

# Tertiary Lymphoid Structures in Brain Metastases of Lung Cancer: Prognostic Significance and Correlation With Clinical Outcomes

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**Abstract.** *Background/Aim:* The prognosis of patients with brain metastases (BMs) originating from lung cancer remains poor, despite advancements in treatment strategies. The role of tertiary lymphoid structures (TLSs) within the tumor immune microenvironment of BMs has not been extensively explored. *Patients and Methods:* This study utilized patient-derived clinical samples from 17 patients with histologically confirmed BMs of lung cancer, undergoing surgical resection. Immunohistochemistry was employed to analyze the presence and characteristics of TLS and tumor-infiltrating lymphocytes (TILs) within BM tissues, correlating these with clinical outcomes. *Results:* TLSs, albeit in their immature form, were identified within BM tissues, distinguishing them from their mature

counterparts in primary lung cancer tissues. A significant correlation between TLS density (but not TIL density) and improved postoperative survival was observed, underscoring the potential of TLS density as an independent prognostic marker. Furthermore, TLS density did not correlate with the Graded Prognostic Assessment (GPA) index, suggesting its unique prognostic value beyond conventional predictors. *Conclusion:* Our findings reveal the presence of TLSs in lung cancer-derived BMs and highlight their prognostic significance, independent of the GPA index. The identification of TLS within the unique central nervous system tumor microenvironment offers new insights into the immune landscape of BMs and suggests potential avenues for immunotherapeutic interventions targeting these structures to improve patient outcomes.

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**Key Words:** Tertiary lymphoid structures, brain metastases, lung cancer, tumor immune microenvironment (TIME), prognostic biomarkers.



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Brain metastases (BMs) are the most common malignant tumors affecting the central nervous system (CNS). Treatment of BMs remains ineffective due to the blood-brain barrier (BBB) and the unique microenvironment of the brain, which is characterized by immune tolerance (1, 2). Lung cancer is a common primary tumor associated with BMs, accounting for 20-56% of cases (1). As advances in systemic therapies have led to better control of primary tumors or metastases outside the CNS, the development of targeted therapies for BMs has become a critical priority.

In recent years, the relationship between cancers and the immune system has been attracting attention. The most prominent of these are tertiary lymphoid structures (TLSs). These are ectopic lymph node-like aggregates of lymphocytes

that form in non-lymphoid tissues observed in malignancies including lung cancer (3-5). TLSs have been shown to play critical roles in shaping the tumor immune microenvironment (TIME) and contribute to anti-tumor immunity (6). In fact, the presence of TLSs in non-small cell lung cancer (NSCLC) correlates with a favorable outcome in previous studies (7, 8).

Although CNS has traditionally been considered an immunologically privileged site, recent evidence has revealed that it possesses intrinsic immune surveillance mechanisms (9). In particular, TLSs in glioblastoma (GBM) have been shown to play an important role in the presentation of tumor antigens and the promotion of anti-tumor T cell priming (10, 11). These findings have led to a growing interest in understanding the mechanisms of the TIME of brain tumors. However, the presence and role of TLSs in BMs are largely unknown.

To address these research questions, we decided to analyze BM-associated TLSs using patient-derived clinical samples. Since lung cancer is the most common primary disease of BMs as mentioned above, we used this disease entity as our study population and investigated the correlation between TLS-associated parameters in tumor tissues and clinical parameters, including patient outcome.

## Patients and Methods

**Patients.** This study was approved by the institutional ethics review board of Aichi Cancer Center (ACC) (approval number: IR041168). The requirement to obtain written informed consent from patients was waived owing to the retrospective nature of this study. Instead, a public notice providing information about this study was posted on our center's website. All patient identifiers have been protected according to ethical guidelines. Seventeen consecutive patients with histologically confirmed BMs of lung cancer who underwent surgical resection at ACC from April 2018 to December 2022 were enrolled. A patient with postoperative complication (n=1) was excluded from this study.

**Immunohistochemistry.** The immunohistochemical staining procedure for formalin-fixed, paraffin-embedded samples of metastatic lung cancer brain tumors has been described previously (12). Briefly, 4- $\mu$ m tissue sections were used for hematoxylin and eosin (HE) staining and immunohistochemistry (IHC). Tissue sections were deparaffinized in xylene, rehydrated through a graded series of ethanol dilutions, and subjected to antigen retrieval using heat-induced epitope retrieval methods. One section from each series was stained with HE, while the remaining sections were immunostained with the following primary monoclonal antibodies (mAbs): rabbit anti-CD4 mAb (clone EPR6855, Abcam, Cambridge, UK), mouse anti-CD20 mAb (clone L26, Nichirei, Tokyo, Japan), mouse anti-BCL6 mAb (clone LN22, Nichirei), and mouse anti-CD8 mAb (clone C8/144B, DAKO, Glostrup, Denmark). The following secondary antibody complexes were then used: Histofine Simple Stain MAX-PO (mouse, Nichirei) for CD8 staining, and Histofine Simple Stain MAX-PO (rabbit, Nichirei) and Histofine Simple Stain AP (mouse, Nichirei) for the triple staining of CD4, CD20, and BCL6. Chromogenic reaction was performed using the First Red II Substrate Kit (Nichirei), HistoGreen Substrate Kit (Cosmo Bio, Tokyo, Japan), and Histofine DAB Substrate Kit (Nichirei). Nuclei were contrast stained with hematoxylin.

**Image analysis.** Image analysis was performed using QuPath (ver 0.5.0), an open-source software platform for digital pathology and bioimage analysis (13). It provides a comprehensive set of tools for the analysis and interpretation of whole slide images (WSIs) and other large biomedical images. The stained tissue sections were digitally scanned using a NanoZoomer-SQ whole slide imaging system (Hamamatsu Photonics, Hamamatsu, Japan) with a 20X 0.75 NA objective lens, according to a standardized protocol. The scanned images were then imported into QuPath for quantitative analysis. The "create threshold" algorithm was used to acquire individual tumor tissue regions. Immunostained cells were identified as follows: First Red II for CD4 and CD8, DAB for CD20, and HistoGreen for Bcl-6. After the manual configuration of color thresholds (color deconvolution), each immunostained cell was detected and quantified using QuPath's positive cell detection algorithm. Immune cell density was measured as the number of cells per 1 mm<sup>2</sup> of tumor area. Lymphocytes accumulated around radiation necrosis were excluded from the analysis.

**Statistical analysis.** All statistical analyses were performed using EZR software (version 1.64, Saitama Medical Center, Jichi Medical University, Saitama, Japan) as previously described (14, 15). Statistical significance between two groups was determined using Mann-Whitney *U*-test. Log-rank test with Kaplan-Meier method was used for survival time analysis. Statistical significance was defined as  $p < 0.05$ .

## Results

**Clinical characteristics of patients with BMs derived from lung cancer.** We first investigated the patient characteristics (Table I and Table II). Patients underwent surgical BM resection at a mean age of 64. Mean postoperative overall survival (OS) was 14 months. In more details, the 17 cohorts consisted of nine males (53%) and eight females (47%). Histopathological diagnoses of the surgical specimens of BMs were 15 adenocarcinomas, one squamous cell carcinoma, and one small cell lung cancer. Regarding the postoperative OS, those who survived less than one year were 10 patients (59%) and the others (41%) survived over one year. Those who received preoperative radiation were 14 patients (82%), and 10 patients (59%) received postoperative radiation. Those who received preoperative chemotherapy were 13 patients (76%), and 10 patients (59%) received postoperative chemotherapy. We observed no significant differences regarding these characteristics. There were five cases of primary tumor specimen available for simultaneous analysis.

**Lung cancer-derived BM tissues consist of immature TLSs.** To investigate the distribution of tumor-infiltrating lymphocytes (TILs) in BMs of lung cancer, we first examined whether we could identify the cells positive for CD20, Bcl-6, CD4, and CD8 in the BM tissues. In the primary lesions of lung cancers, we observed mature TLSs that were characterized by well-organized structures and the presence of germinal centers (GCs) containing CD20+ Bcl-6+ B cells (Figure 1A). In contrast, although we sporadically

Table I. Summary of patient characteristics.

Characteristics (n=17)	Mean (range)
Age at surgery (years)	64 (42-77)
KPS	76 (60-90)
GPA index	2.1 (0.5-4.0)
Postoperative OS (months)	14 (2-36)
Postoperative PFS (months)	8.2 (1-36)
OS from initial diagnosis (months)	46.4 (4-133)
OS from metastasis diagnosis (months)	32.1 (4-118)
Period from initial diagnosis to BM resection (months)	14 (0-57)

KPS: Karnofsky performance status; OS: overall survival; PFS: progression-free survival; GPA: graded prognostic assessment.

identified aggregates of CD20+ B lymphocytes at the peritumoral lesions in the BM tissues, they lacked organized structures and the formation of GCs (Figure 1B). These cell aggregates were considered immature TLSs, and we decided to analyze them in this study.

Next, we examined quantitative differences between TLSs in the primary tumors and BMs using five cases for which both primary tumors and BM specimens were available. The mean TLS density in the primary lung cancer tissues was 3.426/mm<sup>2</sup> (n=5), and that of BM tissues was 0.8323/mm<sup>2</sup> (n=17) ( $p=0.0022$ ; Figure 1C). Regarding TILs, the mean TIL density in the lung cancer tissues was 6,201/mm<sup>2</sup> (n=5), while that of in BM tissues was 572/mm<sup>2</sup> (n=17) ( $p=0.000304$ ; Figure 1D).

*TLS density, but not TIL density, in BMs correlates with patients' survival after surgical resection.* We then assessed the prognostic significance of TLS and TILs in the BM tissues. To this end, all the patients were divided into two groups based on the median densities of TILs and TLSs (high: n=8, low: n=9) in the BM tissues, respectively. The TLS density was significantly associated with a favorable clinical outcome after surgical resection (Figure 2A). In contrast, although the TIL density has been previously reported as a favorable prognostic factor in NSCLC (16), we observed no significant association between the postoperative survival and the TIL density in BMs (Figure 2B).

*TLS density in BMs is a prognostic factor of lung cancer-derived BM patients independent of GPA index.* The Graded Prognostic Assessment (GPA) is a widely used prognostic index to estimate survival in patients with BMs (17, 18). As we observed a positive correlation between the TLS density and patient outcome (Figure 2A), we investigated the correlation between the TLS density and the GPA index. To address this issue, all the patients were divided into two groups based on the median value of GPA index (high: n=8, low: n=9). The group with a higher GPA index showed

Table II. Details of patient characteristics.

Characteristics (n=17)	Numbers (%)
Sex	
Male	9 (53%)
Female	8 (47%)
Type of primary lung cancer	
Adenocarcinoma	15 (88%)
Squamous cell carcinoma	1 (6%)
Small cell lung cancer	1 (6%)
KPS	
≥80	10 (59%)
≤70	7 (41%)
GPA index	
0-1	3 (18%)
1.5-2	6 (35%)
2.5-3	6 (35%)
3.5-4	2 (12%)
Postoperative OS (months)	
0-6	5 (29%)
7-12	5 (29%)
13-24	3 (18%)
>24	4 (24%)
Postoperative PFS (months)	
0-6	10 (59%)
7-12	3 (18%)
13-24	3 (18%)
>24	1 (6%)
OS from initial diagnosis (months)	
0-12	2 (12%)
13-24	5 (29%)
25-36	0 (0%)
>36	10 (59%)
OS from metastasis diagnosis (months)	
0-12	4 (24%)
13-24	5 (29%)
25-36	3 (18%)
>36	5 (29%)
Preoperative RT	
Yes	14 (82%)
No	3 (18%)
Postoperative RT	
Yes	10 (59%)
No	7 (41%)
Preoperative CT	
Yes	13 (76%)
No	4 (24%)
Postoperative CT	
Yes	10 (59%)
No	7 (41%)

RT: Radiation therapy; CT: chemotherapy.

significantly longer postoperative OS ( $p=0.00436$ ; Figure 3A), as well as postoperative PFS ( $p=0.003$ ) and OS ( $p=0.0028$ ) from metastasis diagnosis (data not shown). These data suggested that the GPA index serves as a good prognostic indicator. However, we observed no statistically significant association between the TLS density and the GPA

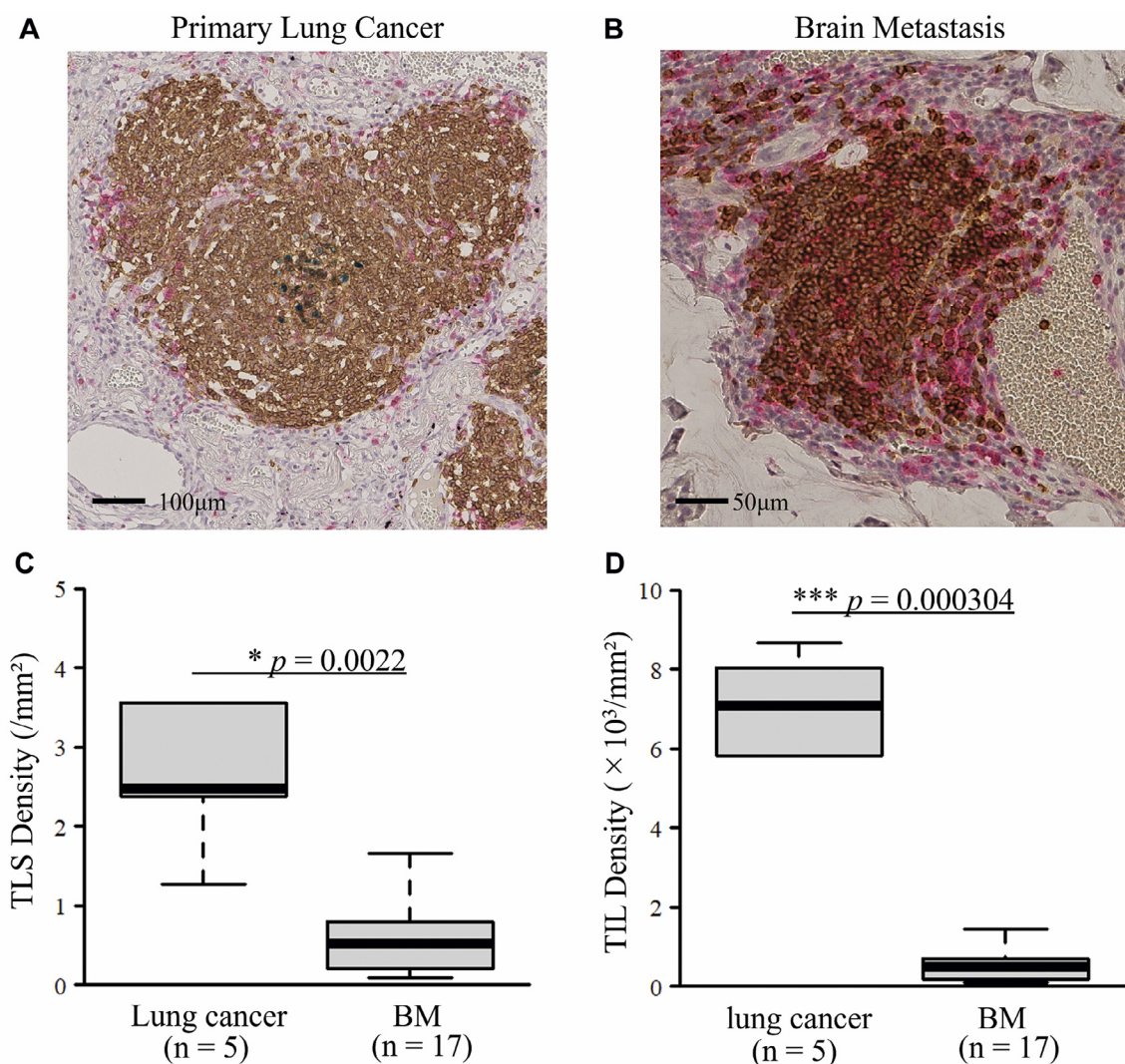


Figure 1. Lung cancer-derived brain metastases (BMs) consist of immature tertiary lymphoid structures (TLSs). (A and B) Representative pathological images of TLSs in primary lung cancer and BMs. The sections were stained with CD4 (red), CD20 (brown), and Bcl-6 (green). (A) A TLS in a primary lung cancer. Magnification: 10×, scale bar: 100 μm. (B) A TLS in a BM tissue. Magnification: 20×, scale bar: 50 μm. (C and D) Comparison of the densities of TLSs (C) and tumor-infiltrating lymphocytes (TILs; D) in the primary lung cancers (n=5) and the BMs (n=17). p-Values were determined using the Mann–Whitney U-test. \*p<0.05; \*\*\*p<0.001.

index ( $p=0.36$ ; Figure 3B). The TIL density showed no significant association with the GPA index, either ( $p=0.6304$ ; Figure 3C). Although neither the TLS density nor the TIL density showed any association with GPA index (Figure 3B and C), the TLS density alone (Figure 2A) may serve as a prognostic factor of lung cancer-derived patients with BMs, independent of GPA index.

## Discussion

In this study, we identified TLSs in the BMs of lung cancer using surgically resected specimens and compared the morphological and quantitative characteristic of TLSs in

BMs with those in primary lung cancers (Figure 1). Furthermore, we demonstrated that the TLS density-based grading (high vs. low) significantly correlated with postoperative survival of the patients (Figure 2A). However, the TLS density itself did not correlate with GPA index, a well-established prognostic indicator for the patients with BMs (Figure 3B). These findings suggest that TLS density-associated parameters may serve as independent prognostic factors for the patients with BMs of lung cancer, providing additional information beyond the GPA index.

TLSs are ectopic lymphoid organs emerging in non-lymphoid tissues during chronic inflammation, such as chronic diseases and cancers (6, 19, 20). The development

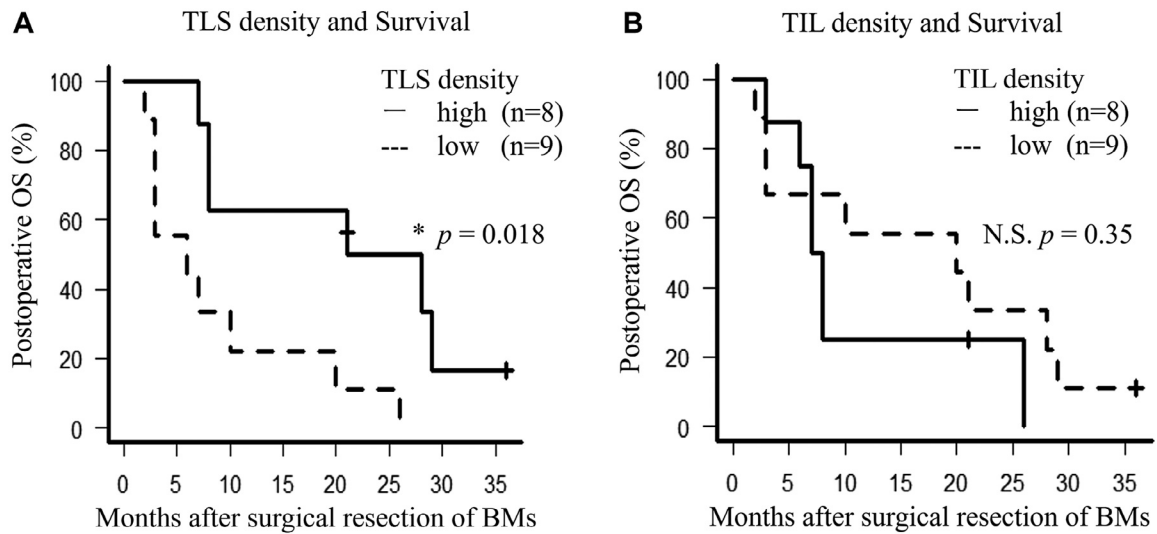


Figure 2. Tertiary lymphoid structure (TLS) density, but not tumor-infiltrating lymphocyte (TIL) density, in brain metastases (BMs) correlates with patients' survival after surgical resection. Kaplan–Meier plots displaying postoperative overall survival (OS) of the patients with BMs stratified into high- (n=8) and low-density groups (n=9) based on TLS (A) and TIL (B). *p*-Values were determined by log-rank test. \* $p < 0.05$ ; N.S.: Not significant.

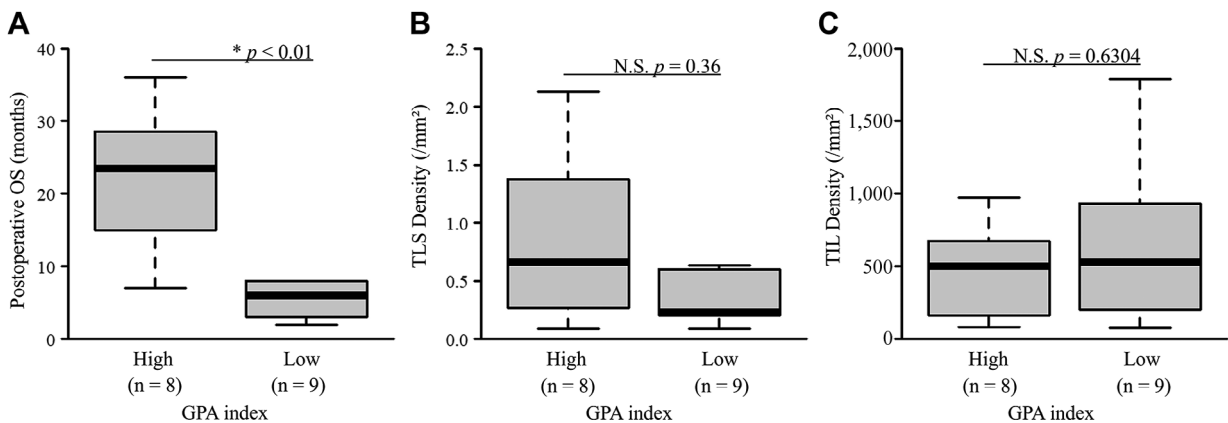


Figure 3. Tertiary lymphoid structure (TLS) density in brain metastases (BMs) is a prognostic factor in patients with lung cancer-derived BM independent of Graded prognostic assessment (GPA) index. The cohorts were classified into two groups based on the GPA index for the correlations between the GPA index, an established prognostic index of BMs, and postoperative overall survival (OS) (A), the TLS density of BMs (B), and the TIL density of BMs (C). *p*-Values were determined using the Mann–Whitney *U*-test. \* $p < 0.05$ ; N.S.: Not significant.

of TLSs is considered a multistep process that occurs in parallel with tumor growth and involves the recruitment and organization of immune cells in the TIME. During the immature stage of TLS formation, the structure is loose and lacks a well-defined organization. As the inflammatory stimulus persists, immature TLSs can develop into mature TLSs, characterized by a more organized structure, including GCs or distinct B cell and T cell zones (19). TLSs in BMs have rarely received attention since the CNS has been regarded as an immune-privileged site. However, recent studies have highlighted the importance of the immune system in brain tumors, and TLSs have been unveiled to

support antitumor responses by priming T and B cells at the tumor site, fostering antigen presentation and generating a local lymphoid niche in malignancies (5). To investigate the existence of TLSs in BMs, we first examined the distribution of TILs in both BMs and primary tumors and confirmed the presence of aggregates of lymphocytes mainly in the peritumoral regions (Figure 1A and B). Interestingly, while the TLSs in primary tumors exhibited an organized structure with GCs (Figure 1A), the structure of TLSs in the BMs was loose and lacked GC formation (Figure 1B). These findings suggest that TLSs in the BMs observed in the current study appeared to share the characteristics of immature TLSs. To

clarify the precise characteristics of BM-associated TLSs, we are now in the process of conducting flow cytometry and single cell mRNA analysis using additional clinical samples.

The correlation between TIL density and patient survival has been extensively studied along with TLSs (4, 16). Higher TIL density was generally associated with favorable outcomes in solid tumors including GBM (21). Recently, several studies have shown that the density of tumor-associated TLSs correlates with improved patient outcome, which led to a growing interest in the roles of TLSs in TIME (20). Based on these findings, we hypothesized that the density of TILs and TLSs in BMs of lung cancer would correlate with the patients' outcome. We first tried to exclude the impact of preoperative chemotherapies and radiation therapies as potential confounding factors; we found no significant differences in patient outcome based on these factors (data not shown). When we directed our focus to the density of TILs and TLSs, we found that the density of both TILs and TLSs in primary tumors were significantly higher than in BMs as we expected (Figure 1C and D). Nevertheless, the TLS density in BMs still significantly correlated with postoperative overall survival of the patients (Figure 2A), while the TIL density showed no correlation (Figure 2B) in contrast to the results of a previous study (22). These data suggested that the TLSs may play a more important role in the anti-tumor immune response than diffusely distributed TILs, even though they were still in immature stage of development. These findings indicate that the TLS density would be useful as prognostic factor in BMs of lung cancer.

The GPA index is a potent prognostic factor that has been widely used to estimate the survival of the patients with BMs (17, 18). Formerly, it consisted of four parameters: age ( $\leq 50$ , 41-60,  $>60$ ), Karnofsky Performance Status ( $<70$ , 70-80, 90-100), number of brain metastases (1, 2-3,  $>3$ ), and presence or absence of extracranial metastases (17). Recently, it has been refined to incorporate additional factors, such as EGFR mutation and ALK rearrangement, as well as PD-L1 expression, because these factors significantly influence the choice of treatment in patients with lung cancer (lung-molGPA) (18). In our study, GPA index significantly correlated with postoperative OS (Figure 3A), as well as OS from the diagnosis of BMs and postoperative progression-free survival (PFS; data not shown). However, the GPA index did not correlate with the TLS density (Figure 3B) or even the TILs density (Figure 3C). These findings suggest that, while the GPA index remains a valuable tool for prognostication in patients with BM, the TLS density would also serve as an additional, complementary prognostic marker for them. Further research is needed to elucidate the mechanisms underlying TLS formation in BMs as to how the TLS densities can be integrated into existing prognostic models for better survival of patients with BM.

*Study limitations.* First, the sample size of 17 patients is relatively small. Larger studies are needed to draw more

definitive conclusions. Second, this study was a retrospective study and conducted at a single institution, which may limit the generalizability of the findings. Prospective, multicenter studies with large patient cohorts will provide more robust evidence and help to validate the relationship between the TLS density and clinical outcomes in patients with BMs of lung cancer.

## Conclusion

In conclusion, this is the first report to identify TLSs in the BMs of lung cancer and illustrate their morphological characteristics while comparing them with TLSs in the primary tumors. We also provide new insights into the potential roles of TLS density in BMs as a novel prognostic biomarker. Our findings shed light on the previously unknown role of TLSs in the TIME of the CNS and promote further research into the immune landscape of BMs. This research may lead to the development of novel immunotherapeutic strategies targeting TLSs to improve patient outcomes.

## Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

## Authors' Contributions

SN, SK, MO, MF, KY, and TN conceptualized the project. SK, MO, and MF acquired the funding. MO, MF, and RS supervised the project and conducted the project administration. MO and HM provided resources. SN, MO, MF, and TN developed the methodology. SN, MO, SH, NS, and RS acquired the raw data. SN, SK, and MF carried out formal analyses. SN and SK wrote the original draft. SN, SK, MO, and MF edited the manuscript. All the Authors have reviewed and approved the manuscript to submit.

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