Heterogeneity in tertiary lymphoid structures predicts distinct prognosis and immune microenvironment characterizations of clear cell renal cell carcinoma

Wenhao Xu,1,2 Jiahe Lu,1,2,3 Wang-Rui Liu,4 Aihetaimujiang Anwaier,1,2 Yuhao Wu,5 Xi Tian,1,2 Jia-Qi Su,1,2 Yuan-Yuan Qu,1,2 Jianfeng Yang,6 Hailiang Zhang,1,2 Dingwei Ye1,2

ABSTRACT

Background Tertiary lymphoid structures (TLS) are organized aggregates of immune cells that develop postnatally in non-lymphoid tissues and are associated with pathological conditions. TLS typically comprise B-cell follicles containing and are encompassed by T-cell zones and dendritic cells. The prognostic and predictive value of TLS in the tumor microenvironment (TME) as potential mediators of antitumor immunity have gained interest. However, the precise relationship between localization and maturation of TLS and the clinical outcome of their presence in clear cell renal cell carcinoma (ccRCC) is yet to be elucidated.

Methods Immunohistochemistry and multispectral fluorescence were used to evaluate the TLS heterogeneity along with TME cell-infiltrating characterizations. A thorough investigation of the prognostic implications of the TLS heterogeneity in 395 patients with ccRCC from two independent cohorts was conducted. Associations between TLS heterogeneity and immunologic activity were assessed by quantifying the immune cell infiltration.

Results Infiltrated TLS were identified in 34.2% of the ccRCC samples (N=395). These TLS were found to be tumor-proximal, tumor-distal, or both in 37.8%, 74.1%, and 11.9% of the TLS-positive cases, respectively. A higher proportion of early TLS was found in tumor-distal TLS (p=0.016), while tumor-proximal TLS primarily comprised secondary follicle-like structures (p=0.004). In the main study cohort (Fudan University Shanghai Cancer Center, N=290), Kaplan-Meier analyses revealed a significant correlation between the presence of tumor-proximal TLS and improved progression-free survival (PFS, p=0.001) and overall survival (OS, p=0.002). Conversely, the presence of tumor-distal TLS was associated with poor PFS (p=0.02) and OS (p=0.021). These findings were further validated in an external validation set of 105 patients with ccRCC. Notably, the presence of mature TLS (namely secondary follicle-like TLS, with CD23+ germinal center) was significantly associated with better clinical outcomes in patients with ccRCC. Furthermore, novel nomograms incorporating the presence of tumor-proximal TLS demonstrated remarkable predictability for the 8-year outcomes of resected ccRCC (area under the curve >0.80). Additionally, ccRCC samples with tumor-distal TLS enriched with primary follicle-like TLS exhibited higher programmed death-ligand 1 tumor-associated macrophages levels and regulatory T cells infiltration in the tumor-distal region, indicative of a suppressive TME.

Conclusion This study for the first time elucidates the impact of TLS localization and maturation heterogeneities on the divergent clinical outcomes of ccRCC. The findings reveal that most TLS in ccRCC are located in the tumor-distal area and are associated with immature, immunosuppressive characterizations. Furthermore, our findings corroborate previous research demonstrating that tumor-proximal TLS were associated with favorable clinical outcomes.

INTRODUCTION

Clear cell renal cell carcinoma (ccRCC) is the most common and malignant histological form of kidney cancer in adults, accounting for over 75% of all kidney cancers.1–4 Immunotherapy with immune checkpoint inhibitors (ICIs), such as cytotoxic T-lymphocyte-associated protein 4 and programmed cell death protein 1 (PD-1) inhibitors, has shown promising results in inhibiting the progression, recurrence, and metastasis of ccRCC.5–7 However, ccRCC is characterized by high heterogeneity, where tumor cells exhibit distinct genetic phenotypes and mutation profiles, leading to varying therapeutic sensitivities and prognoses.8–10 Consequently, the screening and identification of effective biomarkers for prognostic prediction, treatment selection, and treatment efficacy present significant challenges in the diagnosis and management of ccRCC.11,12 Therefore, investigating the potential of biomarker derived from
The concept of tumor microenvironment (TME) has evolved to recognize that cancer behavior is influenced not only by the genetic characteristics of tumor cells but also by the surrounding environment necessary for tumor survival, proliferation, and metastasis. In ccRCC, the immunosuppressive TME is infiltrated by various immune cells, including cluster of differentiation (CD) 8 T cells, tumor-associated macrophages (TAMs), natural killer (NK) cells, and B cells. Research conducted by Haydn T Kissick’s laboratory identified an antigen-presenting cell (APC)-dense niche within kidney tumors that harbors stem-like CD8 T cells, allowing them to differentiate into terminally differentiated PD-1+ T-cell immunoglobulin ligand 1 tumor-associated macrophages and regulatory T-cell infiltration in ccRCC samples with the presence of tumor-distal TLS enriched with immature structures.

The concept of tumor microenvironment (TME) has evolved to recognize that cancer behavior is influenced not only by the genetic characteristics of tumor cells but also by the surrounding environment necessary for tumor survival, proliferation, and metastasis. In ccRCC, the immunosuppressive TME is infiltrated by various immune cells, including cluster of differentiation (CD) 8 T cells, tumor-associated macrophages (TAMs), natural killer (NK) cells, and B cells. Research conducted by Haydn T Kissick’s laboratory identified an antigen-presenting cell (APC)-dense niche within kidney tumors that harbors stem-like CD8 T cells, allowing them to differentiate into terminally differentiated PD-1+ T-cell immunoglobulin ligand 1 tumor-associated macrophages and regulatory T-cell infiltration in ccRCC samples with the presence of tumor-distal TLS enriched with immature structures.

Tumor-proximal and tumor-distal TLS were both identified in ccRCC. Our findings revealed that tumor-proximal (or intratumoral) TLS indicate favorable prognosis whereas tumor-distal (or peritumoral) TLS indicate poor prognosis. A higher proportion of early TLS was observed in the tumor-distal region, whereas tumor-proximal TLS were predominantly composed of mature structures. The positive presence of mature TLS with germinal center cells was significantly correlated with better clinical outcomes in patients with ccRCC. Additionally, a prominent increase was observed in the proportion of programmed death-ligand 1 tumor-associated macrophages and regulatory T-cell infiltration in ccRCC samples with the presence of tumor-distal TLS enriched with immature structures.
The clinicopathological indicators, including age at initial diagnosis, sex, pathological tumor, node, and metastasis (TNM) stage, the American Joint Committee on Cancer (AJCC) stage, and the International Society of Urological Pathology (ISUP) grade, were collected for all patients. The baseline clinicopathological characteristics of 395 patients with ccRCC from the FUSCC training and external validation cohorts were combined. Following surgery, patients were followed-up every 3 months for the first 2 years after surgery and subsequently, every 6 months until recurrence or death. The patients underwent physical, laboratory, and radiological examinations strictly according to the specified protocols.

**H&E and immunohistochemistry assays**

The histopathological records of unspecific lymphocytic infiltration on H&E slides were examined following established protocols, as previously reported. Two experienced pathologists independently assessed the slides for ccRCC tissue or adjacent normal kidney tissues selected for staining. The slides were placed on a baking machine at 56°C for approximately 1 hour. Subsequently, the sections were dewaxed using varying concentrations of xylene and alcohol, followed by incubation in hematoxylin staining solution and 0.5% eosin solution at room temperature. After fixing the sections, a neutral gum seal was applied to the tissue area to capture images. Confirmation of tumor-distal or tumor-proximal localization of TLS was performed. TLS were then identified as lymphocytes aggregates exhibiting histological features resembling lymphoid tissues with B cells (CD20, ab64088, Abcam, USA), T cells (CD3, ab16669, Abcam), follicular DCs (FDCs) (CD21, ab7290, Abcam), and germinal center (GC) cells (CD23, ab16702, Abcam) in the tumor-proximal or tumor-distal areas. Immunohistochemical staining involved several steps, including baking, dewaxing, removal of endogenous peroxidase, antigen repair, washing, sealing, primary antibody incubation, secondary antibody incubation, washing, stained with dianaminobenzidine solution (DAB), dehydration, neutral gum sealing, and photography. The specific methodology was performed as previously described. TLS density was calculated by determining the number of TLS per square millimeter in the tumor-distal and tumor-proximal regions.

TLS localization heterogeneity was determined by identifying at least one occurrence of TLS in H&E slices. There were those located within the tumorous invasive margin, while tumor-distal TLS were defined as those located in normal tissue at a distance of >10 mm from the invasive margin. This identification process was performed independently by a urological surgeon and two pathologists. Based on the different TLS localization, the samples were classified as TLS using the following criteria: tumor-proximal TLS samples were designated as having at least one occurrence of tumor-proximal TLS; tumor-distal TLS samples were categorized as having at least one occurrence of tumor-distal TLS, but no tumor-proximal TLS; ‘both’ samples were classified as having both tumor-proximal and tumor-distal TLS.

For the TLS maturation analysis, slides were costained for CD3, CD20, CD21, and CD23. All dense lymphocytic aggregates, regardless of CD3/CD20/CD21/CD23 signals, were captured as multispectral high power field (HPF) images on the Vectra 3.0 imaging system (PerkinElmer). Horseradish peroxidase (HRP)-conjugated secondary antibodies were detected using DAB for immunohistochemistry (IHC) or tyramide signal amplification with two sets of the Opal 7-plex kit for multiplex IHC (mIHC). Each HPF image was evaluated to the TLS maturation stage as follows: early TLS (E-TLS) represented dense lymphocytic aggregates with mixed B and T cells but lacking CD21 and CD23 signals (no FDCs, and no GC); primary follicle-like TLS (PFL-TLS) were dense lymphocytic aggregates with CD21 but no CD23 signals (FDCs but no GC); secondary follicle-like TLS (SFL-TLS) were characterized by the presence of CD21+CD23+ FDCs, indicating the presence of a GC (both FDC and GC reactions). TLS-positive samples in this study were classified as follows: SFL-TLS positive sample with at least one occurrence of SFL-TLS; PFL-TLS positive sample with at least one occurrence of PFL-TLS but no SFL-TLS; E-TLS positive sample with neither PFL-TLS nor SFL-TLS. For example, if a patient’s sample carries one SFL-TLS (tumor-proximal TLS) and two E-TLS (tumor-distal TLS), the patient is defined as belonging to the ‘SFL-TLS’ patients group and ‘both’ patients group, and average immune cells frequency from all three TLS regions are extracted and analyzed.

**mIHC and immunofluorescence staining assays**

After determining the TLS localization in H&E slices with at least one occurrence of TLS, all slides were costained for CD3, CD20, CD21, and CD23 for the TLS maturation analysis via IHC assay. Meanwhile, 58 slides with at least three TLS regions identified by H&E analysis were randomly selected for multiplex immunofluorescence (mIF) analysis. The Akoya Opal Polaris 7-Color Automation IHC kit (NEL871001KT) with two panels (panel 1: CD8, cytokeratin (CK), CD68, CD163, PD-1, programmed death-ligand 1 (PD-L1), and 4’,6-diamidino-2-phenylindole (DAPI); panel 2: CD3, CK, CD56, CD20, CD4, forhead box P3 (FoxP3), and DAPI) was used to perform mIHC. This resulted in 12 primary antibody signals, as well as CD163S, PD-1, programmed death-ligand 1 (PD-L1), and 4’,6-diamidino-2-phenylindole (DAPI) signals, were captured as multispectral high power field (HPF) images on the Vectra 3.0 imaging system (PerkinElmer). Horseradish peroxidase (HRP)-conjugated secondary antibodies were detected using DAB for immunohistochemistry (IHC) or tyramide signal amplification with two sets of the Opal 7-plex kit for multiplex IHC (mIHC). Each HPF image was evaluated to the TLS maturation stage as follows: early TLS (E-TLS) represented dense lymphocytic aggregates with mixed B and T cells but lacking CD21 and CD23 signals (no FDCs, and no GC); primary follicle-like TLS (PFL-TLS) were dense lymphocytic aggregates with CD21 but no CD23 signals (FDCs but no GC); secondary follicle-like TLS (SFL-TLS) were characterized by the presence of CD21+CD23+ FDCs, indicating the presence of a GC (both FDC and GC reactions). TLS-positive samples in this study were classified as follows: SFL-TLS positive sample with at least one occurrence of SFL-TLS; PFL-TLS positive sample with at least one occurrence of PFL-TLS but no SFL-TLS; E-TLS positive sample with neither PFL-TLS nor SFL-TLS. For example, if a patient’s sample carries one SFL-TLS (tumor-proximal TLS) and two E-TLS (tumor-distal TLS), the patient is defined as belonging to the ‘SFL-TLS’ patients group and ‘both’ patients group, and average immune cells frequency from all three TLS regions are extracted and analyzed.
tyramide signal amplification (TSA) principle, secondary antibodies labeled with HRP and corresponding reactive Opal fluorophores.28 29

Negative controls were included by including tissue slides that were bound with primary and secondary antibodies but not fluorophores to evaluate autofluorescence. The multiplex stained slides were scanned using a Vectra Polaris Quantitative Pathology Imaging System (Akoya Biosciences) at 20 nm wavelength intervals ranging from 440 nm to 780 nm. The scans were conducted with a fixed exposure time and an absolute magnification of 200×. Subsequently, all scans for each slide were superimposed to obtain a single image. The resulting multilayer images were imported into inForm V.2.4.8 (Akoya Biosciences) for quantitative image analysis.28 29 Opal is a multilabel restaining scheme developed using TSA technology that can stain tissue samples, particularly FFPE samples, enabling multi-label identification using different primary antibodies of the same genus origin within a single sample. When combined with the inForm image analysis software, the Vectra multispectral imaging analysis system produced images with a high signal-to-noise ratio, excluding background spontaneous fluorescence, facilitating accurate batch quantitative analysis. This allowed for the precise quantification of various biomarkers in each cell and analysis of the percentage of specific cell types relative to the number of DAPI-positive cells. Opal covalently binds tyramide labeled with various colors to the target protein through sequential staining, followed by the removal of the previous round of antibodies via microwave heating.

Different cell types were distinguished by the established staining markers. During Akoya Vectra Polaris Tumor Immune Microenvironment (AP-TIME) software processing, membrane radius, nuclear pixel threshold, nuclear integrity, cytoplasmic/membrane pixel threshold, cytoplasmic/membrane integrity and other information were set accordingly, after which the negative or positive of a certain cell was determined by the software according to the average intensity of fluorescence thresholds of different channels. The algorithm appendix of the data processing is shown in online supplemental table 2. The quantification of various cell populations was performed by measuring the number of stained cells per square millimeter and calculating the percentage of positively-stained cells among all nucleated cells.28 29 These cell populations included CD4+FoxP3+ regulatory T (Treg) cells, CD8+ T cells, PD-1+CD8+ T cells, CK’CD68’ TAMs, CD68’CD163’ M2 macrophages, PD-L1’CD68’CD163’ M2 macrophages, CD68’CD163’ M1 macrophages, and CD56bright/dim NK cells, following the manufacturer’s instructions. Additionally, Opal mIHC was used to assess the presence of abundances of tumor-infiltrating lymphocytes (TILs), E-TLS, PFL-TLS, SFL-TLS, and Combined Positive Score (CPS) of PD-L1 expression using a multispectral imaging system (Vectra Polaris, Shanghai, China).27-29 By recognizing the staining markers in the tissue images, the system automatically adjusted the fluorescence intensity and identified and labeled the TLSs in the images. Independent quantitative data of immune cells infiltration within each TLS in the images were obtained, and at the same time, further spatial analysis was conducted on the interior of the TLS. All the quantitative data were derived from immune cells proportions against the spatial background within the TLS.

For the analysis of DCs in the context of TLS maturation, slides were costained for CD20, CD21, CD23, CD45, DC-lysosome-associated membrane glycoprotein (LAMP), pan-CK, and DAPI. The imaging and analysis were conducted on all dense lymphocytic aggregates and regions with DC-LAMP signals, examining their associations with specific TLS maturation stages using a 7-color multispectral immunofluorescence (IF).23 Within TLS, antigen-presenting mature DCs expressing DC-LAMP’ (DC-LAMP/CD208, dendritics, DDX0191) along with other established markers, exhibiting variable frequencies across different maturation stages.30 31

Cox regression analysis and nomogram construction
The primary endpoint was progression-free survival (PFS), while overall survival (OS) was considered as the secondary endpoint. Survival analysis was conducted using the Kaplan-Meier method. The cut-off value was determined using the ‘survminer’ package in R and assessed using the log-rank test with 95% CIs. Univariate and multivariate Cox logistic regression analyses were performed to identify independent predictors, estimating HRs and 95% CIs. As a supplement to survival analysis, Cox logistic regression analysis was performed on patients from the FUSCC and external validation cohorts for predicting PFS and OS using ‘rms’ and ‘survival’ R packages. Nomograms were developed based on multivariate Cox regression analysis, integrating TLS presence with various clinicopathological predictors. These nomograms transformed the complex regression equation into a graph on the same plane, depicting the proportional relationships. The nomograms were used to illustrate the relationship between each variable in the predictive model and accurately evaluate the corresponding 2-year, 5-year, and 8-year recurrence or survival rates of patients with ccRCC.

Statistical analysis
Statistical and graphical analyses were performed using SPSS software (V.23.0), GraphPad Prism software (V.8.0), or R software (V.3.3.2). One-way analysis of variance (ANOVA) was used for comparing multiple groups (≥2), while the Student’s t-test was used to assess the statistical differences between the two groups. A time-dependent receiver operating characteristic (ROC) curve was used to study the generalization performance of nomograms from the perspective of threshold selection using the area under the curve (AUC) value. All hypothesis tests were two-sided, and statistical significance was set at p value<0.05.
RESULTS
Demographic data and clinicopathological characteristics
The baseline clinicopathological characteristics of 395 patients with ccRCC from the FUSCC training and external validation cohorts are summarized in Table 1. In the FUSCC validation cohort, the median PFS and OS were 42 months and 64.5 months, respectively, as of the last day of follow-up on November 28, 2022. In the FUSCC external validation cohort, the corresponding durations were 51 months and 62 months, respectively. Approximately two-thirds of patients were men (66.2% in the FUSCC and 61.0% in the validation cohorts). The median age was 57 years (range: 21–86 years) in the FUSCC cohort and 54 years (range: 28–83 years) in the external validation cohort. Significant differences were observed in age, metastasis stage (M stage), AJCC stage, and ISUP grade between the FUSCC training and external validation cohorts, as indicated by the \( \chi^2 \) test results (\( p<0.05 \)). The external validation cohort had younger patients (<60 years) and significantly lower disease malignancy.

<table>
<thead>
<tr>
<th>Clinicopathological characteristics</th>
<th>FUSCC training cohort (n=290)</th>
<th>External validation cohort (n=105)</th>
<th>( \chi^2 )</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥60 years</td>
<td>188 (64.8)</td>
<td>38 (36.2)</td>
<td>15.83</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>&lt;60 years</td>
<td>102 (35.2)</td>
<td>67 (63.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>192 (66.2)</td>
<td>64 (61.0)</td>
<td>0.933</td>
<td>0.334</td>
</tr>
<tr>
<td>Female</td>
<td>98 (33.8)</td>
<td>41 (39.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1–T2</td>
<td>221 (76.2)</td>
<td>87 (82.9)</td>
<td>1.985</td>
<td>0.159</td>
</tr>
<tr>
<td>T3–T4</td>
<td>69 (23.8)</td>
<td>18 (17.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pN stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>248 (85.5)</td>
<td>92 (87.6)</td>
<td>0.284</td>
<td>0.594</td>
</tr>
<tr>
<td>N1</td>
<td>42 (14.5)</td>
<td>13 (12.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>229 (79.0)</td>
<td>96 (91.4)</td>
<td>8.212</td>
<td>0.004</td>
</tr>
<tr>
<td>M1</td>
<td>61 (21.0)</td>
<td>9 (8.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AJCC stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I–II</td>
<td>205 (70.7)</td>
<td>87 (82.9)</td>
<td>45.921</td>
<td>0.015</td>
</tr>
<tr>
<td>III–IV</td>
<td>85 (29.3)</td>
<td>18 (17.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISUP grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1–G2</td>
<td>140 (48.3)</td>
<td>82 (78.1)</td>
<td>27.847</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>G3–G4</td>
<td>150 (51.7)</td>
<td>23 (21.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence</td>
<td>99 (34.1)</td>
<td>36 (34.3)</td>
<td>0.001</td>
<td>0.978</td>
</tr>
<tr>
<td>Absence</td>
<td>191 (65.9)</td>
<td>69 (65.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor-distal TLS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence</td>
<td>73 (25.2)</td>
<td>27 (25.7)</td>
<td>0.012</td>
<td>0.913</td>
</tr>
<tr>
<td>Absence</td>
<td>217 (74.8)</td>
<td>78 (74.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor-proximal TLS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence</td>
<td>38 (13.1)</td>
<td>13 (12.4)</td>
<td>0.036</td>
<td>0.850</td>
</tr>
<tr>
<td>Absence</td>
<td>252 (86.9)</td>
<td>92 (87.6)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P value<0.05 is marked in bold.
AJCC, American Joint Committee on Cancer; ccRCC, clear cell renal cell carcinoma; FUSCC, Fudan University Shanghai Cancer Center; ISUP, International Society of Urological Pathology; pM, pathological metastasis; pN, pathological node; pT, pathological tumor; TNM stage, tumor size, lymph nodes affected, metastases; TLS, tertiary lymphoid structures.
Characteristics of TLS localization heterogeneity in ccRCC

The presence, localization (tumor-proximal or tumor-distal), and maturation (E-TLS, PFL-TLS, or SFL-TLS) of TLS were initially assessed based on the clinicopathological characteristics analyzed by H&E staining on diagnostic samples from 395 patients with ccRCC from two independent sets. The TLS proportion and size were determined for each individual, revealing their presence in tumor-proximal and tumor-distal regions. The TLS size and shape varied, with most exhibiting an oval or irregular appearance (figure 1A). Larger overviews of the H&E-stained slices are presented in online supplemental figure S1. Each H&E section needed to contain both ccRCC and paracancer normal tissues.

As presented in table 1, TLS presence in the ccRCC samples were 34.1% and 34.3% in the two cohorts comprising 395 samples. In the FUSCC cohort, the tumor-proximal and tumor-distal presence of TLS was 13.1% and 25.2%, respectively, while in the external validation cohort, these percentages were 12.4% and 25.7%, respectively. In the TLS-positive group, defined by the presence of at least one dense lymphocytic cluster, 37.8% exhibited only tumor-distal TLS presence, 74.1% showed only tumor-distal TLS, and 11.9% displayed both tumor-proximal and tumor-distal presence of TLS (figure 1B,C).

Impact of the tumor-proximal or tumor-distal localization of TLS on the prognosis of ccRCC

Previous reports have proposed that the association between the two TLS localization subtypes and disease progression could differ.32 33 However, in most cancers, the relationship between tumor-distal or tumor-proximal TLS location and clinical outcomes remains unclear. In this study, survival analyses were conducted on two real-world cohorts with clinical follow-up and clinicopathological data (training set from FUSCC, n=290, and validation set from the external cohort, n=105). Among the 290 Chinese patients with ccRCC from the FUSCC cohort, the presence of tumor-proximal TLS was associated with improved long-term survival (OS: p<0.001, HR=0.456) and suppressed disease progression (PFS: p=0.002, HR=0.387). Conversely, tumor-distal TLS-positive presence was significantly associated with poor long-term survival (OS: p=0.021, HR=1.444) and progressive disease (PFS: p=0.020, HR=1.486). Similarly, in the external validation set, the presence of tumor-proximal TLS was significantly associated with prolonged OS (p=0.012, HR=0.263) and restricted PFS (p=0.042, HR=0.167).
Tumor-distal TLS-positive presence was also significantly associated with poor OS (p=0.009, HR=2.024) and progressive PFS (p=0.043, HR=1.993) in 105 patients with ccRCC (figure 2). The overall presence of TLS did not predict clinical outcomes in either cohort (online supplemental figure S2). Taken together, these findings revealed that heterogeneous TLS localization allowed distinct prognostic stratification of patients with ccRCC in the FUSCC and external sets.

**Characteristics of TLS maturation heterogeneity in ccRCC**

An IHC assay was implemented to define TLS maturation in ccRCC and evaluate lymphocytic aggregates that have histological features analogous to lymphoid tissues with CD3⁺ T cells, CD20⁺ follicular B cells, CD21⁺ FDCs, and CD23⁺ GC cells in serial sections (figure 3A). Analogous to the stages of secondary lymphoid organs follicles, lymphocytic clusters without FDCs as E-TLS (the first phase of TLS maturation), FDC-existing TLS without GC as PFL-TLS (the transitional phase of TLS maturation), and GC-existing as SFL-TLS (the final phase of TLS maturation) were defined. The presence of mature antigen-presenting DCs (DC-LAMP⁺) can be detected throughout TLS development and maturation, and their infiltration is associated with improved clinical outcomes. The abundance of DC-LAMP⁺ (mature DCs) in TLS at different stages of maturation was explored using a 7-color multispectral IF technique (figure 3B). Significantly higher levels of mature DCs were observed in the SFL-TLS cluster than in the E-TLS cluster (p<0.001), while no significant difference was observed compared with PFL-TLS (figure 3B). The effect of heterogeneity in TLS localization on TLS maturation was investigated to further explore the heterogeneity in TLS composition, particularly the presence of ‘suppressive’ TLSs in ccRCC. A significantly higher proportion of E-TLS in the tumor-distal TLS cluster (p=0.016, n=26) was observed, while the tumor-proximal TLS cluster mainly comprised SFL-TLS (p=0.004, n=26) (figure 3C).

**Impact of TLS maturation on ccRCC prognosis**

Previous findings indicate that tumor-proximal TLS are mainly mature while tumor-distal are primarily immature. Therefore, TLS maturation stages could be a confounding factor contributing to the differences in prognostic associations and TLS localization warranting further investigation. The mature and immature TLS density (which can be assessed as GC⁺ and GC⁻ clusters using IHC) was then assessed. The ccRCC samples were divided regardless of their location within the tumor. Initially, the Kaplan-Meier method was employed to evaluate the prognostic value of the presence of mature TLS (namely SFL-TLS, with CD23⁺ GC cells). As presented in figure 4, presence of SFL-TLS was significantly associated with better clinical outcomes (PFS, p=0.014, HR=0.483; OS, p=0.019, HR=0.440) compared with ccRCC samples with only immature TLS in the FUSCC training set. In the external validation set, among patients with ccRCC, a significant association was observed between the positive presence of SFL-TLS and longer PFS (p=0.028, HR=0.320), while its relationship with OS was marginally significant (p=0.056, HR=0.181). Additionally, univariate Cox logistic regression analysis for PFS and OS in 395 patients with ccRCC from the FUSCC and external validation cohorts was performed. As shown in online supplemental table S1, the Cox regression analysis revealed a significant association between the presence of SFL-TLS and reduced risk of progression and longer survival (PFS, p=0.018, HR=0.462; OS, p=0.023, HR=0.438) in 290 patients with ccRCC from the FUSCC cohort. However, no significant relationship was observed between the presence of SFL-TLS and clinical outcomes for patients with ccRCC in the external validation cohort (PFS, p=0.054, HR=0.319; OS, p=0.091, HR=0.280).

**A tumor-proximal TLS nomogram predicts ccRCC prognosis**

Univariate and multivariate Cox regression analyses were performed on 395 patients with ccRCC from two cohorts. In the univariate Cox regression analysis, conventional prognostic predictors, such as the pathological TNM stage, ISUP grade, AJCC stage, and the presence of tumor-proximal and tumor-distal TLS exhibited significant correlations with PFS and OS in patients with ccRCC. In the multivariate Cox regression analysis of the FUSCC cohort, pathological tumor stage (pT stage), pathological metastasis stage (pM stage), ISUP grade, and the presence of tumor-proximal TLS remained predictors for both PFS (pT stage: p=0.005; pM stage: p=0.001; ISUP grade: p=0.001; tumor-proximal TLS: p=0.029) and OS (pT stage: p=0.004; pM stage: p=0.001; ISUP grade: p=0.027; tumor-proximal TLS: p=0.030). Similarly, in the external validation cohort, pT stage, pM stage, ISUP grade, and the presence of tumor-proximal TLS were also associated with PFS (pT stage: p=0.036; pM stage: p=0.002; ISUP grade: p=0.029; tumor-proximal TLS: p=0.029) and OS (pT stage: p=0.001; pM stage: p=0.011; ISUP grade: p=0.088; tumor-proximal TLS: p=0.039) among 105 patients with ccRCC (table 2).

Subsequently, a nomogram was developed to predict the probability of 2-year, 5-year, and 8-year PFS based on the multivariate Cox regression analysis using the FUSCC training cohort (figure 5A). The nomogram incorporated the presence of tumor-proximal TLS as one of the four variables. The high risk score associated with tumor-proximal TLS in the nomogram demonstrated a significant correlation with aggressive PFS (p=7.4e-23, HR=5.92) in the cohort of 290 patients with ccRCC (figure 5B). The AUC value of the time-dependent ROC curve was >0.80 (2-year AUC=0.82; 5-year AUC=0.82, and 8-year AUC=0.84), indicating high sensitivity and specificity of the PFS-predicting nomogram. Furthermore, on including tumor-proximal TLS, pT stage, pM stage, and ISUP grade, the elevated risk score from the OS-predicting nomogram was significantly associated with shorter OS (p=2.8e-23, HR=6.14) in the same cohort of 290 patients with ccRCC (figure 5C,D). The AUC values were validated in the external validation cohort (PFS, p=0.054, HR=0.319; OS, p=0.091, HR=0.280).
Figure 2  Influence of tumor-proximal or tumor-distal localization of TLS on the prognosis of patients with ccRCC from the FUSCC and external validation sets. (A,B) Kaplan-Meier and log-rank tests identifying the predictive value of tumor-proximal or tumor-distal localization of TLS for the prognosis in the training set (n=290) from the FUSCC cohort and the external validation set (n=105). ccRCC, clear cell renal cell carcinoma; FUSCC, Fudan University Shanghai Cancer Center; TLS, tertiary lymphoid structures.
of the time-dependent ROC curve demonstrated superior discriminative ability with 2-year, 5-year, and 8-year AUC of 0.78, 0.82, and 0.83, respectively. Additionally, the nomogram involving tumor-proximal TLS was tested in the external validation cohort of 105 patients with ccRCC, revealing a higher risk score as a significant predictor of progressive PFS (p=3.2e-13, HR=6.47, 5-year AUC=0.82) and poorer OS (p=1.4e-9, HR=6.32, 8-year AUC=0.83) (figure 5E). These findings collectively demonstrated the sensitive and independent prognostic abilities of tumor-proximal TLS in patients with ccRCC and underscored the remarkable predictive performance of the nomograms involving tumor-proximal TLS, enabling the identification of patients at high risk of long-term tumor recurrence and poor clinical outcomes.

Heterogeneity in TLS maturation and cellular components assessed by mIHC

Heterogeneity in the relationship between TLS maturation stages and cancer has been observed, suggesting potential prognostic implications.23 34 Therefore, a more comprehensive understanding of TLS heterogeneity, encompassing cellular composition, location, maturation, and their impact on antitumor immune responses,
In this study, the TLS heterogeneity was characterized in heterogeneous ccRCC samples using mIHC in 58 cases of ccRCC tissues. Two antibody panels (panel 1: CD8, CK, CD68, CD163, PD-1, PD-L1, and DAPI; panel 2: CD3, CK, CD56, CD20, CD4, FoxP3, and DAPI) were used with the Akoya Opal Polaris 7-Color Automation IHC kit (NEL871001KT), allowing for the visualization of 12 primary antibody stains. Our analysis focused on assessing the infiltration ratio of adaptive immune responder cells within the ccRCC TME that are involved in recognizing, responding to, and eliminating tumor cells during tumor progression (online supplemental figure S3). Panel 1 provides insights into the abundance of CD8+ T cells, PD-1+CD8 + T cells, CD68+CD163 − M1 macrophages, CD68 +CD163 + M2 macrophages, CK-surrounded CD68 +CD163 + TAMs, PD-L1 +CD68 +CD163 + M2 macrophages, and CPS of PD-L1 expression, all of which were further analyzed (figure 6A). Panel 2 allowed for the evaluation of CD3+ T cells, CD4+ T cells, CD4+FoxP3+ Treg cells, CD20+ B cells, and CD56bright/dim NK cells within the TME (figure 6B). Furthermore, the analysis of cellular composition within TLS maturation stages revealed heterogeneity in TILs, as shown in figure 6C–E. Interestingly, at a low magnification of a tissue sample, E-TLS and SFL-TLS were observed to be adjacent to each other in the same location; however, distinct differences were evident in their external contour structure and internal cellular components.

The localization and maturation heterogeneities of tumor-specific TLS are associated with different TME characteristics

A set of patients with different TLS localization and maturation stages was selected to examine the cellular components involved in TLS development and their predictive value on the TME immune status, and the abundance of lymphocytes within the TLS and tumor immune escape in ccRCC was assessed.19 A series of lymphocytes classification for tumor-proximal and tumor-distal infiltration was established, and their proportion per hundred DAPI-positive cells

---

Figure 4 Influence of SFL-TLS presence on progression-free survival and overall survival in patients with ccRCC from the FUSCC validation and external validation cohorts. The mature and immature TLS density was assessed (which can be assessed as GC+ and GC− clusters using immunohistochemistry staining), and ccRCC samples were divided irrespective of their location within the tumor. Kaplan-Meier and log-rank tests identifying the predictive value of the presence of mature TLS (namely SFL-TLS, with CD23+ GC cells) for prognosis in the training set (n=290) from the FUSCC and the external validation cohorts (n=105). ccRCC, clear cell renal cell carcinoma; FUSCC, Fudan University Shanghai Cancer Center; GC, germinal center; SFL, secondary follicle-like; TLS, tertiary lymphoid structures.
algorithmically, including Treg cells, PD-L1+CD8+ T cells, and PD-L1+ tumor-associated M2 macrophages (PD-L1+ TAMs) was quantitatively determined using an algorithm (figure 7). The inForm image analysis software was used with the Vectra multispectral imaging analysis system to obtain images with a high signal-to-noise ratio (excluding background spontaneous fluorescence) and conduct accurate batch quantitative analysis, provide accurate quantification of various biomarkers in each cell, and analyze the percentage of specific cell types in the number of DAPI-positive cells. The resulting data were subjected to non-hierarchical clustering analysis, generating a heatmap that visualized the fractions of lymphocytes and CD8+ T cells infiltrating in the tumor-proximal and tumor-distal regions. Furthermore, the heatmap depicted the heterogeneity of TLS in terms of localization and maturation stages (figure 7A and online supplemental figure S4). In vertical order by TLS localization, distinct patterns of lymphocyte infiltration highlight notable differences among multiple lymphocyte subpopulations. Specifically, differential infiltration of tumor-distal PD-L1+CD68+CD163+ M2 macrophages, tumor-proximal CD4+FoxP3+ Treg cells, and tumor-proximal PD-L1+CD8+ T cell in the tumor-distal TLS patients group were observed compared with those in the tumor-proximal TLS patients group (figure 7A). This observation of TLS heterogeneity provides valuable indirect evidence for a theoretical basis and addresses questions regarding causality in relation to prognostic effects. Therefore, the heterogeneity in TLS maturation stages was applied to distinguish the variations in lymphocyte infiltration and the TME status. In vertical order by step-by-step TLS maturation stages, distinct patterns of the immune cells infiltration also reflect novel differences among patients group (online supplemental figures 5,6). As shown in figure 7B–G, the abundance of TILs based on the heterogeneity of TLS maturation was compared. Remarkably elevated infiltration states were observed in the tumor-distal area within the PFL-TLS patients group for CD68+CD163+ M2 macrophages, PD-L1+CD68+CD163+ M2 macrophages, CD4+FoxP3+ Treg cells, and CD8+ T cells using one-way ANOVA test. A higher proportion of CD68+CD163+ M1 macrophages and CD8+ T cells in tumors were observed.

### Table 2 Multivariate Cox logistic regression analysis of PFS and OS for 395 patients with ccRCC from the FUSCC and external validation cohorts

<table>
<thead>
<tr>
<th></th>
<th>FUSCC training cohort (n=290)</th>
<th></th>
<th>External validation cohort (n=105)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P value</td>
<td>HR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td><strong>PFS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1.003 (0.99 to 1.017)</td>
<td>0.646</td>
<td>1.008 (0.986 to 1.03)</td>
<td>0.487</td>
</tr>
<tr>
<td>Pathological T stage (ref. T1/T2)</td>
<td>1.728 (1.182 to 2.526)</td>
<td><strong>0.005</strong></td>
<td>2.148 (1.051 to 4.391)</td>
<td><strong>0.036</strong></td>
</tr>
<tr>
<td>Pathological M stage (ref. M0)</td>
<td>4.189 (2.768 to 6.339)</td>
<td><strong>&lt;0.001</strong></td>
<td>4.279 (1.736 to 10.544)</td>
<td><strong>0.002</strong></td>
</tr>
<tr>
<td>ISUP grade (ref. G1/G2)</td>
<td>1.696 (1.24 to 2.321)</td>
<td><strong>0.001</strong></td>
<td>2.127 (1.082 to 4.184)</td>
<td><strong>0.029</strong></td>
</tr>
<tr>
<td>Tumor-proximal TLS (ref. absence)</td>
<td>0.426 (0.198 to 0.916)</td>
<td><strong>0.029</strong></td>
<td>0.252 (0.073 to 0.868)</td>
<td><strong>0.029</strong></td>
</tr>
<tr>
<td>Tumor-distal TLS (ref. absence)</td>
<td>1.25 (0.859 to 1.818)</td>
<td>0.243</td>
<td>1.237 (0.653 to 2.343)</td>
<td>0.514</td>
</tr>
<tr>
<td><strong>OS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1.006 (0.992 to 1.021)</td>
<td>0.417</td>
<td>1.034 (1.005 to 1.065)</td>
<td>0.023</td>
</tr>
<tr>
<td>Pathological T stage (ref. T1/T2)</td>
<td>1.807 (1.203 to 2.715)</td>
<td><strong>0.004</strong></td>
<td>5.953 (2.405 to 14.736)</td>
<td><strong>&lt;0.001</strong></td>
</tr>
<tr>
<td>Pathological M stage (ref. M0)</td>
<td>4.659 (2.994 to 7.25)</td>
<td><strong>&lt;0.001</strong></td>
<td>4.323 (1.397 to 13.38)</td>
<td>0.011</td>
</tr>
<tr>
<td>ISUP grade (ref. G1/G2)</td>
<td>1.463 (1.044 to 2.048)</td>
<td><strong>0.027</strong></td>
<td>2.077 (0.897 to 4.806)</td>
<td>0.088</td>
</tr>
<tr>
<td>Tumor-proximal TLS (ref. absence)</td>
<td>0.33 (0.121 to 0.898)</td>
<td><strong>0.030</strong></td>
<td>0.103 (0.012 to 0.891)</td>
<td><strong>0.039</strong></td>
</tr>
<tr>
<td>Tumor-distal TLS (ref. absence)</td>
<td>1.137 (0.764 to 1.693)</td>
<td>0.526</td>
<td>0.859 (0.342 to 2.155)</td>
<td>0.745</td>
</tr>
</tbody>
</table>

*P value<0.05 is marked in bold.

ccRCC, clear cell renal cell carcinoma; FUSCC, Fudan University Shanghai Cancer Center; ISUP, International Society of Urological Pathology; OS, overall survival; PFS, progression-free survival; TLS, tertiary lymphoid structures.
DISCUSSION

TLS have emerged as a focal point in cancer research, particularly due to their association with the treatment prognosis. Numerous studies have investigated TLS in various cancer types and have consistently found a positive correlation with favorable therapeutic outcomes.35-36 However, limited information exists regarding TLS in renal cell carcinoma. Furthermore, the existing studies on TLS in this context have yielded contradictory findings.20-21 Therefore, a more comprehensive analysis of clinical data and assessment of TLS characteristics are needed. In this study, three significant findings were presented that could serve as a foundation for future clinical examinations and prognosis prediction.

Our initial investigation into TLS histology in ccRCC revealed their heterogeneity. Previous studies on other cancers have outlined two distinct localizations and three maturation stages of TLS.35-36 Herein, tumor-proximal (or intratumoral) and tumor-distal (or peritumoral) TLS in ccRCC were identified. Interestingly, it was observed that tumor-proximal TLS in ccRCC primarily exhibited characteristics of SFL-TLS, while tumor-distal TLS predominantly displayed features of E-TLS. This finding contrasts
with the study by Masuda et al., where no PFL-TLS or SFL-TLS are observed in ccRCC. Considering the relatively low presence of PFL-TLS (16.5%) and SFL-TLS (7.8%) in our ccRCC samples, limited patient numbers and small sample sectioning methods such as pathological tissue microarray scan could overlook PFL-TLS or SFL-TLS. However, the underlying mechanisms driving the differences in TLS maturation between different locations remain unclear. It would be valuable to investigate the prognostic significance of TLSs in patients with ccRCC.

Figure 6  Heterogeneity of TLS maturation and cellular components assessed by mIHC. (A–B) The cellular composition of TLS in heterogeneous ccRCC samples was detected using mIHC in 58 ccRCC tissue samples using two panels of 12 antibody markers for further analysis. (C–E) Distinct differences in tumor-infiltrated lymphocytes revealed heterogeneity of cellular composition in TLS maturation stages (early TLS, primary follicle-like TLS, and secondary follicle-like TLS). ccRCC, clear cell renal cell carcinoma; CD, cluster of differentiation; CK, cytokeratin; DAPI, 4’,6-diamidino-2-phenylindole; E-TLS, early TLS; FoxP3, forkhead box P3; mIHC, multiplex immunohistochemistry; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; PFL-TLS, primary follicle-like TLS; SFL-TLS, secondary follicle-like TLS; TLS, tertiary lymphoid structures.
undergoing treatment with antiangiogenic drugs and combination therapy, considering the potential impact of abundant vasculature within ccRCC on FDC and lymphocyte recruitment.38

Our second finding suggests that tumor-proximal TLS and tumor-distal TLS characterized by different maturity stages and localization exhibit opposing prognostic trends in ccRCC. This observation might help explain the paradoxical role of TLS in renal carcinoma. Our data revealed that the presence of TLS does not predict ccRCC prognosis; however, a more detailed examination of TLS localization holds clinical significance. The presence of tumor-proximal TLS indicates a better prognosis, consistent with the findings of in ccRCC and the general understanding of other cancer types.32 39 40 Conversely, the presence of tumor-distal TLS, primarily in the immature stage, is associated with a poorer prognosis in ccRCC, consistent with the observations of Masuda et al.30 This divergence in prognostic value highlights the heterogeneity of TLS in ccRCC and the importance of characterizing TLS features. Interestingly, patients with tumor-distal TLS demonstrated a significantly higher risk of recurrence and poorer clinical outcomes in breast and liver cancers.32 41 In contrast, studies on other cancer types, such as colorectal and lung cancers, emphasize the positive prognostic importance of mature TLS, adding further pathological relevance to their function.19 24 34 42

Therefore, investigating whether TLS maturation could be a confounding factor for the differing prognostic associations in ccRCC rather than solely focusing on the localization could be a valuable direction for future research.

At present, using TLS location as a diagnostic indicator is easier, faster and less expensive in clinical practice, since determining the maturation stages of TLS requires serial sectioning and IHC staining.

Our third key finding involved mIHC staining to analyze immune cell populations and further elucidate the differences in the tumor environment between

Figure 7  The localization and maturation heterogeneities of tumor-specific TLS associated with different tumor microenvironment characteristics. (A) Non-hierarchical clustering in the heatmap reveals the fraction of lymphocytic infiltration (proportion per hundred cells) in the tumor-proximal and tumor-distal regions, as well as the heterogeneity of TLS in terms of the localization and maturation stages. (B–G) The inForm image analysis software combined with the Vectra multispectral imaging analysis system was used to obtain images with a high signal-to-noise ratio (excluding background spontaneous fluorescence) and conduct accurate batch quantitative analysis, provide accurate quantification of various biomarkers in each cell, and analyze the percentage of specific types of cells in the number of DAPI positive cells. Differential percentage of the cell number/total cell number was compared by the heterogeneity of TLS maturation using one-way ANOVA. ANOVA, analysis of variance; CPS, Combined Positive Score; E-TLS, early TLS; DAPI, 4′,6-diamidino-2-phenylindole; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; PFL-TLS, primary follicle-like TLS; SFL-TLS, secondary follicle-like TLS; TAM, tumor-associated macrophages; TLS, tertiary lymphoid structures; Treg cells, regulatory T cells.
tumor-proximal and tumor-distal TLS in ccRCC. Higher tumor-distal PD-L1+ M2 macrophage and tumor-proximal Treg infiltration were observed in tumor-distal TLS relative to tumor-proximal TLS, and both cells represent an immunosuppressive environment. Furthermore, increased tumor-proximal PD-1+CD8+ T-cell infiltration was observed in the tumor-distal TLS compared with tumor-proximal TLS. These T cells, together with the presence of PD-L1+ M2 macrophage and Treg cells, are likely to be exhausted and have been associated with an immune-cold TME or adaptive immune resistance. These findings might help explain the poorer prognosis associated with distal TLS in ccRCC, and targeting the reinvigoration of PD-1+CD8+ T cells and challenging the suppressive TME could enhance patients’ sensitivity to current immunotherapy strategies. Additionally, previous studies have demonstrated that pre-existing tumor immunity in renal cell carcinoma predicts a better response to immune checkpoint blockade therapy, which might be associated with the APC-dense niche within the TME. Thus, it would be intriguing to include an assessment of the APC-dense niche and T-cell factor-1+ CD8 T cells in future investigation, particularly when examining the SFL-TLS.

There are certain limitations in this study. First, the potential predictive value of tumor-specific TLS in ICI response for patients with advanced ccRCC has not been investigated. TLSs play a crucial role in creating a favorable microenvironment for immune cell aggregation and humoral immune responses, making them a promising candidate for effectively predicting ICI tolerance in patients with solid tumors. However, this aspect was not explored in our study. Second, despite the inclusion of a large number of patients with ccRCC and the use of independent cohorts, the retrospective nature of the study cannot be overlooked, which would reduce the clinical significance of our findings. Therefore, future prospective cohorts are necessary to validate the hypotheses proposed in this study. In addition, the identification of patient samples based on the TLS maturation stages was performed as follows: SFL-TLS positive sample had at least one occurrence of SFL-TLS; PFL-TLS positive sample had at least one occurrence of PFL-TLS but no SFL-TLS; E-TLS positive sample had neither PFL-TLS nor SFL-TLS. We acknowledge that such a method may produce statistical bias and mechanically fail to demonstrate the most intuitive distinction between immune cell components at various TLS maturation stages. However, the statistical procedure may be more practical for clinical practice based on a pathological section from a single patient. For instance, clinicians can readily determine the overall clinicopathological characteristics of SFL-TLS patients, which is beneficial for prognosis and treatment response prediction. Lastly, while induced TLS therapies are considered a promising new strategy for cancer treatment, they are predominantly used in preclinical studies. For example, anti-angiogenesis therapy combined with PD-L1 blocking could regulate angiogenic vascularity, induce TLS, and enhance cytotoxic T-cell activity, resulting in additional survival benefits. This study failed to further explore the mechanism by which TLS localization and maturation heterogeneity affects abnormal ccRCC TME phenotypes, and studies, wherein the TME mimic ccRCC in vivo, are yet to be conducted. Exploration using the immunosound-in-situ xenografted tumor model or patient-derived tumor organoid/patient-derived tumor xenograft model might help direct future research.

In conclusion, this study revealed the predictive value of TLS heterogeneity in terms of the maturation status and localization in relation to ccRCC progression and immunological response for the first time. Most TLS reside in the tumor-distal area and are associated with an immature, immunosuppressive phenotype. The tumor-proximal, mature TLS could help evaluate the prognostic patterns and the unique TME characteristics of each patient. These findings could help identify immunophenotypes and improve the effectiveness of immunotherapeutic approaches for ccRCC.

Author affiliations
1Department of Urology, Fudan University Shanghai Cancer Center; Department of Oncology, Shanghai Medical College, Fudan University, Shanghai, People’s Republic of China
2Shanghai Genitourinary Cancer Institute, Shanghai, People’s Republic of China
3School of Cellular and Molecular Medicine, University of Bristol, Bristol, UK
4Department of Interventional Oncology, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, People’s Republic of China
5Institute of Photomedicine, Shanghai Skin Disease Hospital, School of Medicine, Tongji University, Shanghai, People’s Republic of China
6Department of Surgery, Shanghai Nan Branch of Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, People’s Republic of China

Twitter Wang-Rui Liu @Michale7_Liu

Acknowledgements We extend our sincere appreciation to all who participated in this study. Furthermore, we would like to express our sincere gratitude to Shanghai KR Pharmtech for their invaluable technical guidance and support in implementing multiplex immunohistochemistry assay based on next-generation pathology technology.


Funding This work was supported by grants from the National Natural Science Foundation of China (No.81802525, and 821172817), the Natural Science Foundation of Shanghai (No. 20ZR1413100), Beijing Xisike Clinical Oncology Research Foundation (No. Y-2R2020MS-0948), the Shanghai Anticancer Association EVAS PROJECT (No. SACA-CY21A06 and SACA-CY21B01), and Shanghai Municipal Health Bureau (No.2020CXJD03).

Competing interests None declared.

Patient consent for publication Consent obtained directly from patient(s).

Ethics approval The study design and test procedures followed the Helsinki Declaration II. The ethics approval and consent to participate the urology and pathology departments in this study were approved by the ethics committee of Fudan University Shanghai Cancer Center (No: 050432-4-2108*, FUSCC, Shanghai, China). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. The data sets used and/or analyzed in the study are available from the corresponding author upon reasonable request.

Supplemental material This content has been supplied by the author(s). It has not been vetted by BMJ Publishing Group Limited (BMJ) and may not have been peer-reviewed. Any opinions or recommendations discussed are solely those of the author(s) and are not endorsed by BMJ. BMJ disclaims all liability and responsibility arising from any reliance placed on the content. Where the content includes any translated material, BMJ does not warrant the accuracy and reliability of the translations (including but not limited to local regulations, clinical guidelines, terminology, drug names and drug dosages), and is not responsible for any error and/or omissions arising from translation and adaptation or otherwise.

Open access This is an open access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited, appropriate credit is given, any changes made indicated, and the use and license their derivative works are appropriately credited.

Open access statement Data are available upon reasonable request. The data sets used and/or analyzed in the study are available from the corresponding author upon reasonable request.

Supplemental material This content has been supplied by the author(s). It has not been vetted by BMJ Publishing Group Limited (BMJ) and may not have been peer-reviewed. Any opinions or recommendations discussed are solely those of the author(s) and are not endorsed by BMJ. BMJ disclaims all liability and responsibility arising from any reliance placed on the content. Where the content includes any translated material, BMJ does not warrant the accuracy and reliability of the translations (including but not limited to local regulations, clinical guidelines, terminology, drug names and drug dosages), and is not responsible for any error and/or omissions arising from translation and adaptation or otherwise.

Open access This is an open access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited, appropriate credit is given, any changes made indicated, and the use and license their derivative works are appropriately credited.

REFERENCES


