Article

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Prognostic Significance of a 9-Gene Signature Related to Tertiary Lymphoid Structures in Male Stomach Adenocarcinoma Patients

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Background: The role of tertiary lymphoid structures (TLSs) in stomach adenocarcinoma (STAD) remains unclear despite their known potential effects on tumor progression and prognosis.
Methods: Data were collected from 362 patients with STAD from The Cancer Genome Atlas (TCGA) database. Using single-sample genomic enrichment analysis, TLSs were quantified based on a 9-gene signature, and the patients were categorized into TLS-signature high (TLS-high) and TLS-signature low (TLS-low) groups. The association of TLS signature with prognosis, tumor microenvironment (TME) immune status, tumor mutation burden, and gene mutation status was evaluated. The GSE26253 cohort served as an external dataset to validate the prognostic predictive effect of the TLS signature in patients with STAD.
Results: The TLS-high group exhibited notably lower overall survival (OS) among male patients with STAD from the TCGA cohort (p = 0.01). Multivariate analysis revealed that the TLS signature was a significant independent negative predictor of OS in male patients with stage I–III STAD (hazard ratio (HR): 2.68; 95% confidence interval (CI): 1.19–6.00; p = 0.02). The TLS-high patients exhibited increased infiltration of immune cell subsets; however, cancer-immunity cycle analysis revealed both antitumor and protumor responses within the TME. Correlation analyses indicated that TLS was more strongly associated with immunosuppression-related cells than with antitumor immune cells. Furthermore, expressions of immunosuppressive cell-recruitment factors, immunosuppressive factors, and immune checkpoint receptors were higher in the TLS-high group than in the TLS-low group. Nonetheless, among male patients with stage I–III STAD who received adjuvant therapy, multivariate analysis identified TLS trait as a significant independent positive predictor of relapse-free survival in the GSE26253 cohort (HR: 0.61; 95% CI: 0.38–0.97; p = 0.04). Interaction of the TLS signature with adjuvant therapy exerted a significant positive effect on OS in these patients (HR: 0.41; 95% CI: 0.17–0.97; p = 0.04).
Conclusion: In the TCGA cohort, the TLS signature acted as an independent adverse prognostic factor for male patients with stage I–III STAD and was associated with immunosuppressive TME, which interacted to affect patient prognosis. However, adjuvant therapy may affect the prognostic predictive effect of TLS in male patients with stage I–III STAD.

Keywords: stomach adenocarcinoma; tertiary lymphoid structures; gene signature; immune microenvironment

Introduction

Stomach adenocarcinoma (STAD) comprises the bulk of gastric cancer (GC), and its incidence accounts for 95% of GC [1,2]. Although the global morbidity and related mortality of STAD are declining, it remains one of the top three deadliest cancers in the world. Moreover, the number of males affected by GC is usually much higher than that of females, and in the East Asian population alone, the number of male patients is twice that of female patients [3]. The prognosis of male patients with GC is poorer than that of female patients, and the risks are higher [4]. The trajectory of cancer progression is considerably affected by sex, influencing both biological processes and clinical results [5]. However, the current widely used models or biomarkers have been unable to address the impact of sex-based differences. Therefore, it is essential to determine indicators with strong prognostic significance for male patients diagnosed with STAD.

In recent years, immunotherapy has offered a promising direction for patients with GC. However, this benefit has been found only in specific patient populations with advanced STAD [6]. The reasons for treatment failure are complex, with the presence of an immunosuppressive tu-

mor microenvironment (TME) being one of the key reasons [7,8]. Resident stromal cells and recruited immune cells in the TME regulate its immune status, affecting tumor development and the response to cancer treatment [9]. Hence, further identification of biomarkers associated with immunosuppressive TME is crucial for enhancing the precision of immunotherapy in male patients with STAD.

Tertiary lymphoid structures (TLSs), vital components of the TME, have been newly proposed and are known to elicit an enhanced immune response [10]. TLSs are well-organized tumor-infiltrating lymphocyte clusters that mainly comprise B-cell zones, T-cell zones, dendritic cell zones, and high endothelial venules and affect the clinical outcomes of patients with tumors [10]. Prior research has observed a correlation between TLSs and positive clinical results in cancers, including ovarian cancer, cutaneous melanoma of the skin, and endometrial carcinoma of the uterine corpus. Nonetheless, certain studies have reported that tumor-associated TLSs have an adverse impact on patient prognosis, as evidenced in hepatocellular carcinoma, breast cancer, and bladder cancer [11–13]. In studies focusing on GC, the presence of TLS areas has been strongly correlated with clinical outcomes in patients with GC. For instance, one study documented that TLSs were a positive prognostic factor for Epstein-Barr virus-associated GC [14]. Another study noted that the increased density of TLSs favors better overall survival (OS) in patients with GC [15]. Yet another study suggested that TLSs in GC may be involved in antitumor immune responses and that these responses are correlated with the degree of formation of TLSs in addition to the histological type of the tumor [16]. This finding suggests that the formation and function of TLSs play an essential part in diverse types of GC. However, TLSs have mainly been observed in immunoassay studies of GC in patients with poor prognosis [17–19]. The dynamics of TLSs during the clinical progression of male STAD have not been fully elucidated. Therefore, this study aimed to quantify the TLSs in male patients with STAD and explore their significance in the clinical progression of male STAD.

One previous study evaluating TLSs has often used immunohistochemistry (IHC) and hematoxylin and eosin (H&E) staining [20]. However, those approaches can only detect a limited number of markers at a time, which may not entirely represent the heterogeneity of TLSs. RNA-seq, based on the sequence and abundance of RNA molecules, permits a more comprehensive analysis of the immune infiltrate. In addition, RNA-seq is a more sensitive and quantitative method that detects low-abundance transcripts. Recently, a 9-gene TLS signature, including C-C motif chemokine receptor 6 (*CCR6*), CD1d molecule (*CD1D*), CD79b molecule (*CD79B*), cholesteryl ester transfer protein (*CETP*), eukaryotic translation initiation factor 1A, Y-Linked (*EIF1AY*), linker for activation of T cells (*LAT*), prostaglandin D2 synthase (*PTGDS*), retinol-binding protein 5 (*RBP5*), and src kinase associated phosphoprotein 1 (*SKAP1*), was identified, which primarily represented the gene expression in B cells and other immune cells within TLSs, such as T cells and Myeloid-derived suppressor cells (MDSCs). This signature predicted the clinical outcomes for patients with melanoma treated using immunotherapy [21]. Another classical gene signature used to quantify TLSs is the 12-chemokine signature, primarily linked to TLSs [22]. Nevertheless, the 9-gene TLS signature provides a more encompassing representation of TLS-associated gene expression profiles than the 12-chemokine signature.

Thus, this study was undertaken to ascertain the prognostic implications of the 9-gene TLS signature in male patients with STAD by leveraging data from The Cancer Genome Atlas (TCGA) and the Gene Expression Omnibus (GEO) databases, to provide critical insights into the prediction of patient prognosis. The survival curves of male patients with STAD were compared between the TLS-low and TLS-high groups, and univariate and multivariate regression analyses were further performed in male patients within stage I–III STAD. Moreover, the association of TLSs with TME immune status, tumor mutation burden (TMB), and mutation status of genes was investigated. The combination of TLS characteristics and prognostic gene mutation status may help to achieve personalized treatment in the future.

Materials and Methods

Data Sources and Processing Methods

Fragments per kilobase of transcript per million mapped reads (FPKM) values and the corresponding clinical information for 362 patients with STAD were retrieved from the UCSC Xena database (https://xenabrowser.net/datapages/?cohort=TCGA%20Stomach%20Cancer%20(STAD)&removeHub=https%3A%2F%2Fxena.treehouse.gi.ucsc.edu%3A443). Subsequently, these FPKM values were converted to transcripts per kilobase million (TPM) values. The single nucleotide variant (SNV) data were sourced from the TCGA database (https://portal.gdc.cancer.gov/projects/TCGA-STAD). In addition, an external validation data GSE26253 cohort (Samsung Medical Center [SMC] cohort) that includes 432 patients, this data was obtained from the GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26253).

The 9-gene TLS signature (*CCR6*, *CD1D*, *CD79B*, *CETP*, *EIF1AY*, *LAT*, *PTGDS*, *RBP5*, and *SKAP1*) was derived from metastasized melanoma cancer study [21]. Single-sample gene set enrichment analysis (ssGSEA) represents an extension of the GSEA approach, with a distinct advantage being its capacity to assess the enrichment of predefined gene sets within individual samples [23]. This attribute renders ssGSEA particularly suitable for analyz-

ing tumors with high heterogeneity. The ssGSEA was used to calculate the TLS signature score for each sample based on their mRNA levels. Furthermore, the R package 'GSVA' (https://www.bioconductor.org/packages/release/bioc/html/GSVA.html) was used to perform this analysis [24]. According to the standardized ssGSAE scores, the patients were stratified into two groups: a TLS-low group representing the lower tertile, and a TLS-high group representing the upper tertile.

Survival Analysis

To determine the clinical significance of TLSs, we compared the survival outcomes and clinical characteristics of patients with TLS-low and high groups. These clinical characteristics included age, gender, tumor grade, and tumor stage. Moreover, Kaplan-Meier analysis and Cox proportional hazards regression models were obtained by using the 'survival' (https://cran.r-project.org/web/packages/survival/index.html) and 'survminer' (https://cran.r-project.org/web/packages/survminer/index.html) R packages.

Estimation of TME Immune Characteristics

The metagenes corresponding to 28 distinct types of immune cells were derived from the Molecular Signatures Database (MSigDB) (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp) [25]. We subsequently performed ssGSEA to quantify the infiltration levels of 28 immune cells within the TME. The resulting ssGSEA scores were standardized (ranging from 0 to 1); these values indicated the minimum and maximum infiltration abundance for each immune cell type, respectively.

Following the initial analysis, we evaluated the immunological characteristics of the TME in STAD patients by calculating the immunogram score (IGS) for the cancer-immunity cycle (CIC), as described previously [26]. The following eight axes were identified as IGS1, indicating the presence of T-cell immunity within the tumor; IGS2, related to tumor antigenicity; IGS3, reflecting priming and activation of the immune response; IGS4, focused on trafficking and infiltration of immune cells; IGS5, associated with the recognition of tumor antigens; IGS6 to IGS8, reflecting suppressive factors that hinder the immune system's ability to kill cancer cells. The gene sets of IGS1, IGS3, and IGS6 were downloaded from previous publications [27–29]. An IGS score of 3 represents an intermediate level, with a higher score on the IGS1-5 axis indicating a stronger anti-tumor response and a lower score on the IGS6-8 axis indicating a more suppressed anti-tumor response. We then evaluated the relationship among the TLS signature with the major anti-tumor immune cell subsets, the main immunosuppressive cell subsets [30,31], immunosuppressive cell recruitment factors [32], immunosuppressive factors [33], and immune checkpoint receptors [34].

Mutation Genes Analysis

We used the R package 'maftools' (https://www.bioconductor.org/packages/release/bioc/html/maftools.html) to calculate each patient's TMB score. Survival analysis was performed across TMB-low and TMB-high groups (with 2.68 as the cutoff value). Furthermore, the TMB score was used to analyze the top 20 mutation genes in the TLS-low and high groups, respectively. In addition, the patients were categorized into two strata on the status of the given gene—wild-type or mutant—and then clinical outcomes were analyzed between the low and high TLS groups.

Statistical Analysis

All data processing, analyses, and figure plotting were performed by R software (v4.2.2) (R Foundation for Statistical Computing, Vienna, Austria) and SPSS software (v26.0) (IBM Corp, Armonk, NY, USA). Continuous variables (such as age) were converted to categorical variables, expressed as quantity and percentage. Prior to conducting statistical analyses, the Kolmogorov—Smirnov test and the Shapiro—Wilk test were used to assess the normality of the continuous variables. For the non-normal continuous variables, the Wilcoxon rank-sum test was performed to assess the differences between the groups. For categorical variables, a Chi-square test was performed to assess the differences between the groups. $p < 0.05$ was considered to indicate statistical significance.

Results

TLS Signature as An Independent Poor Prognostic Factor in Male Patients with Stage I–III STAD

The entire cohort of patients with STAD from the TCGA cohort was stratified into two groups using the standardized ssGSEA scores derived from a 9-gene TLS signature: TLS-low (N = 119) and TLS-high (N = 243) (Fig. 1A). Furthermore, GSEA was performed to prove that it was a TLS signature-related model, which revealed a significant enrichment of the TLS signature in the TLS-high group (**Supplementary Fig. 1**, NES: 2.62, *p-adjust* < 0.001). Considering that sex had a high impact on the occurrence and development of STAD, it was included as a key factor in the ssGSEA score analysis (Fig. 1B,C). The OS was then compared between the TLS-low and TLS-high groups in these three scenarios, and the clinical characteristics are listed in Table 1. In the entire patient cohort with STAD, the TLS-high group had a poorer OS than the TLS-low group, although not statistically significant (Fig. 2A, $p = 0.05$). Moreover, in female patients with STAD, there was no significant difference between the two groups (Fig. 2B). However, in male patients with STAD, the TLS-high group was significantly associated with poorer OS (Fig. 2C, $p = 0.01$).

To further determine the clinical significance of TLSs in male patients with STAD, Cox proportional hazards re-

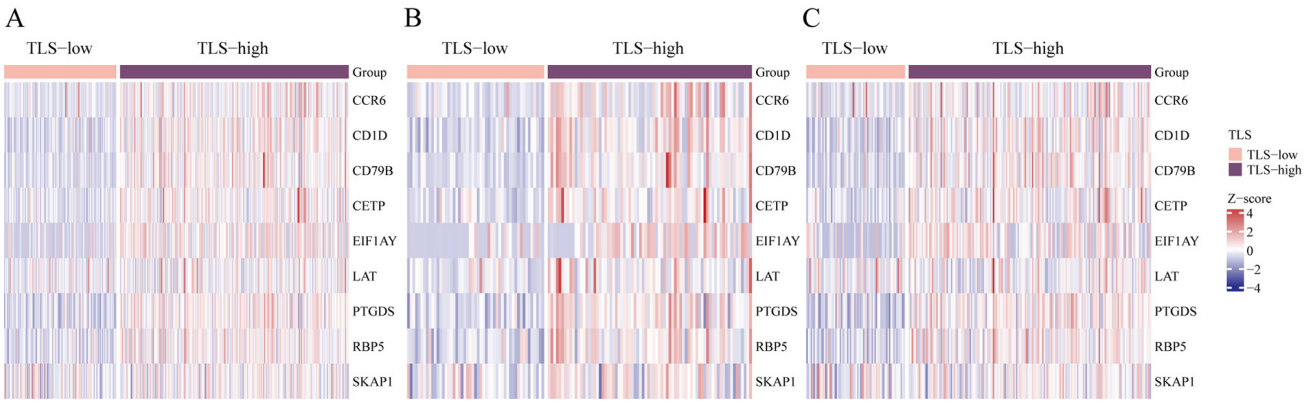

Fig. 1. The expressions of 9 genes in the TLS-low and high groups of STAD from the TCGA cohort. (A) Total patients: TLS-low group (N = 119) and TLS-high group (N = 243). (B) Female: TLS-low group (N = 51) and TLS-high group (N = 76). (C) Male: TLS-low group (N = 68) and TLS-high group (N = 167). TLS, tertiary lymphoid structure; STAD, stomach adenocarcinoma; TCGA, The Cancer Genome Atlas; TLS-low, TLS-signature low; TLS-high, TLS-signature high; *CCR6*, C-C motif chemokine receptor 6; *CD1D*, CD1d molecule; *CD79B*, CD79b molecule; *CETP*, cholesteryl ester transfer protein; *EIF1AY*, eukaryotic translation initiation factor 1A, Y-Linked; *LAT*, linker for activation of T cells; *PTGDS*, prostaglandin D2 synthase; *RBP5*, retinol-binding protein 5; *SKAP1*, src kinase associated phosphoprotein 1.

gression models were established for the univariate and multivariate analysis. Given the small sample size of TLS-low male patients with tumor stage IV (N = 3), only male patients with tumor stage I–III were included in the subsequent studies for accurate analysis. The data showed that TLS signature (hazard ratio (HR): 2.97; 95% confidence interval (CI): 1.32–6.65; $p = 0.008$) and adjuvant therapy (HR: 0.14; 95% CI: 0.03–0.58; $p = 0.007$) were significant predictive factors of OS in univariate analysis (Table 2). Likewise, multivariate analysis identified that TLS signature was a significant independent prognostic factor, negatively predicting OS (HR: 2.68; 95% CI: 1.19–6.00; $p = 0.02$) (Table 3).

The TLS-High Group Was Found to Be Associated with An Immunosuppressive TME

In the TCGA cohort of male patients with stage I–III STAD, the TLS-high group (N = 134) demonstrated higher abundance levels of 28 immune-associated cell types than the TLS-low group (N = 62) (Fig. 3A). To evaluate the immune status of these patients' TME, the characteristics of the CIC were compared between the two groups. The radar map showed that the TLS-high group exhibited higher IGS1, IGS3, GS4, and IGS5 but lower IGS6 and IGS7 (Fig. 3B), suggesting that the TME of this group may elicit both antitumor and protumor immune responses. In addition, correlation analysis revealed that within immunosuppressive cell subsets, the TLS signature displayed strong and positive associations with several cell types: B regulatory cells (Breg cells) (Pearson R = 0.672, $p < 0.0001$), T regulatory cells (Treg cells) (Pearson R = 0.778, $p < 0.0001$), MDSCs (Pearson R = 0.746, $p < 0.0001$), plasmacytoid dendritic cells (pDCs) (Pearson R = 0.726, $p < 0.0001$), and M2 macrophages (Pearson R = 0.655, $p < 0.0001$). Moreover, a significant yet moderate positive correlation was observed with tumor-associated neutrophils (TANs) (Pearson R = 0.521, $p < 0.0001$) (Fig. 3C and **Supplementary Table 1**). Of the major antitumor immune cell subsets, the TLS signature showed significant strong positive correlations with activated B cells (Pearson R = 0.746, $p < 0.0001$), activated $CD8^+$ T cells (Pearson R = 0.602, $p < 0.0001$), M1 macrophages (Pearson R = 0.602, $p < 0.0001$), T follicular helper cells (Pearson R = 0.705, $p < 0.0001$), and type 1 T helper cells (Pearson R = 0.703, $p < 0.0001$); a significant moderate correlation with activated dendritic cells (Pearson R = 0.552, $p < 0.0001$), and a significant weak positive correlation with activated $CD4^+$ T cells (Pearson R = 0.383, $p < 0.0001$, Fig. 3C, **Supplementary Table 1**). Furthermore, Cox regression analysis signified the presence of separate interaction effects between TLS signature scores and Breg cells (HR: 3.60; 95% CI: 1.17–11.07; $p = 0.03$), Treg cells (HR: 4.37; 95% CI: 1.44–13.28; $p = 0.009$), MDSCs (HR: 3.71; 95% CI: 1.24–11.16; $p = 0.02$), pDCs (HR: 3.79; 95% CI: 1.36–10.60; $p = 0.01$), TANs (HR: 5.70; 95% CI: 1.42–22.89; $p = 0.01$), and M2 macrophages (HR: 4.64; 95% CI: 1.40–15.35; $p = 0.01$) (**Supplementary Table 2**). The TLS signature was more strongly correlated with immunosuppressive cell subsets in male patients with stage I–III STAD in the TCGA cohort.

To further determine the relationship between TLSs and immunosuppressive properties, the expressions of immunosuppressive cell recruitment factors, immunosuppressive factors, and immune checkpoint receptors were compared between TLS-low and high groups. This analysis revealed that the mRNA expression levels of most of these cytokines were elevated in the TLS-high group relative to

Table 1. Differences in the clinical characteristics among low and high TLS groups of STAD patients in the TCGA cohort (N = 362).

Characteristics	Total (N = 362)		χ^2, p	Female (N = 127)		χ^2, p	Male (N = 235)		χ^2, p
	TLS-low	TLS-high		TLS-low	TLS-high		TLS-low	TLS-high	
All cases (N, %)	119 (32.9%)	243 (67.1%)		51 (40.2%)	76 (59.8%)		68 (28.9%)	167 (71.1%)	
Age			0.66, 0.42			0.51, 0.48			1.94, 0.16
≤65	49 (41.2%)	111 (45.7%)		22 (43.1%)	28 (39.4%)		27 (39.7%)	83 (49.7%)	
>65	70 (58.8%)	132 (54.3%)		29 (56.9%)	48 (63.2%)		41 (60.3%)	84 (50.3%)	
Tumor grade			5.23, 0.15			2.17, 0.36			4.31, 0.21
G1	4 (3.4%)	6 (2.5%)		2 (3.9%)	3 (3.9%)		2 (2.9%)	3 (1.8%)	
G2	50 (42.0%)	78 (32.1%)		21 (41.2%)	22 (28.9%)		29 (42.6%)	56 (33.5%)	
G3	61 (51.3%)	154 (63.4%)		28 (54.9%)	51 (67.1%)		33 (48.5%)	103 (61.7%)	
GX	4 (3.4%)	5 (2.1%)		0 (0.0%)	0 (0.0%)		4 (5.9%)	5 (3.0%)	
Tumor stage			12.30, 0.02			2.81, 0.61			12.98, 0.01
I	26 (21.8%)	23 (9.5%)		7 (13.7%)	5 (6.6%)		19 (27.9%)	18 (10.8%)	
II	33 (27.7%)	76 (31.3%)		18 (35.3%)	26 (34.2%)		15 (22.1%)	50 (29.9%)	
III	47 (39.5%)	99 (40.7%)		19 (37.3%)	33 (43.4%)		28 (41.2%)	66 (39.5%)	
IV	8 (6.7%)	27 (11.1%)		5 (9.8%)	6 (7.9%)		3 (4.4%)	21 (12.6%)	
Not reported	5 (4.2%)	18 (7.4%)		2 (3.9%)	6 (7.9%)		3 (4.4%)	12 (7.2%)	
T stage			2.32, 0.13			0.05, 0.82			3.70, 0.05
TX–T2	40 (33.6%)	63 (25.9%)		13 (25.5%)	18 (23.7%)		27 (39.7%)	45 (26.9%)	
T3–T4	79 (66.4%)	180 (74.1%)		38 (74.5%)	58 (76.3%)		41 (60.3%)	122 (73.1%)	
N stage			5.11, 0.02			1.61, 0.21			3.50, 0.06
NX–N0	50 (42.0%)	73 (30.0%)		21 (41.2%)	23 (30.3%)		29 (42.6%)	50 (29.9%)	
N1–N3	69 (58.0%)	170 (70.0%)		30 (58.8%)	53 (69.7%)		39 (57.4%)	117 (70.1%)	
Vital stage			4.60, 0.03			0.02, 0.90			7.02, 0.008
Alive	82 (68.9%)	139 (57.2%)		33 (64.7%)	50 (65.8%)		49 (72.1%)	89 (53.3%)	
Dead	37 (31.1%)	104 (42.8%)		18 (35.3%)	26 (34.2%)		19 (27.9%)	78 (46.7%)	
Adjuvant therapy			1.50, 0.47			0.44, 0.80			1.12, 0.57
No	72 (60.5%)	157 (64.6%)		31 (60.8%)	47 (61.8%)		41 (60.3%)	110 (65.9%)	
Yes	18 (15.1%)	26 (10.7%)		8 (15.7%)	9 (11.8%)		10 (14.7%)	17 (10.2%)	
Not reported	29 (24.4%)	60 (24.7%)		12 (23.5%)	20 (26.3%)		17 (25.0%)	40 (24.0%)	

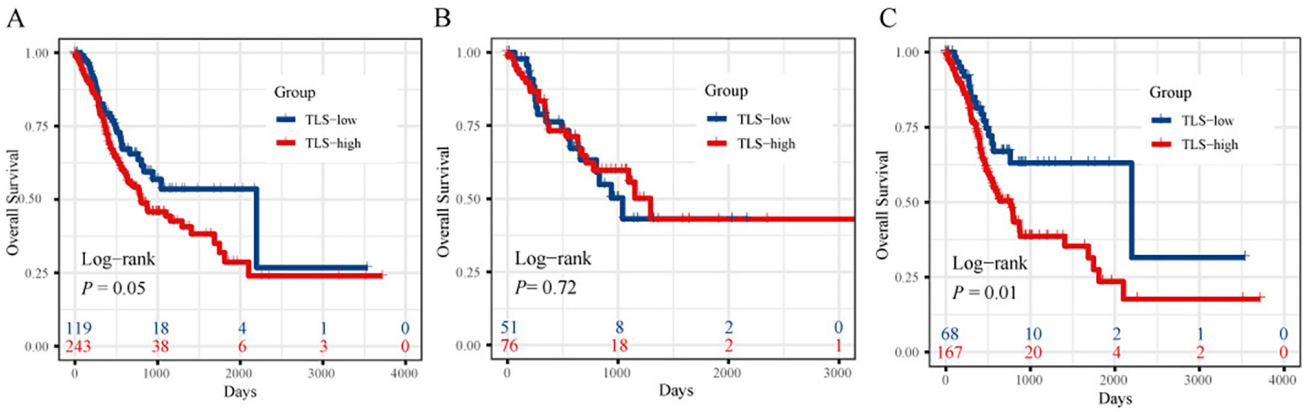

Fig. 2. Survival curve for the low and high TLS groups in STAD patients from the TCGA cohort. (A) In 362 patients, those in the TLS-high group exhibited a poorer overall survival (OS) compared to those in the TLS-low group, although this difference did not reach statistical significance. (B) In 127 female patients, no obvious change was observed in the OS among the two groups. (C) In 235 male patients, the TLS-high group demonstrated a significant association with a poorer OS.

the TLS-low group (Fig. 3D–F). Collectively, these findings assert that within male patients with stage I–III STAD from the TCGA cohort, the TME of the TLS-high group is marked by chronic inflammation, immunosuppression, and tumor promotion. These characteristics likely contribute to the poorer prognosis noted in this patient subset.

Table 2. Univariate Cox analyses of the prognosis of stage I–III STAD male patients from the TCGA cohort (N = 158).

Overall survival	Group	Univariate analysis				
		β	SE	Wald χ^2	HR (95% CI)	p-value
TLS signature	Low[1]					
	High	1.09	0.41	6.98	2.97 (1.32 to 6.65)	0.008
Age	≤65[1]					
	>65	–0.11	0.30	0.14	0.89 (0.50 to 1.61)	0.71
Tumor grade	GX–G1[1]					
	G2–G3	0.99	1.01	0.95	2.68 (0.37 to 19.47)	0.33
T stage	T1–T2[1]					
	T3–T4	0.27	0.34	0.64	1.32 (0.67 to 2.58)	0.42
N stage	NX–N1[1]					
	N2–N3	0.66	0.36	3.36	1.93 (0.96 to 3.92)	0.07
Adjuvant therapy	No[1]					
	Yes	–1.97	0.73	7.33	0.14 (0.03 to 0.58)	0.007

1: control group.

Abbreviations: β, regression coefficient; SE, standard error; HR, hazard ratio; CI, confidence interval.

Table 3. Multivariate Cox analysis of prognosis of stage I–III STAD male patients from the TCGA cohort (N = 158).

Overall survival	Group	Multivariate analysis				
		β	SE	Wald χ^2	HR (95% CI)	p-value
TLS signature	Low[1]					
	High	0.98	0.41	5.70	2.68 (1.19 to 6.00)	0.02
Adjuvant therapy	No[1]					
	Yes	–1.88	0.73	6.61	0.15 (0.04 to 0.64)	0.01

1: control group.

Relationship of TMB and TLS in Male Patients with Stage I–III STAD

Considering that high TMB may play a critical role in the immunotherapy response [35], the "maftools" R package was utilized to perform an in-depth analysis of the SNV data for male patients with stage I–III STAD within the TCGA cohort. As depicted in Fig. 4A, the TLS signature score showed a significant negative correlation with TMB (Spearman R = –0.28, $p < 0.001$). Moreover, the comparative analysis indicated that patients in the TLS-low group exhibited a significantly higher TMB than those in the TLS-high group (Fig. 4B, Wilcoxon test $p = 0.01$). To more deeply investigate the influence of TMB on the prognosis of these patients, survival analysis was performed across TMB-low and TMB-high groups (with 2.68 as the cutoff value). The data implied that the survival prognosis was more favorable in the TMB-high group than in the TMB-low group to a certain degree (Fig. 4C). Subsequently, the TLS signature score was integrated with the TMB to predict patient outcomes. The findings showed that patients with a low TLS signature score (including TLS-low–TMB-low and TLS-low–TMB-high) exhibited better OS than those with a high score (including TLS-high–TMB-low and TLS-high–TMB-high) (Fig. 4D). In addition, the OS of patients with TLS-low–TMB-high was comparable to that of those with TLS-low–TMB-low (Fig. 4D). These results imply that the TLS signature score is a superior prognostic marker for male patients with stage I–III STAD, exhibiting a prognostic value that surpasses that of TMB.

Moreover, the disparities in the distribution of somatic mutations were investigated among TLS-low and TLS-high groups. As depicted in Fig. 5A,B, these two groups demonstrated unique profiles for the top 20 genes showing the highest mutation frequencies, with each group possessing its own specific set of genes. Tumor protein p53 (*TP53*) has been reported to be a mutational cancer driver gene in 66 tumor types [36], and *MUC16* (mucin 16, cell surface associated) is a mutational gene linked to the prognosis of STAD [37]. Therefore, the differences between the TLS-low and TLS-high groups in predicting the clinical outcomes were evaluated based on the mutation status of the two genes. In patients with *TP53* or *MUC16* gene mutation, the TLS-low group exhibited a statistically significant improvement in OS compared with the TLS-high group (Fig. 5C,D, $p = 0.04$ and $p = 0.03$). Conversely, significant differences in OS were not noted between the TLS-low and TLS-high groups among patients with wild-type *TP53* or *MUC16* genes (Fig. 5C,D). Succinctly, these findings provide a novel framework for elucidating the mechanisms underlying the predictive utility of TLSs in estimat-

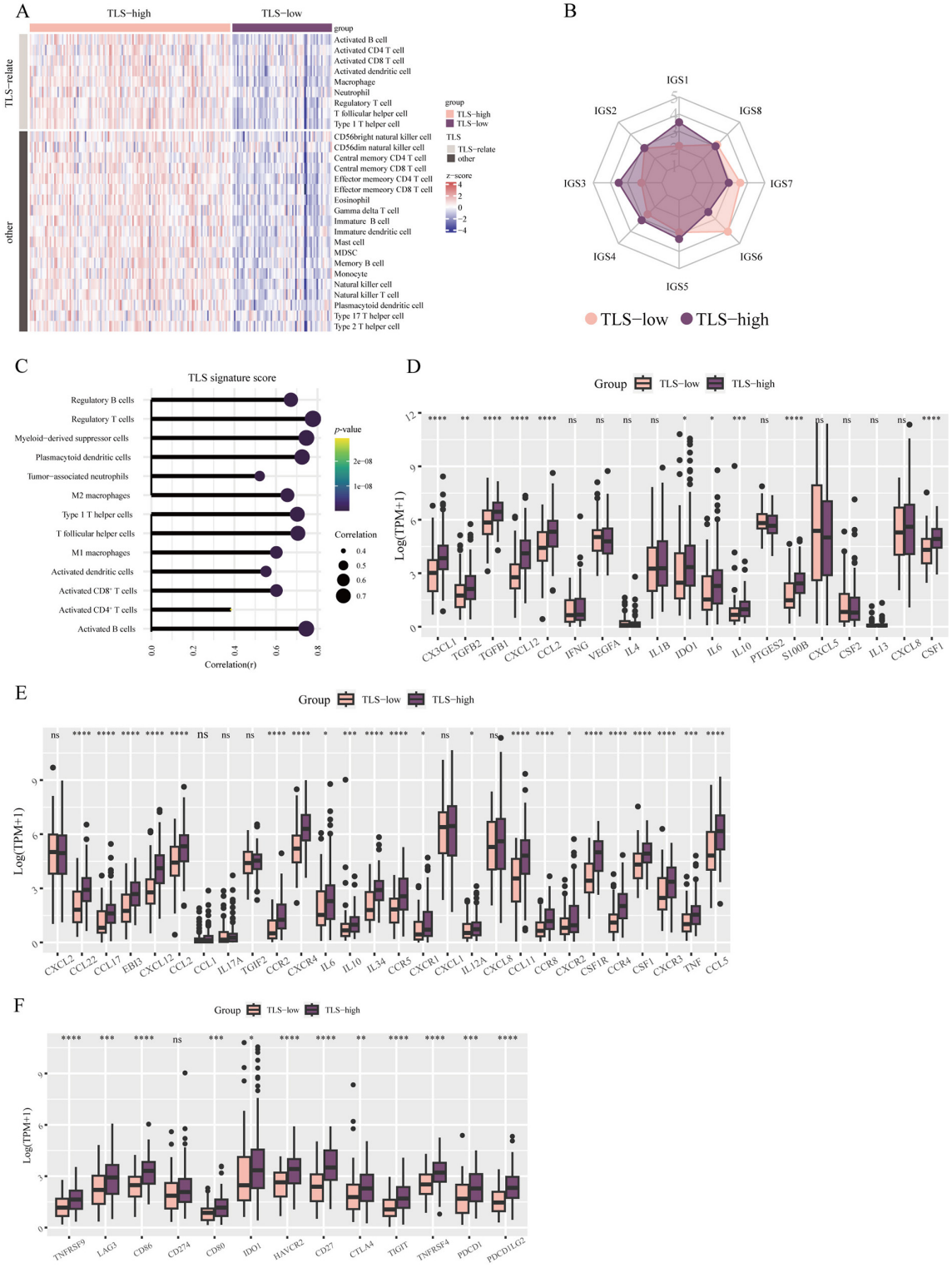

Fig. 3. Correlation of TLS signature with tumor immune microenvironment in stage I–III male STAD patients from the TCGA cohort. (A) The abundance levels of 28 types of immune-associated cells were significantly higher in the TLS-high group than in the TLS-low group. (B) The radar map indicated that the TME of the TLS-high group may have both anti- and pro-tumor immune responses. (C) The correlational analysis diagram revealed that the TLS signature enrichment score was more strongly correlated with major immunosuppressive cell subsets (upper panel) than with major antitumor immune cell subsets (lower panel). The difference in the expression of immunosuppressive cell recruitment factors (D), immunosuppressive factors (E), and immune checkpoint receptors (F) between the TLS subgroups. Wilcoxon test, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns, not significant. IGS, immunogram score.

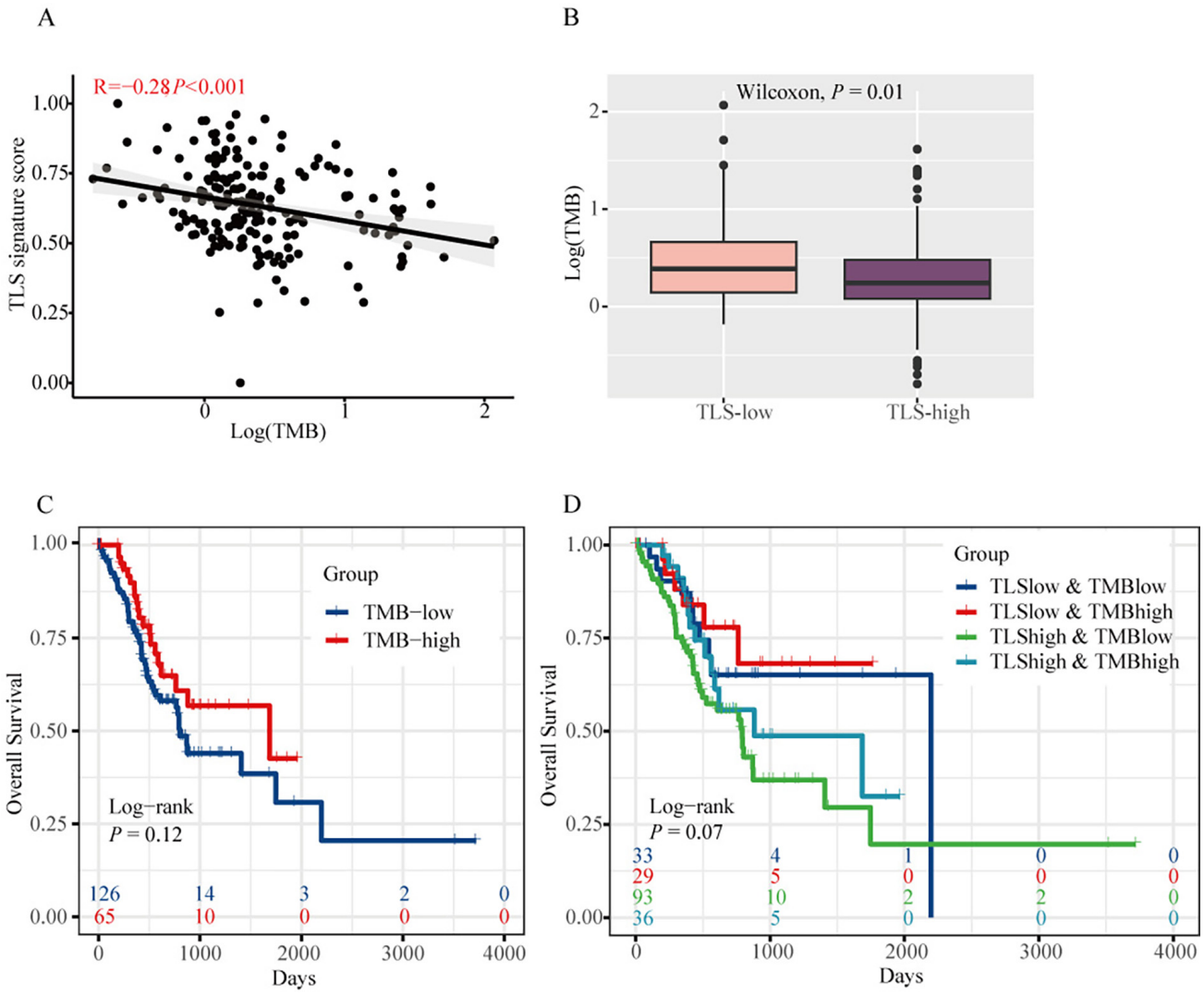

Fig. 4. Relationship between TLS signature and tumor mutation burden (TMB) in stage I–III male STAD patients from the TCGA cohort. (A) The scatter plot revealed a weak negative correlation between the TLS signature score and TMB. (B) The TLS-low group suffered from significantly higher TMB than the TLS-high group. (C) Kaplan-Meier survival curves were constructed to compare the OS between patients with low TMB and those with high TMB (2.68 as cutoff value). (D) Differences in OS among the TLS low and TMB low (N = 33), TLS low and TMB high (N = 29), TLS high and TMB low (N = 93), and TLS high and TMB high (N = 36).

ing the prognosis of male patients with stage I–III STAD within the TCGA cohort.

External Data Analysis for the Predictive Effect of the TLS Signature on Male Patients with Stage I–III STAD

An extended analysis was conducted to determine the prognostic predictive efficacy of the TLS signature in male patients with stage I–III STAD. This analysis utilized the SMC cohort data to further validate the predictive power of the TLS signature on patient survival outcomes. Patients were similarly categorized into two groups: the TLS-low and TLS-high groups. The comparative analysis of the clinical characteristics of the two groups of patients is presented in Table 4. In the SMC cohort, there was no significant difference in both OS (Fig. 6A) and recurrence-free survival (Fig. 6B) between the TLS-high and TLS-low groups. Consistent results were observed in the univariate Cox analysis (HR: 0.66; 95% CI: 0.42–1.05; p = 0.08) (Table 5). However, in multivariate Cox analysis, the TLS signature emerged as an independent positive prognostic factor for male patients with stage I–III STAD in the SMC cohort (HR: 0.61; 95% CI: 0.38–0.97; p = 0.04) (Table 6).

In addition, all patients in the SMC cohort received adjuvant therapy following surgery, whereas only a few in the TCGA cohort received this treatment. As adjuvant therapy can modulate patient prognosis and lead to reprogramming of the tumor immune microenvironment (TIME), it was incorporated as a survival variable in the analysis of the TCGA cohort. Patients who received adjuvant therapy had

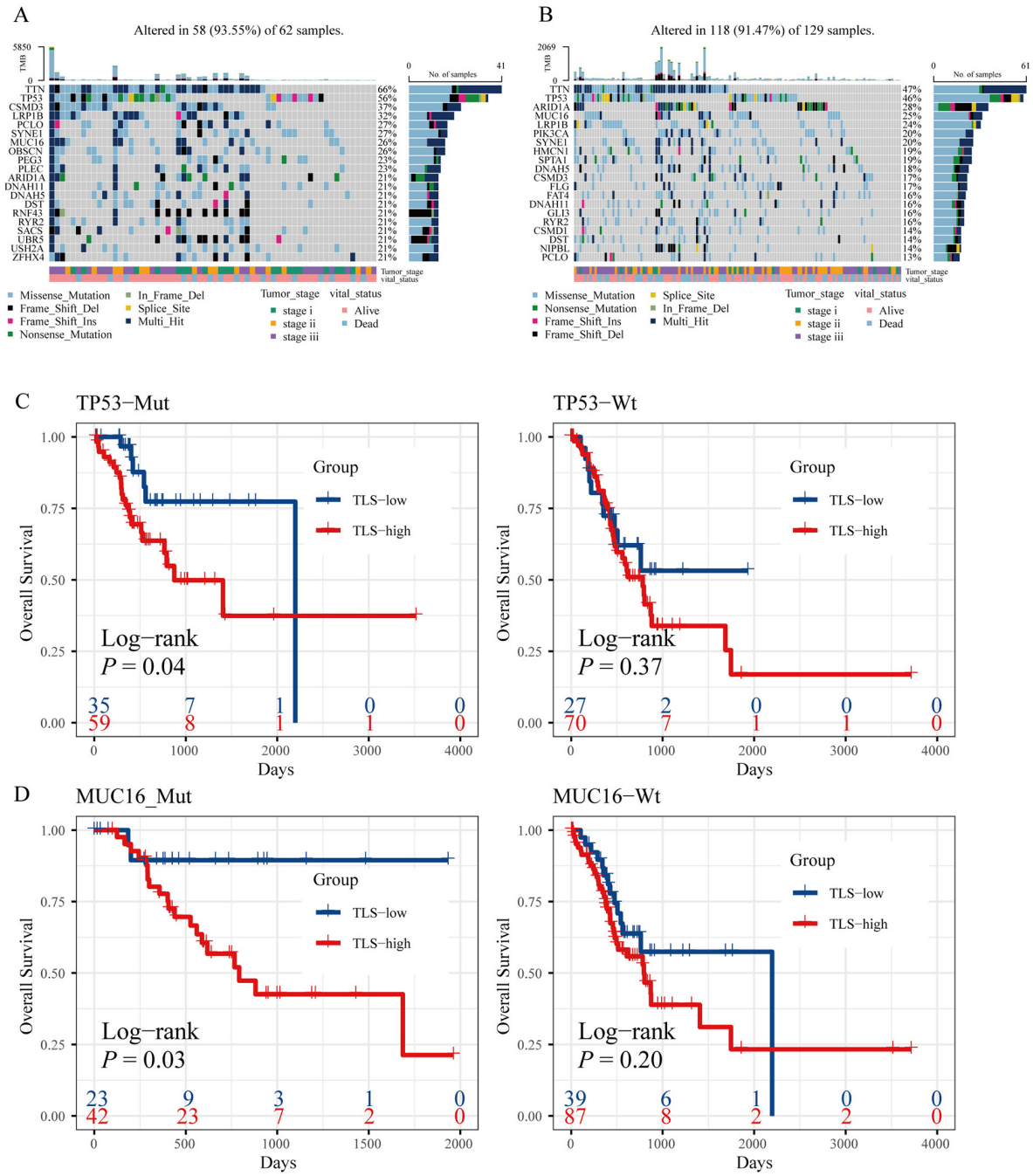

Fig. 5. Correlation of TLS signature with the mutation status of genes in stage I–III male STAD patients from the TCGA cohort. The top 20 genes with the highest mutation frequency in the TLS-low group on the left panel (A) and TLS-high group (right panel) (B) in stage I–III male STAD patients. Difference in the OS of patients with *TP53* (C) or *MUC16* (D) gene mutation or wide type between the low and high TLS groups. TTN, Titin; TP53, Tumor protein p53; CSMD3, Cub and sushi multiple domains 3; LRP1B, Ldl receptor related protein 1B; PCLO, Piccolo presynaptic cytomatrix protein; SYNE1, Spectrin repeat containing nuclear envelope protein 1; MUC16, mucin 16, cell surface associated; OBSCN, Obscurin, cytoskeletal calmodulin and titin-interacting rhogef; PEG3, Paternally expressed 3; PLEC, Plectin; ARID1A, At-rich interaction domain 1A; DNAH11, Dynein axonemal heavy chain 11; DNAH5, Dynein axonemal heavy chain 5; DST, Dystonin; RNF43, Ring finger protein 43; RYR2, Ryanodine receptor 2; SACS, Sacsin molecular chaperone; UBR5, Ubiquitin protein ligase e3 component n-recognin 5; USH2A, Usherin; ZFHX4, Zinc finger homeobox 4; PIK3CA, Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; HMCN1, Hemicentin 1; SPTA1, Spectrin alpha, erythrocytic 1; FLG, Filaggrin; FAT4, Fat atypical cadherin 4; GLI3, Gli family zinc finger 3; CSMD1, Cub and sushi multiple domains 1; NIPBL, Nipbl cohesin loading factor.

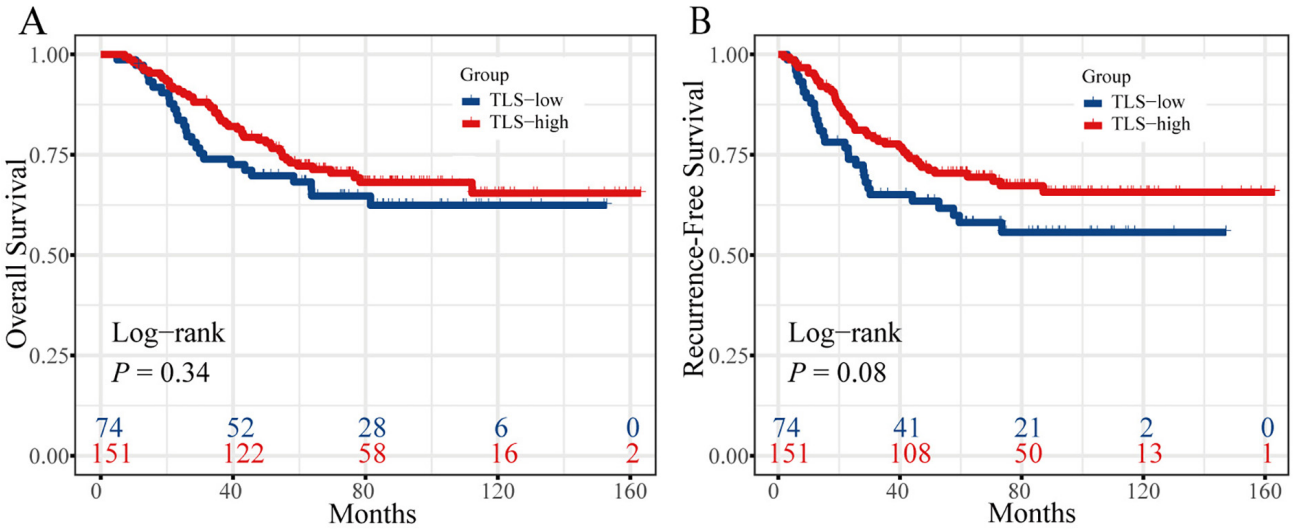

Fig. 6. Survival curves for the low and high TLS groups in stage I–III male STAD patients from Samsung Medical Center (SMC) cohort (N = 225). (A) The difference in the OS between the high and low TLS groups was not statistically significant. (B) To an extent, the TLS-high group exhibited better recurrence-free survival (RFS) compared to the TLS-low group.

significantly better OS than those who did not (Fig. 7, p = 0.002). Moreover, both univariate and multivariate Cox regression analyses indicated that adjuvant therapy was a significant independent positive factor for male patients with stage I–III STAD (Table 2, HR: 0.14; 95% CI: 0.03–0.58; p = 0.007) (Table 3; HR: 0.15; 95% CI: 0.04–0.64; p = 0.01). As fewer patients in the TCGA cohort received adjuvant therapy, the survival data of this cohort was combined with that of the SMC cohort. The results of multifactorial Cox regression revealed that TLS was a significant independent poor prognostic factor (HR: 2.14; 95% CI: 1.03–4.45; p = 0.04), whereas adjuvant therapy was a significant independent positive prognostic factor (HR: 0.45; 95% CI: 0.20–0.97; p = 0.04). Furthermore, the interaction between TLS and adjuvant therapy positively affected patient prognosis (HR: 0.41; 95% CI: 0.17–0.97; p = 0.04), revealing that this therapy may influence the prognostic predictive effect of TLS (**Supplementary Table 3**).

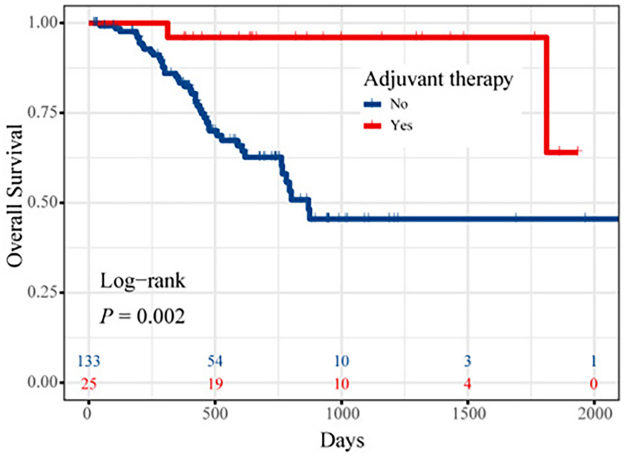

Fig. 7. Survival curves for adjuvant therapy recipients and non-recipients among stage I–III male patients with STAD in the TCGA cohort (N = 158). The group receiving adjuvant therapy demonstrated significantly better OS compared to the group that did not receive adjuvant therapy.

Discussion

TLSs are ectopic lymphoid structures present in many solid tumors and are often linked to favorable clinical outcomes [38]. Nonetheless, they can also negatively impact the prognosis in some cases, such as those with elevated levels of Treg cells within TLSs [39]. Although most previous studies had used H&E staining and IHC to assess TLSs, quantifying TLSs accurately using these techniques is not easy owing to the influence of sensitivity and specificity. As an emerging technology, transcriptomic analyses can quantitatively analyze TLSs using specific gene signatures. Specifically, the 9-gene TLS signature identified in metastatic melanoma (*CCR6*, *CD1D*, *CD79B*, *CETP*, *EIF1AY*, *LAT*, *PTGDS*, *RBP5*, and *SKAP1*) can be effectively used to analyze TLSs in the TME quantitatively [40]. Studies have shown that *PTGDS* [41] and *SKAP1* [42] in this TLS gene signature are associated with poor prognosis and immunotherapy outcomes in GC. Therefore, the TLS signature may serve as a potential biomarker for predicting the clinical outcome of patients with STAD.

Epidemiological studies have shown that sex affects the prognosis of various primary malignant tumors, with males exhibiting higher mortality and shorter survival periods than females [5]. Our study observed that in male patients with STAD, the TLS-high group was significantly linked to poor prognosis. In contrast, in female patients

Table 4. Differences in the clinical characteristics between the low and high TLS groups in stage I–III male STAD patients among the SMC cohort (N = 225).

Recurrence-free survival	TLS		χ^2	*p*-value
	Low group	High group		
All case (N, %)	74 (32.9%)	151 (67.1%)		
Age			0.61	0.44
≤65	66 (89.2%)	129 (85.4%)		
>65	8 (10.8%)	22 (14.6%)		
Location			3.83	0.28
Cardia	10 (13.5%)	20 (13.2%)		
Distal	47 (63.5%)	81 (53.6%)		
Mid	16 (21.6%)	41 (27.2%)		
Whole	1 (1.4%)	9 (6.0%)		
Lauren			10.65	0.004
Diffuse	31 (41.9%)	94 (62.3%)		
Intestinal	41 (55.4%)	49 (32.5%)		
Mixed	2 (2.7%)	8 (5.3%)		
Tumor stage			1.97	0.37
I	8 (10.8%)	26 (17.2%)		
II	40 (54.1%)	70 (46.4%)		
III	26 (35.1%)	55 (36.4%)		
Subgroup			1.32	0.25
MP	4 (5.4%)	15 (9.9%)		
EP	70 (94.6%)	136 (90.1%)		
Death			0.50	0.48
No	48 (64.9%)	105 (69.5%)		
Yes	26 (35.1%)	46 (30.5%)		
Recurrence			1.96	0.16
No	44 (59.5%)	104 (68.9%)		
Yes	30 (40.5%)	47 (31.1%)		

MP, mesenchymal phenotype; EP, epithelial phenotype.

Table 5. Univariate Cox analysis of prognosis of stage I-III male patients STAD among SMC cohort (N = 225).

Recurrence-free survival	Group	Univariate analysis				
		β	SE	Wald χ^2	HR (95% CI)	*p*-value
TLS signature	Low[1]					
	High	–0.41	0.23	3.09	0.66 (0.42 to 1.05)	0.08
Age	≤65[1]					
	>65	0.21	0.33	0.42	1.23 (0.65 to 2.34)	0.52
Location	Cardia[1]					
	Distal	0.28	0.38	0.54	1.32 (0.63 to 2.81)	0.46
	Mid	0.33	0.41	0.62	1.39 (0.62 to 3.11)	0.43
	Whole	–1.22	1.06	1.33	0.29 (0.04 to 2.35)	0.25
Lauren	Diffuse[1]					
	Intestinal	–0.10	0.24	0.18	0.91 (0.57 to 1.44)	0.67
	Mixed	–1.51	1.01	2.22	0.22 (0.03 to 1.61)	0.14
Subgroup	MP[1]					
	EP	–0.79	0.33	5.94	0.45 (0.24 to 0.86)	0.02

1: control group.

with STAD, the prognosis did not differ significantly between TLS-high and TLS-low groups. Furthermore, in male patients with stage I–III STAD, the TLS signature was an independent predictor of poor prognosis. These findings suggest that the role of the TLS signature in predicting patient prognosis is likely influenced by sex dif-

Table 6. Multivariate Cox analysis of prognosis of stage I–III male patients STAD among SMC cohort (N = 225).

Recurrence-free survival	Group	Multivariate analysis				
		β	SE	Wald χ^2	HR (95% CI)	p-value
TLS signature	Low[1]					
	High	–0.50	0.24	4.40	0.61 (0.38 to 0.97)	0.04
Subgroup	MP[1]					
	EP	–0.91	0.33	7.56	0.40 (0.21 to 0.77)	0.006

1: control group.

ferences. One research study has consistently established that the incidence rate of GC among male patients tends to be higher than that of their female counterparts [43]. Furthermore, a recent study has documented that in addition to genetic and behavioral factors, the leading cause is immune cell and molecular variation between the sexes [44]. For example, M2 macrophages and monocytic MDSCs are more abundant in the TME of males, and their anti-inflammatory factors (such as interleukin-6) can enhance the formation of an immunosuppressive TME, thereby inhibiting the antitumor response [45]. In contrast, the TME of females elicits a more robust immune response. Recent studies have shown that TLSs in patients with GC exhibit significant heterogeneity and that their maturation states are largely influenced by the TIME [17,46]. Tumors containing immature TLS (iTLS) and mature TLS (mTLS) display functional differences. iTLS-enriched tumors possess more immunosuppressive cell populations and express all the characteristics and pathways of an immunosuppressive microenvironment. In contrast, mTLS tumors display specifically enhanced apoptosis-related pathways and upregulate antitumor-effector pathways [47]. Therefore, the role of the TLS signature in predicting prognosis in male patients with stage I–III STAD may be related to the immunosuppressive TME.

Subsequently, the differences in the TME were analyzed in the two groups of male patients with stage I–III STAD. Immune interactions are critical features of tumorigenesis and therapeutic targets for STAD. Immune cells, cytokines, and other factors are the immune components present within the TME [48]. The interactions among these components, namely, those that promote antitumor responses and those that promote protumor responses, play a critical role in shaping the trajectory of antitumor immunity [49]. In this study, a marked increase was noted in the infiltration of immune-associated cells within the TLS-high group, which was statistically significant compared with the TLS-low group. This finding suggests that the role of the TLS signature could be associated with the involvement of the TME, thereby regulating tumor initiation and progression. In addition, a strong link was observed between the TLS signature and immunosuppressive cell subsets (Breg cells, Treg cells, MDSCs, pDCs, M2 macrophages, and TANs) in male patients with stage I–III STAD. This immunosuppressive TME prevents effector immune cells from entering tumors and further inactivates antitumor immune responses [50–52]. Breg cells have been suggested to suppress T-cell responses and stimulate Treg cells by producing anti-inflammatory cytokines (interleukin (*IL*)-*10*, *IL-35*, and transforming growth factor beta (*TGF-*β)) [53,54]. Enriching Treg cells, MDSCs and M2 macrophages inhibits the proliferation and functionality of antitumor effector cells [49,53–55]. TANs are crucial components of the stromal microenvironment, exerting a considerable influence on the process of carcinogenesis. Li *et al.* [56] have claimed that TANs could promote GC progression by secreting *IL-17a* and activating Janus kinase 2/Signal transducer and activator of transcription 3 (*JAK2/STAT3*) signaling. In addition, pDCs are linked to immunosuppressive TME by recruiting Treg cells or inducing the expression of immune regulatory molecules [57]. This phenomenon not only poses an immense obstacle to tumor immunotherapy but also exhibits a strong association with compromised survival outcomes. This study revealed that the TLS-high group exhibited higher mRNA levels of immunosuppressive cell recruitment factors, immunosuppressive factors, and immune checkpoint receptors. Based on the obtained data, TLSs could be speculated to play a vital role in regulating the immunosuppressive TME. TLSs recruit and facilitate the secretion of immunosuppressive factors from immunosuppressive cells, ultimately promoting tumor immune evasion.

Although a previous study has shown that TMB is a key predictor of tumor immune response, its role in STAD remains controversial [58]. The results from this study established that a higher TLS signature score indicated a lower TMB and meant a poorer prognosis; in contrast, a high TLS signature score but not a high TMB played a leading role in predicting prognosis. A recent study has reported that a high TMB is not a reliable predictor of immune checkpoint blockade response across all cancer types, supporting our conclusion [59]. As cancer often has a genetic predisposition, detecting mutation driver genes may aid in monitoring cancer occurrence and determining the therapeutic options [60]. This study revealed that patients with *TP53* or *MUC16* gene mutation in the TLS-low group exhibited a considerably longer OS than those in the TLS-high group. A recent study has shown that antiangiogenic, anti-*VEGFR2* systemic therapy is associated with superior OS in patients with metastatic GC having *TP53* mutations [61].

MUC16 is known to regulate the immune response to the cancer. Signaling pathways involved in immune response, antigen processing, cell cycle checkpoints, and DNA replication and repair are upregulated in patients with GC with *MUC16* mutations and may benefit from immune checkpoint blockade [37]. Based on the evidence discussed, integrating the TLS signature and the mutation status of genes associated with prognosis can aid in selecting the patient population that may benefit from immunotherapy.

Several studies have documented that adjuvant chemotherapy exerts a positive impact on prognosis by eliminating micrometastasis, improving local control, and enhancing OS rates for patients [62,63]. According to the findings of a retrospective analysis, compared to monotherapy with fluoropyrimidine for stage IIIB–IIIC STAD, the dual-drug chemotherapeutic regimen (fluoropyrimidine plus platinum) resulted in better disease-free survival and OS [63]. Similarly, in bladder cancer, chemotherapy positively impacted prognosis in the patients with the nodal stage (N1+2) or tumor stage subgroups (T3+4), improving OS [64]. In the TCGA-STAD cohort, the prognosis was significantly enhanced in patients who received adjuvant therapy. This therapy was a significant independent positive prognostic factor for male patients with stage I–III STAD. Moreover, the interaction between TLS and adjuvant therapy positively influenced the prognosis of these patients. On the contrary, within the TME, TLSs promoted the infiltration of immune cells into solid tumors. These infiltrating immune cells can impact patient prognosis and are modulated by adjuvant therapy [65]. A recent study found that in Chinese patients with GC who responded to treatment with oxaliplatin and Teysuno, both the density and maturity of TLSs were increased [66]. Mechanistically, chemotherapy can improve tumor antigenicity and trigger the apoptosis of tumor cells, resulting in the recruitment and reprogramming of immune cells [65,67]. A study has recently stated that during chemotherapy for GC, the treatment sensitivity is associated with reduced M1 macrophage repolarization owing to the recruitment of natural killer cells and the increased infiltration of effector T cells [68]. Therefore, the inconsistency in the analysis results between the TCGA and SMC cohorts could be attributed to the fact that adjuvant therapy potentially influences the maturity of TLSs by modulating the TIME. This modulation could, in turn, alter the prognostic prediction effectiveness of TLSs in STAD.

This study has several limitations. First, although a systematic bioinformatics analysis was conducted, experimental validation was not performed, which may affect the reliability and validity of the results. Second, as the data were sourced from public databases, specific information about the adjuvant therapies used in the SMC cohort could not be obtained, which limits the potential for further analysis. More comprehensive data must be acquired in the future to analyze the impact of adjuvant therapies on the TIME, particularly on TLSs.

Conclusion

This study has identified that a high TLS signature score is an independent adverse prognostic factor in male patients with stage I–III STAD in the TCGA cohort. Furthermore, the TLS-high group exhibited poorer OS than the TLS-low group, which could potentially be ascribed to an immunosuppressive TME. However, adjuvant therapy may affect the prognostic predictive effect of TLSs in these patients. These findings provide novel insights into personalized therapy for patients with STAD based on the TLS signature score in the future.

Availability of Data and Materials

The associated TCGA and GEO datasets, as well as metadata of the samples, are available at the TCGA database (https://portal.gdc.cancer.gov/projects/TCGA-STAD) and NCBI GEO (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26253). The original contributions of this study are detailed within the article and the Supplementary Material; any further inquiries should be addressed to the corresponding authors.

Author Contributions

Conceptualization: LY, HL and JJJ. Methodology: ZXY, SHH and FQ. Formal analysis and data curation: ZXY, SHH and FQ. Acquisition of data: HLW and FYW. Analysis and interpretation of data: SRL and YKZ. Writing-original draft: ZXY and SHH. Writing-review and editing: LY, HL and JJJ. All authors contributed significantly to editorial changes of important content. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202537193.24.

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