inside from the tumor boundary) [5, 12]. The number of TLS in both areas was recorded, and the area of the boundary and core regions was measured using iViewer software (7.2.7.2). Additionally, the TLS density (/cm^2) in each region was recorded. A schematic diagram is shown in Fig. 1A.

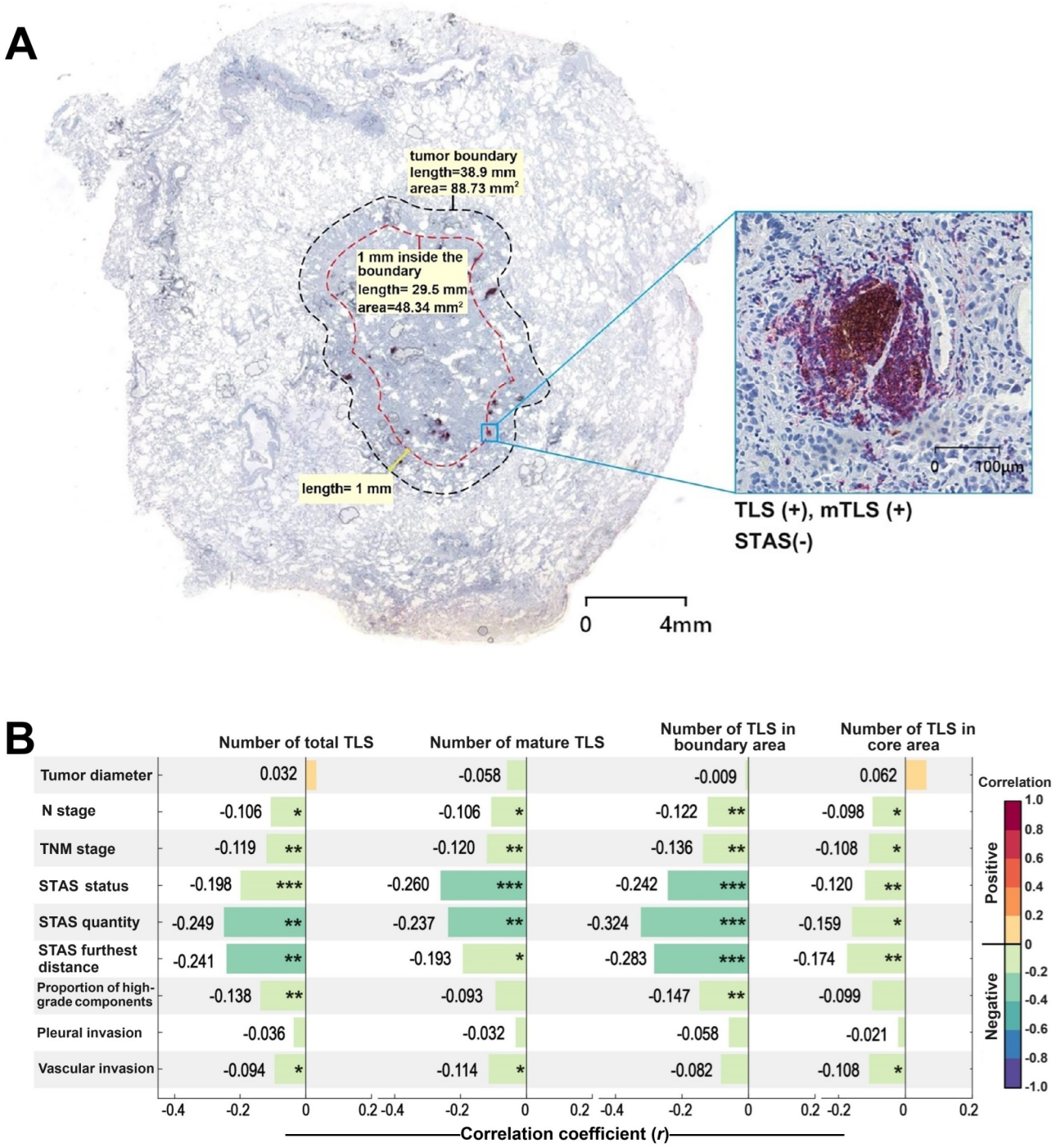

Fig. 1 The correlation between TLS and pathological features of LADC. (**A**) Schematic diagram of the regional division in LADC and the assessment of TLS distribution and density (left scale bar = 4 mm, right scale bar = 100 μm); (**B**) Correlation analysis of different TLS features with pathological features of LADC (*P<0.05, **P<0.01, ***P<0.001)

Evaluation of STAS

The evaluation of STAS and its features in LADC was conducted using iViewer software (7.2.7.2) based on the digitized WSI of HE and immunohistochemistry. The included indicators of STAS features are as follows: (1) STAS status; (2) STAS quantity: the number of alveolar spaces with STAS; (3) STAS furthest distance: the distance from the furthest STAS to the edge of the main tumor in the digitized WSI. Record the aforementioned characteristics of STAS and TLS within the same slice.

14-Gene molecular assay

Paraffin-embedded tumor samples from 142 patients enrolled at Fujian Provincial Hospital were retrospectively collected for 14-Gene molecular quantitative PCR analysis (DetermaRx™, Burning Rock Biotech). The genes analyzed include: BAG1, BRCA1, CDC6, CDK2AP1, ERBB3, FUT3, IL11, LCK, RND3, SH3BGR, WNT3A, ESD, TBP, and YAP1. The specific procedures refer to previous studies [13, 14], and the results were categorized as low, intermediate, or high risk, with higher risk indicating a potentially poorer postoperative prognosis. 14-gene molecular assay has been proved to be useful for prognostic risk stratification in patients with non-squamous NSCLC [13, 14].

Follow-up

Patient follow-up information was collected from the database of each hospital. Overall survival (OS) was defined as the interval between the date of surgery and the date of death or last follow-up. Recurrence-free survival (RFS) was defined as the interval between the date of surgery and the date of initial diagnosis of recurrence or last follow-up.

Statistical analysis

Statistical analyses were conducted using SPSS software (25.0) and graphs were created using GraphPad software (8.0) and Origin software (2021). Normally distributed continuous data were presented as mean ± standard deviation, and the comparisons between groups were conducted using the *t* test. Skewed distributed continuous data were presented as median (quartiles), and the comparisons between groups were performed using the nonparametric *Mann-Whitney U* test. Counting data were expressed as n (%), and the comparisons between groups were conducted using the chi-square or *Fisher's* exact test. The correlations between TLS quantity and continuous variables were calculated using Spearman rank analysis, and the correlations between TLS quantity and categorical variables were calculated using Kendall rank correlation test. A correlation coefficient (r) > 0 indicates a positive correlation, while $r < 0$ indicates a negative correlation. Univariate and multivariate *Cox* regression analyses (forward LR) were used for survival analysis. $P < 0.05$ was taken as statistically significant.

Results

Clinical characteristics and TLS features

A total of 388 patients were included in the study. Among them, 226 cases (58.2%) were TLS-positive, and 162 cases (41.8%) were TLS-negative. The baseline characteristics of all enrolled patients are presented in Table 1.

Correlation between TLS and pathological features of LADC

As shown in Fig. 1B, TLS were negatively correlated with multiple adverse pathological features. The numbers of total TLS, TLS in the boundary area, TLS in the core area and mTLS were all significantly negatively associated with STAS status, quantity, and furthest distance. Among them, the number of TLS in the boundary area showed the strongest correlation with STAS quantity (r = -0.324, $P < 0.001$).

TLS were associated with a good prognosis in LADC

As shown in Fig. 2, univariate analyses of RFS and OS were conducted by incorporating TLS features and pathological features significantly associated with all TLS features. Subsequently, factors with $P < 0.05$ were included in the multivariate analyses. As illustrated in Table 2, the number of TLS in the boundary area was associated with good RFS, while the number of mTLS was associated with favorable OS.

Spatial distribution of TLS in LADC

We further compared the TLS density between the boundary and core areas in TLS-positive cases. The results are shown in Fig. 3. The TLS density in the boundary area was significantly higher than that in the core area, suggesting that TLS in LADC may be more abundant in the boundary area, which also reflects spatial heterogeneity of the tumor microenvironment.

TLS and 14-Gene molecular risk stratification

We divided patients undergoing 14-Gene molecular assay into low boundary TLS group ($n = 73$) and high boundary TLS group ($n = 69$) based on the median TLS density (5.17/cm^2) in the boundary area of all TLS positive patients. The results are shown in Table 3, indicating that the 14-Gene molecular risk stratification in the high boundary TLS group was significantly lower than that in the low boundary TLS group ($P < 0.05$).

Discussion

The presence and characteristics of TLS reflect the immune status within tumors. They play a crucial role in inhibiting tumor development and metastasis by

Table 1 Clinical characteristics and TLS features of all enrolled patients with LADC ($n=388$)

Variable	TLS-negative ($n=162$)	TLS-positive ($n=226$)	*P* valve
Age (years), mean ± SD	61.18 ± 8.32	61.20 ± 7.52	0.976
Gender, n (%)			0.780
Female	88 (54.3)	126 (55.8)	
Male	74 (45.7)	100 (44.2)	
Smoking history, n (%)			0.616
No	97 (59.9)	141 (62.4)	
Yes	65 (40.1)	85 (37.6)	
Tumor diameter (cm), mean ± SD	2.22 ± 1.09	2.23 ± 0.93	0.929
Lymph node metastasis, n (%)			0.005
No	120 (74.1)	193 (85.4)	
Yes	42 (25.9)	33 (14.6)	
Pathological subtypes, n (%)			0.047
High-grade components < 20%	84 (51.9)	140 (61.9)	
High-grade components ≥ 20%	78 (48.1)	86 (38.1)	
Pleural invasion, n (%)			0.420
No	75 (46.3)	114 (50.4)	
Yes	87 (53.7)	112 (49.6)	
Vascular invasion, n (%)			0.038
No	90 (55.6)	149 (65.9)	
Yes	72 (44.4)	77 (34.1)	
Driver gene mutations, n (%)			0.147
Yes	102 (63.0)	123 (54.4)	
Undetected	41 (25.3)	78 (34.5)	
Unknow	19 (11.7)	25 (11.1)	
Scope of surgery, n (%)			0.913
Lobectomy	147 (90.7)	207 (91.6)	
Segmentectomy	12 (7.4)	14 (6.2)	
Partial resection	3 (1.9)	5 (2.2)	
STAS, n (%)			<0.001
Negative	80 (49.4)	152 (67.3)	
Positive	82 (50.6)	74 (32.7)	
Number of STAS in STAS (+), *M (P25, P75)*	10.0 (7.8, 15.0)	8.0 (6.0, 12.0)	0.002
Maximum distance of STAS in STAS (+), (cm), *M (P25, P75)*	2.1 (1.6, 3.0)	1.8 (1.1, 2.5)	0.007
Number of TLS, *M (P25, P75)*	-		
Total TLS		7.0 (5.0, 9.0)	
TLS in core area	-	4.0 (3.0, 5.0)	
TLS in boundary area	-	3.0 (2.0, 5.0)	
mTLS		2.0 (0.0, 4.0)	
Mature TLS, n (%)			
No	-	68 (30.1)	
Yes	-	158 (69.9)	

TLS: tertiary lymphoid structures; LADC: lung adenocarcinoma; STAS: tumor spread through air spaces

aggregating, activating immune cells, and promoting local immune responses [15, 16]. Meanwhile, STAS is a unique mode of dissemination in lung cancer, reflecting the invasiveness and spreading ability of lung cancer cells, and is also a key factor affecting the prognosis of patients with LADC [3, 17]. Previous studies have revealed the respective impacts of TLS and STAS on the prognosis of lung cancer. However, the relationship between them has not been adequately clarified. Therefore, in this study, we explored the various features of TLS, especially the spatial distribution, and their relationship with the pathological characteristics and prognosis of LADC, particularly focusing on the relationship between TLS and STAS. The results of this study indicated a significant negative correlation between TLS in the boundary area and STAS. Additionally, the TLS in the boundary area and the mTLS were also identified as important factors influencing the prognosis of patients with LADC.

Currently, TLS are considered ectopic lymphoid structures formed in response to inflammation, mainly composed of B cells, T cells, and dendritic cells, with high endothelial venules also being an important component [8, 15, 18]. TLS have been identified in various solid tumors. In this study, multiple features of TLS were associated with a better prognosis in LADC. Similarly, previous studies have demonstrated an association between TLS and favorable prognosis in patients with lung cancer [19–23]. Nevertheless, some TLS with specific features also have the effect of inhibiting the immune microenvironment and promoting tumors. Joshi et al. [24] found that TLS in lung cancer contain Treg cells, which can suppress the endogenous anti-tumor immune response. Additionally, TLS with different distributions and maturation levels play different roles in the TME [7]. Based on the potential diversity of feature-functionality of TLS, we further investigated various TLS characteristics, including absolute count, density, distribution, and maturity, contributing to a deeper understanding of the role of TLS in LADC.

The spatial distribution is one of the indicators for assessing TLS. In most tumors, TLS are detected in the tumor stroma and/or tumor invasive margins, and they are often associated with a favorable clinical prognosis [14, 25, 26]. In this study, TLS in both the core and boundary areas were associated with a better prognosis in LADC. However, the distribution of TLS is uneven, and TLS in different regions are not exactly identical. Vanhersecke et al. [5] demonstrated that TLS predominate in the invasive margin rather than at the core of the tumor in various solid tumors. In this study, the density of TLS in the boundary area of LADC was significantly higher than in the core area. The difference in immune infiltration between the boundary and core areas may be related to the increased likelihood of necrosis and hypoxia in the tumor core [27]. Previously, the focus of much research has been on the relationship between the location of TLS within and outside tumors and prognosis. Giatromanolaki et al. [23] found that high TLS density both inside and outside tumors is associated with

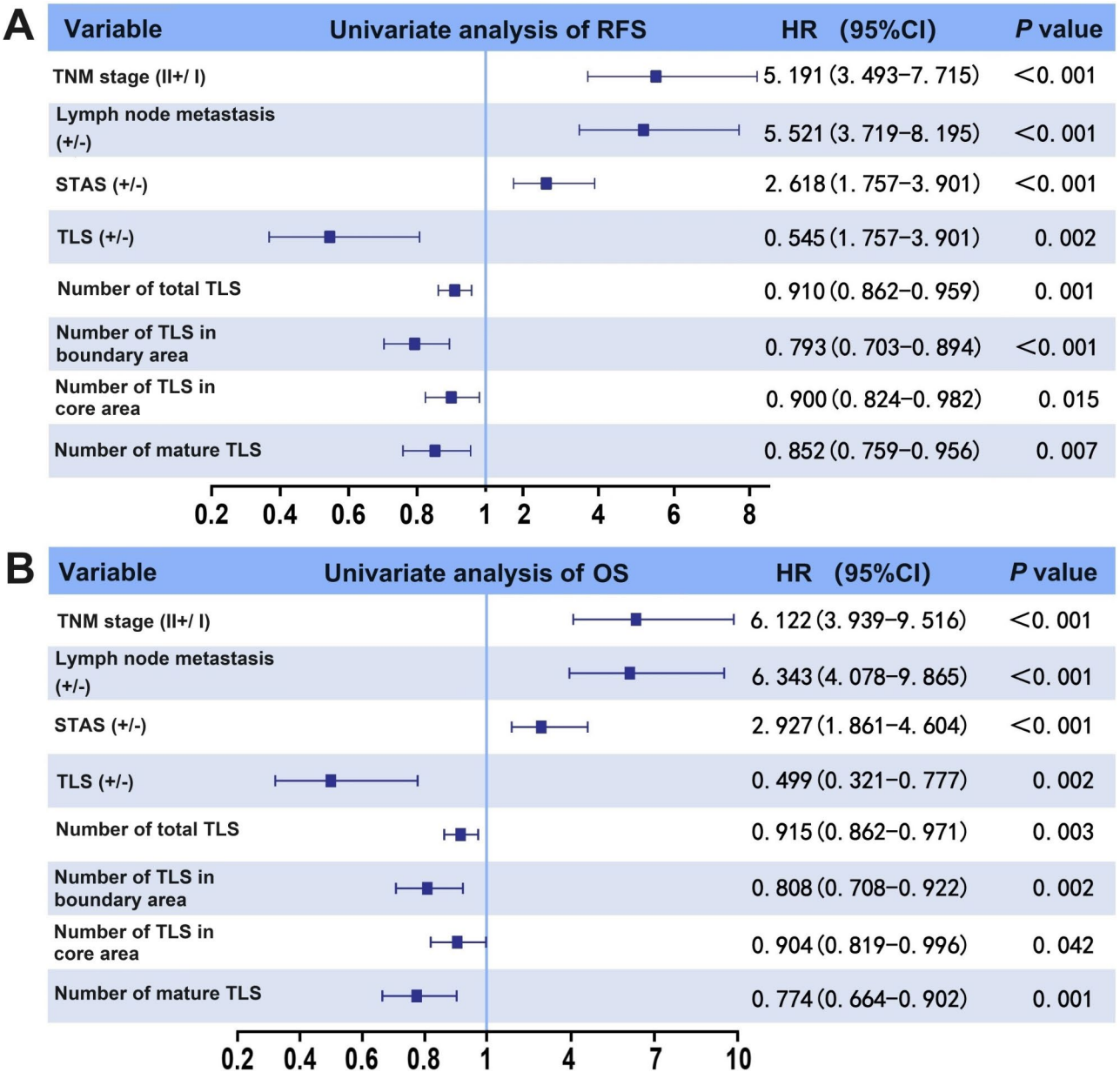

Fig. 2 Forest plots of univariate analyses on the hazard ratio for RFS (**A**) and OS (**B**) in LADC patients

Table 2 Multivariate Cox regression analyses of RFS and OS ($n = 388$)

Factors	B	SE	Wald	HR (95%CI)	*P* value
RFS					
TNM stage (II+/I)	1.515	0.207	53.663	4.551 (3.034–6.826)	<0.001
STAS (+/-)	0.566	0.212	7.15	1.761 (1.163–2.666)	0.007
Number of TLS in boundary area	-0.155	0.061	6.404	0.856 (0.759–0.966)	0.026
OS					
TNM stage (II+/I)	1.674	0.23	52.828	5.331 (3.395–8.372)	<0.001
STAS (+/-)	0.616	0.244	6.404	1.852 (1.149–2.985)	0.011
Number of mature TLS	-0.173	0.082	4.465	0.841 (0.717–0.988)	0.035

RFS: recurrence-free survival; OS: overall survival; STAS: tumor spread through air spaces; TLS: tertiary lymphoid structures

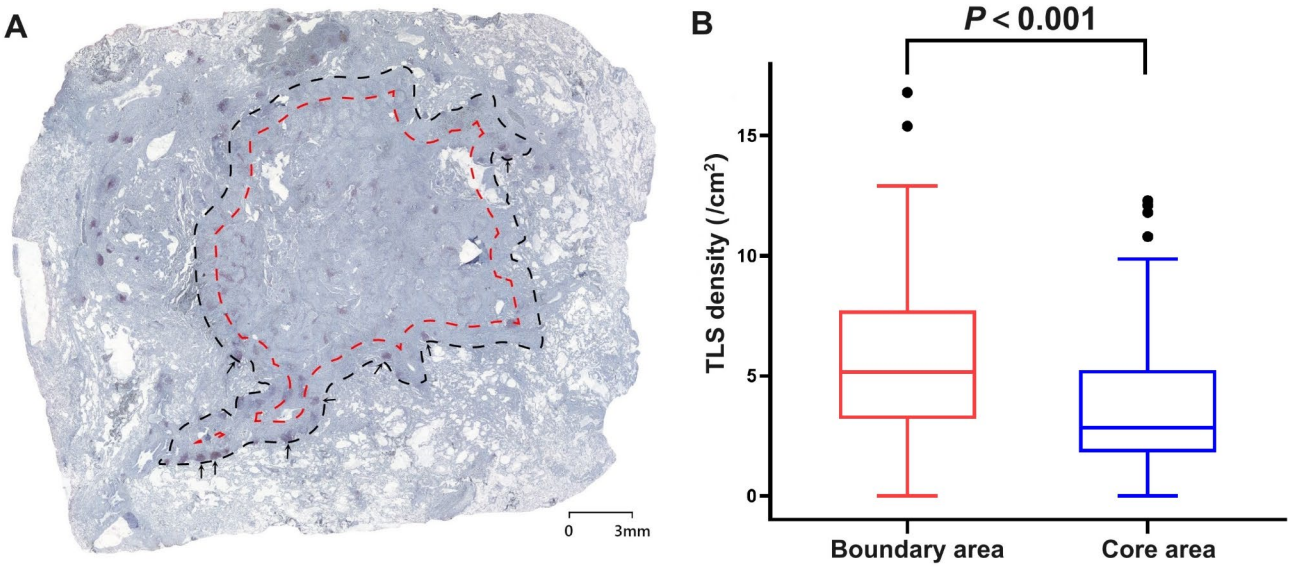

Fig. 3 The distribution of TLS in the boundary and core areas of LADC. (**A**) Representative example of TLS (arrowheads) distribution in the boundary area of LADC (scale bar = 3 mm); (**B**) Comparison of TLS density in different areas of LADC

Table 3 Relationship between TLS density in the boundary area and 14-Gene molecular risk stratification ($n = 142$)

	Low boundary TLS group ($n = 73$)	High boundary TLS group ($n = 69$)	*P*-value
14-Gene molecular assay, n (%)			0.013
Low risk	33 (45.2)	48 (69.6)	
Intermediate risk	25 (34.2)	13 (18.8)	
High risk	15 (20.5)	8 (11.6)	

TLS: tertiary lymphoid structures

better postoperative prognosis in lung cancer. Deng et al. [12] observed a negative correlation between TLS at both the invasive margin and core of lung cancer with distant metastasis and staging. Furthermore, in this study, high-density TLS in the boundary area was associated with low-risk stratification of survival by 14-Gene assay, suggesting that high-density TLS in boundary area may represent a stronger local immune response that inhibits the malignant biological behavior of tumors. However, it is inconclusive about the association between the distribution of TLS and prognosis in most tumors, especially in the boundary areas. There are also differences in the definition of the range of the invasive margin and tumor boundary area in different studies. Therefore, accurate identification and description of the spatial location of TLS is necessary. In addition, STAS is also a pathological feature with spatial attributes. Previous studies have shown that the incidence of STAS in TLS-positive NSCLC was significantly lower than in TLS-negative NSCLC [21]. Furthermore, we compared the relationship between TLS in different regions and STAS. The results showed that TLS in both core and boundary areas were significantly negatively correlated with STAS and its characteristics, with the correlation of TLS in the boundary area being stronger than that in the core area. These results suggest that the tumor boundary has a unique immune microenvironment, influencing the occurrence and development of STAS in LADC. They also emphasize the importance of considering the role of spatial characteristics when studying tumor biology.

Furthermore, maturity reflects the temporal attribute of TLS. mTLS contain CD23 + follicular dendritic cells and some B cells internally, including effector B cells, plasma cells, and memory B cells, most of which are associated with the anti-tumor effects of TLS [28]. In this study, TLS maturity was assessed based on CD23 expression. The results showed that the quantity of mTLS was associated with a better prognosis in LADC, and multivariate analysis suggested it as an independent predictor of OS. Similarly, in the study by He et al. [22], NSCLC patients with TLS+/GC + had significantly better prognosis than those with TLS+/GC-. Survival analysis studies have positive significance for understanding diseases [29, 30]. Additionally, our results indicated a significant negative correlation between the quantity of mTLS and the status, quantity, and farthest distance of STAS in LADC, suggesting that mTLS may inhibit the occurrence and progression of STAS. This provides a basis for the application of the induction of TLS formation and maturation in the postoperative treatment of STAS-positive LADC, especially in sublobar resection.

This study also has limitations. (1) In this study, the boundary area was defined as 1 mm inside from the invasive margin. However, there is currently no clear standard or consistent method to determine the invasive margin,

which may result in the inclusion of data from other spatial locations when studying the boundary area of tumors. Therefore, future efforts to more accurately identify the invasive margin will contribute to a more precise and in-depth understanding of the characteristics of the tumor boundary region. (2) This study only detected CD20 + and CD23 + cells in TLS, and further analysis of markers such as CD4 and CD8 in the future will help to better understand the composition of various TLS and the impact of different compositions of TLS on LADC. (3) This study only explored the correlation between STAS and TLS. In the future, it is also necessary to further investigate their causal relationship and biomarkers using precise in vivo and in vitro models of STAS.

Conclusion

In this study, we focused on the spatial distribution of pathological features in LADC and, for the first time, confirmed the relationship between TLS distribution and STAS as well as clinical outcomes. Overall, TLS showed a negative correlation with STAS, further supporting the association between intratumoural TLS and favorable pathological features, risk stratification, and clinical outcomes in LADC. Additionally, this study underscores the impact of spatial characteristics of the TME components on clinical outcomes. Assessment of spatial heterogeneity in LADC aids in precise risk stratification for patients postoperatively, enabling individualized treatment.

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Author contributions
YD designed the study. YD and MY performed the experiments, analyzed the data, and wrote the manuscript. MX and WZ performed the experiments and analyzed the data. YH and JR collected the data and samples. TG reviewed the data and oversaw the presentation of the data. DS and XP is responsible for the overall content as the guarantor. All authors read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This study was approved by the Ethics Review Committee of Tianjin Chest Hospital, the Ethics Review Committee of Tianjin Jinnan Hospital and the Ethics Review Committee of Fujian Provincial Hospital. Informed consent was waived due to retrospective nature of the study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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