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ORIGINAL RESEARCH

Upregulation of *THBS1* is Related to Immunity and Chemotherapy Resistance in Gastric Cancer

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Purpose: Thrombospondin 1 (*THBS1*) is an endogenous inhibitor of angiogenesis, but it also promotes tumor invasion, metastasis, and immune response in the tumor environment. Previous research has found that *THBS1* is highly expressed in many tumors and has a negative correlation with tumor prognosis. However, research on the relationship between *THBS1* and immune infiltration in GC is less well documented, and the objective of our study was to investigate the role of *THBS1* expression in GC.

Patients and Methods: The expression of *THBS1* in GC was analyzed by Oncomine, TIMER, TGCA, GEO and IHC staining. Analysis of the signaling pathways associated with *THBS1* expression in GC uses GSEA. The relationship between *THBS1* expression and immune infiltration was analyzed by the ESTIMATE algorithm, single-cell transcriptome analysis, TIMER2 database and CIBERSORT algorithm. Finally, the relationship between *THBS1* expression and drug sensitivity was analyzed by the CellMiner database.

Results: *THBS1* was overexpressed in GC and was associated with poor prognosis, and high *THBS1* expression was an independent risk factor. GSEA results showed that high *THBS1* expression in GC was associated with tumorigenesis, adhesion, and significant immune enrichment. *THBS1* expression was most strongly correlated with tumor-associated macrophages (TAMs), M2 macrophages and cancer-associated fibroblast (CAFs) in GC. *THBS1* expression positively correlates with most immune checkpoint members, suggesting that *THBS1* may play an important role in the tumor microenvironment. *THBS1* overexpression was negatively correlated with some drug sensitivities, such as Oxaliplatin.

Conclusion: Upregulation of *THBS1* was positively correlated with poor prognosis and immunosuppression in GC and negatively correlated with anticancer drug sensitivity, suggesting that *THBS1* may serve as a potential target for the treatment of GC.

Keywords: *THBS1*, gastric cancer, drug sensitivity, prognosis, tumor microenvironment

Introduction

Gastric cancer (GC) is the fifth leading tumor and the fourth leading cause of cancer-related deaths worldwide, with more than 1 million new cases and more than 700,000 new deaths each year. The highest prevalence rate is found in East Asia.[1] Present therapeutic approaches for GC include surgery, chemotherapy, radiotherapy, targeted therapy and immunotherapy. Although they have alleviated some of the symptoms of patients, the prognosis for advanced patients remains poor, The 5-year survival rate for GC is 30.4%[2] and less than 5% for advanced GC.[3] GC is characterized by potential biological and genetic heterogeneity.[4] Exploring new diagnostic and therapeutic targets is of great importance.

Thrombospondin (*THBS*) is a multifunctional family of extracellular matrix proteins involved in tissue remodeling and with implications for tumor formation,

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wound healing, and embryonic development.[5] *THBS1* was the first member identified and is thought to be an endogenous angiogenesis inhibitor. *THBS1* exerts different effects by binding to different cellular receptors.[6] Previous studies have shown that *THBS1* is highly expressed in lymphoma,[7] breast cancer,[8] melanoma[9] and oral squamous carcinoma,[10] promoting tumor cell adhesion, proliferation, apoptosis, invasion and metastasis. *THBS1* has been reported to correlate with immune cell infiltration in melanoma.[11] The above implies a potential function of *THBS1* in tumorigenesis. However, the role of *THBS1* expression in GC has been less studied.

In this study, we investigated the prognosis of *THBS1* in GC and its correlation with immune infiltration and anticancer drug sensitivity. The expression of *THBS1* in GC was analyzed by genomic techniques and the correlation of *THBS1* expression with the prognosis of GC. Enrichment analysis of *THBS1* was performed by GSEA. The correlation between *THBS1* and tumor infiltrating immune cells (TIICs) was analyzed by CIBERSORT. Finally, the correlation between THBS1 and drug sensitivity was analyzed by CellMiner. Our work elucidated the role of *THBS1* expression in GC and the mechanism of interaction with tumor immunity, suggesting that *THBS1* may be a therapeutic target for GC.

Materials and Methods

THBS1 Gene Expression and Prognosis Analysis

The analysis was performed by Online Cancer Microarray Database(Oncomine) (https://www.oncomine.org/resource/main.html),[12] UCSC Xena (https://xenabrowser.net/)[13] and Tumor Immune Estimation Resource (TIMER) (https://cistrome.shinyapps.io/timer/).[14] Differential expression of THBS1 was performed by TIMER, TCGA and two Gene Expression Omnibus (GEO) databases (GSE66229 and GSE118916). The correlation between *THBS1* and pathological stages of GC was analyzed analyzed using the "ggboxplot" package in the R. The prognosis of *THBS1* was analyzed according to the TCGA and GSE62254 databases,[15] and statistical analysis was performed using the "survival" and "survminer" packages of the R (version 4.0.4).

Gene Set Enrichment Analysis (GSEA)

We analyzed the changes in *THBS1* mRNA levels in the genomic profile of the KEGG pathway using the GSEA tool (version 4.1.0).[16] In the TCGA-STAD cohort, the median expression of *THBS1* was used as a cut-off point to divide into a low and a high expression group. To determine the potential function of *THBS1*, GSEA examined the top ranked gene enriched pathways in both groups. GSEA was performed via the "clusterProfiler" package of the R.

Correlation Analysis of *THBS1* Expression with the Tumor Microenvironment (TME)

The TCGA-STAD cohort was subjected to ESTIMATE analysis. The ESTIMATE algorithm using the "estimate" package of R was used to estimate the ratio of *THBS1* high and low expression groups in the immune and stromal component of TME. The "survival" and "survminer" packages in R were also used for survival analysis. The TISCH database (http://tisch.comp-genomics.org/)[17] was used to perform scRNA-seq dataset analysis.

Immune Infiltration Analysis

The TIMER was used to analyze immune cells infiltrating by tumors. The correlation of *THBS1* expression with the markers of M1 and M2 macrophages and tumor-associated macrophages (TAMs) in GC was also analyzed. The TCGA-STAD cohort was subjected to CIBERSORT analysis to investigate the effect of *THBS1* expression on 22 types of immune cells. 22 immune cells expression profiles were derived from CIBERSORT (an online database for estimating cell proportions and inferring cell type-specific gene expression profiles),[18] and then the R was used for " vioplot", "ggpubr" and "ggExtra" packages in R were then used for the analysis of variance and correlation analysis. $P<0.05$ was considered statistically significant by the Pearson coefficient test and Wilcoxon rank sum test.

Correlation of *THBS1* expression with tumor immunosuppressive genes was analyzed by the TISIDB database (http://cis.Hku.hk/TISIDB/).[19]

Correlation Analysis of *THBS1* Expression and Drug Sensitivity

The CellMiner database (http://discover.nci.nih.gov/cellminer/)[20,21] was used to perform correlation analysis of *THBS1* expression with drug sensitivity. Data processing and graphing were performed via the "impute", "limma" and "ggpubr" packages in R.

Tissue Samples and Immunohistochemistry Staining (IHC)

This study was approved by the Institutional Research Ethics Committee of Tongji Hospital, Tongji Medical

College, Huazhong University of Science and Technology in accordance with the Declaration of Helsinki. Forty-seven primary GC samples and matched adjacent normal human tissues from January 2014 to August 2014 were included in the study.

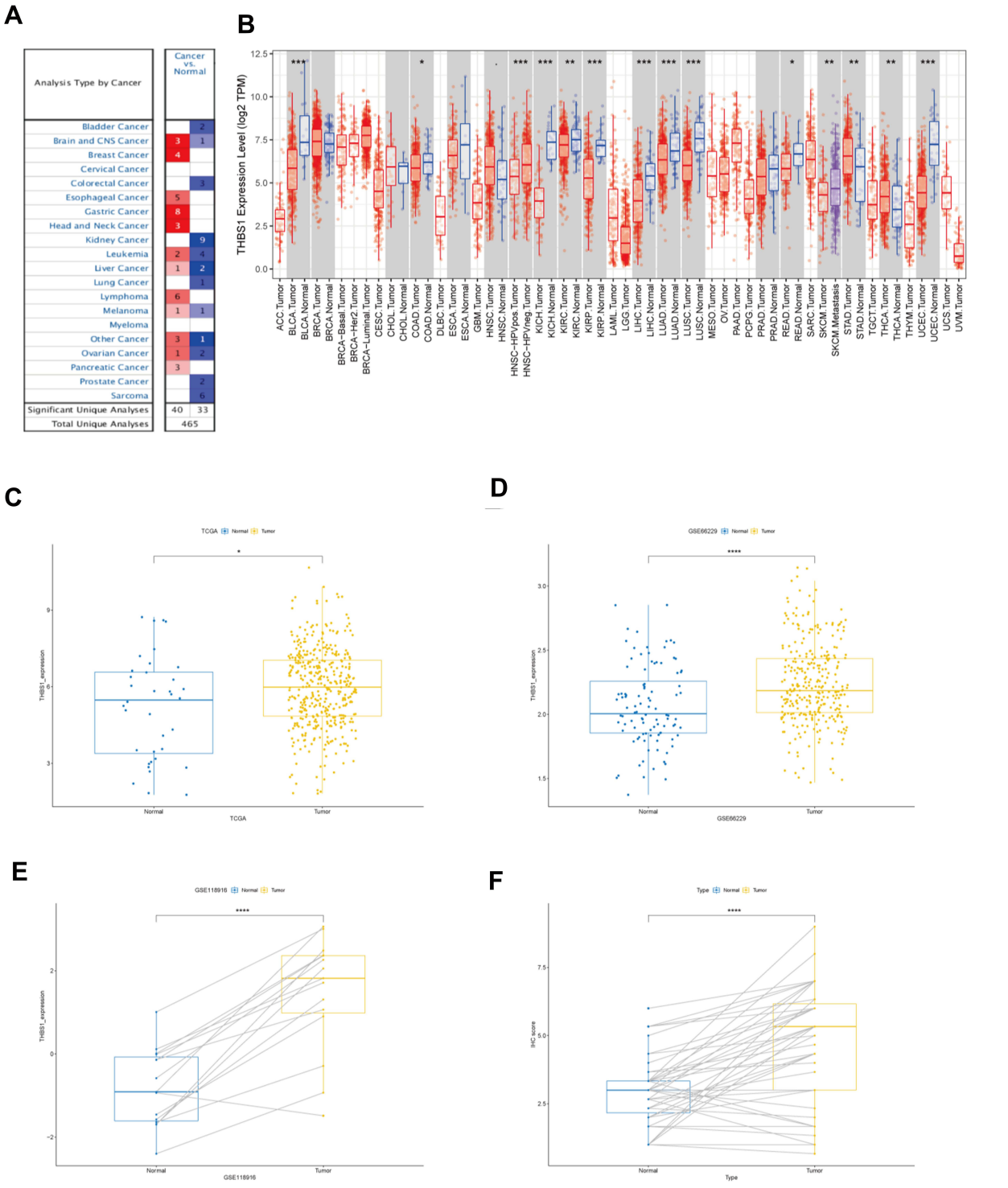

Figure 1 The expression of *THBS1* in gastric cancer. (**A**) *THBS1* in different cancers increased or decreased compared with normal tissues in the Oncomine database. (**B**) The expression of *THBS1* in different tumor types by TIMER database. (**C**) The expression of *THBS1* in GC tissues compared with normal tissues in TCGA. (**D**–**E**) The expression of *THBS1* in GC tissues compared with normal tissues in GSE66229 and GSE118916. (**F**)The protein expression of THBS1 in GC tissues compared with adjacent normal tissues. * *P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

All paraffin tissue sections were dried, dewaxed, hydrated, inactivated with endogenous enzymes and antigen repair, closed with 5% BSA at room temperature for 30 min, and incubated overnight at 4°C with primary antibody (*THBS1*, TA325040, 1:100; Origene, Rockville, MD, USA). After washing 3 times with TBS, the reaction was performed by polymerizing the conjugated secondary antibody and DAB with peroxidase, followed by counterstaining with hematoxylin. Five randomly selected sections were observed by optical microscope. Staining results were scored according to the intensity of staining (negative: 0, weak: 1, moderate: 2, strong: 3) and the fraction of positive cells (0=0–9%; 1= 10–30%; 2=31–50%; 3=51–100%). If IHC score ≥4, it was considered high expression. IHC score <0.4 was considered low expression. The IHC staining results were assessed and scored by two senior pathologists.

Results

Expression Analysis of *THBS1* in GC

The expression of *THBS1* in each tumor was analyzed by Oncomine and TIMER database, and the results showed that *THBS1* expression changed depending on the cancer type. Figure 1 shows that *THBS1* expression was significantly higher in GC tissues than in adjuvant tissues, and 8 datasets showed high expression of *THBS1* in GC. By analyzing TCGA and 2 GEO databases, the results support that *THBS1* is highly expressed in GC tissues.

We then analyzed the protein expression level of *THBS1* in GC by IHC staining, and as shown in Figure 1F, the protein level of *THBS1* in GC tissues was significantly higher than that in adjacent normal tissues. The results of IHC staining are shown in Figure 2. It is suggested that *THBS1* plays a role in GC development.

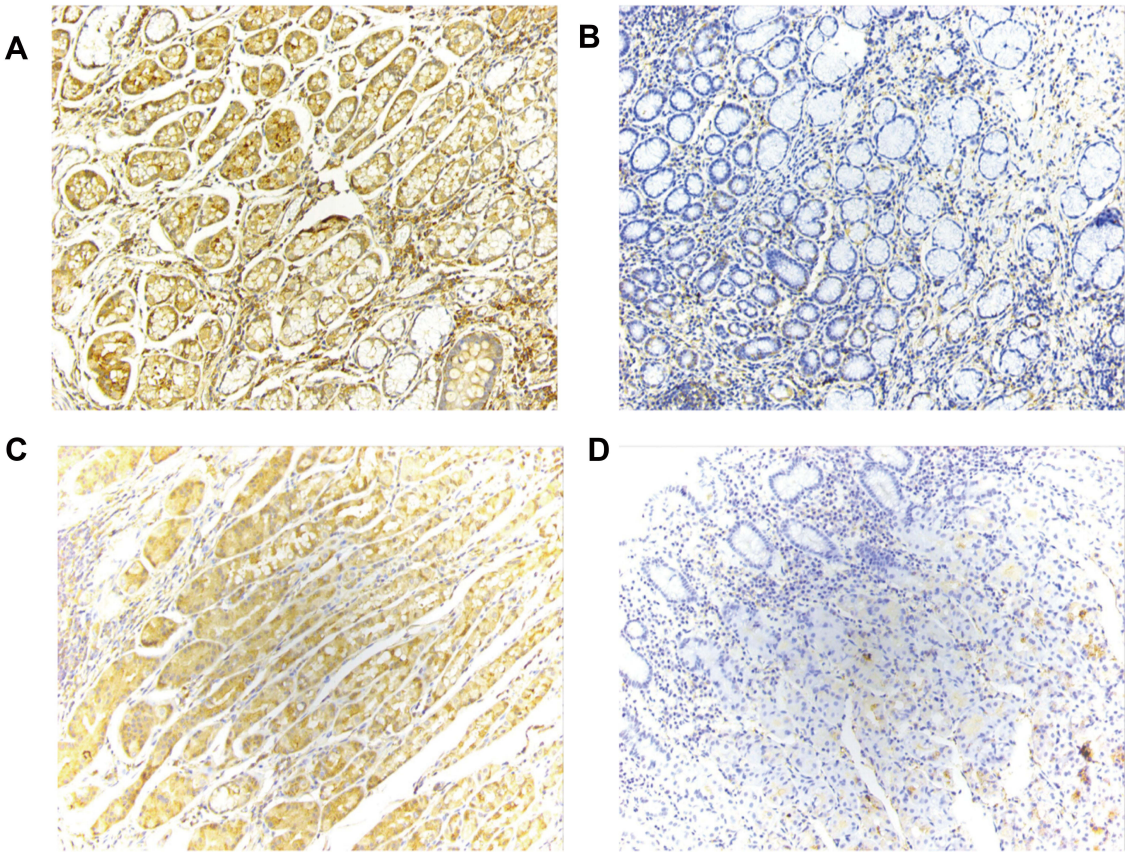

Figure 2 IHC staining of *THBS1* (magnification, x200). (**A**) High expression of *THBS1* in GC tissues. (**B**) Low expression of *THBS1* in GC tissues. (**C**) High expression of *THBS1* in adjacent normal tissues. (**D**) Low expression of *THBS1* in adjacent normal tissues.

Correlation Between *THBS1* Expression and Clinical Outcomes of GC Patients

Clinical information on TCGA-STAD cohort was downloaded through the UCSC Xena database, and 325 patients were included in the analysis, and *THBS1* expression was divided by median into high and low expression groups. The effect of *THBS1* expression on GC prognosis was analyzed by TCGA and GEO databases, and it was shown by Figure 3 that high *THBS1* expression in TCGA was significantly correlated with shorter OS in GC, and high *THBS1* expression in GSE62254 was significantly correlated with shorter OS and DFS in GC. Moreover, univariate and multifactorial Cox regression analyses of *THBS1* expression showed that high *THBS1* expression was an independent risk factor for poor prognosis in GC patients (Table 1).

GSEA Identifies *THBS1*-Related Signaling Pathways in GC

To investigate the function of THBS1, we performed a GSEA analysis (Figure 4). The results suggest that the immune-related ones were TGF-beta signaling pathway, chemokine signaling pathway and viral protein interaction with cytokine and cytokine receptor. The cell adhesion and tumorigenesis related ones are PI3K−Akt signaling pathway, focal adhesion and cell adhesion molecules. GSEA results showed that

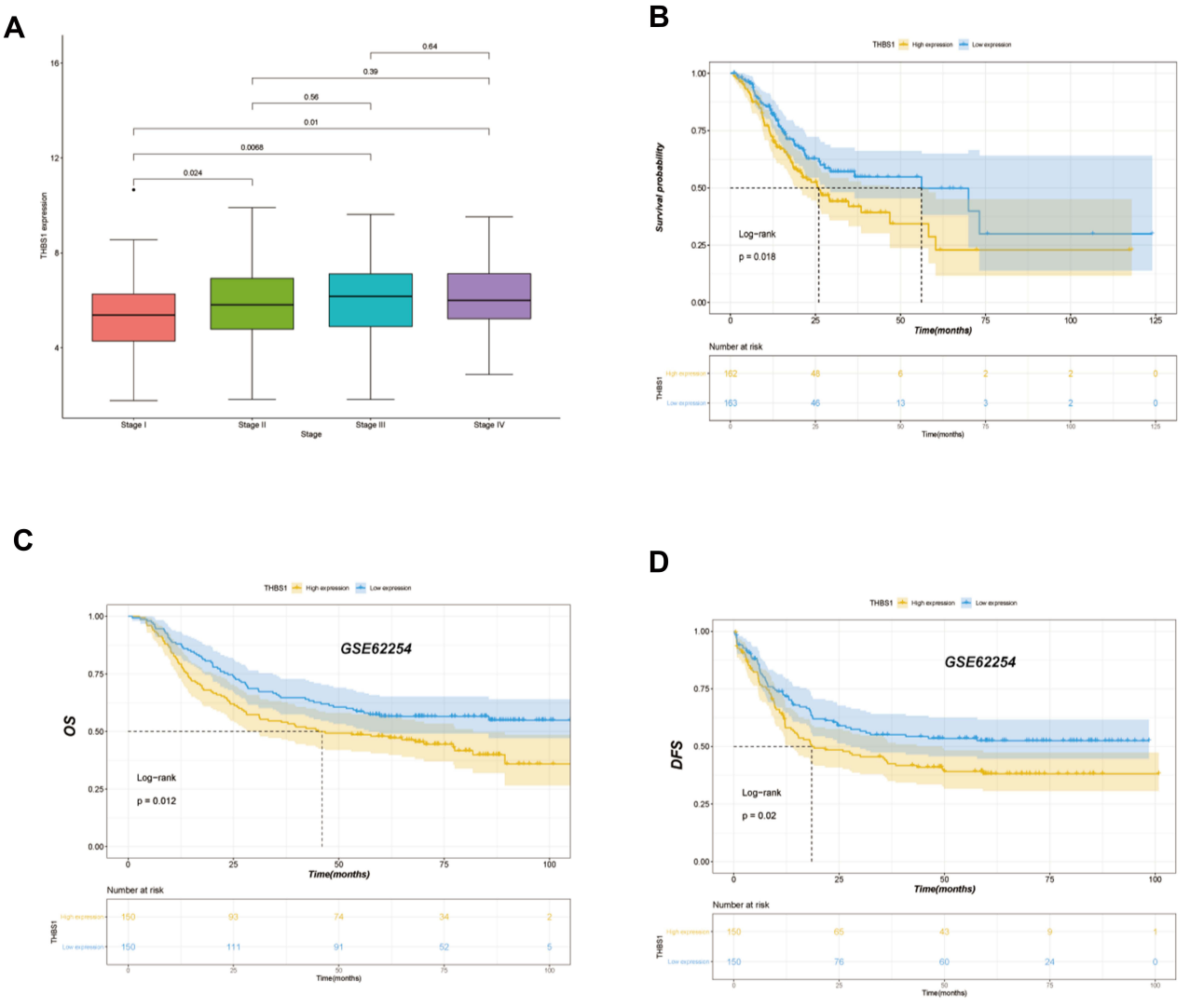

Figure 3 Correlation analysis between *THBS1* expression and prognostic survival in STAD patients. (**A**) Differential expression of *THBS1* in different pathological stage. (**B**) Kaplan-Meier OS curves of survival differences among TCGA-STAD cohort. (**C**) Kaplan-Meier OS curves of survival differences among GSE62254 dataset. (**D**) Kaplan-Meier DFS curves of survival differences among GSE62254 dataset.

Table 1 Univariate and Multivariate Cox Regression Analysis of OS in Gastric Cancer Patients (n=325)

Variables		Univariate Analysis		Multivariate Analysis	
		HR (95% CI)	P-values	HR (95% CI)	P-values
Age	>65 vs ≤65	1.555 (1.099–2.201)	0.013[a]	1.856 (1.296–2.657)	0.001[a]
Gender	Male vs Female	1.404 (0.970–2.033)	0.072		
Clinical stage	III–IV vs I–II	1.864 (1.297–2.678)	0.001[a]	1.106 (0.649–1.882)	0.721
Tumor depth	T3/T4 vs T1/T2	1.698 (1.097–2.629)	0.018[a]	1.351 (0.825–2.212)	0.231
Lymph node	N1/N2/N3 vs N0	1.968 (1.290–3.001)	0.002[a]	1.687 (0.096–2.963)	0.069
Grade	G3 vs G1/G2	1.404 (0.970–2.033)	0.072		
Distant metastasis	M1 vs M0	2.058 (1.108–3.824)	0.022[a]	2.229 (1.154–4.303)	0.017[a]
THBS1 expression	High vs Low	1.513 (1.072–2.134)	0.018[a]	1.560 (1.095–2.222)	0.014[a]

Notes: The TNM staging system is based on the extent of the tumor (T), the extent of spread to the lymph nodes (N) and the presence of metastasis. [a]Statistically significant.

high THBS1 expression in GC was associated with tumorigenesis, adhesion, and immune significant enrichment.

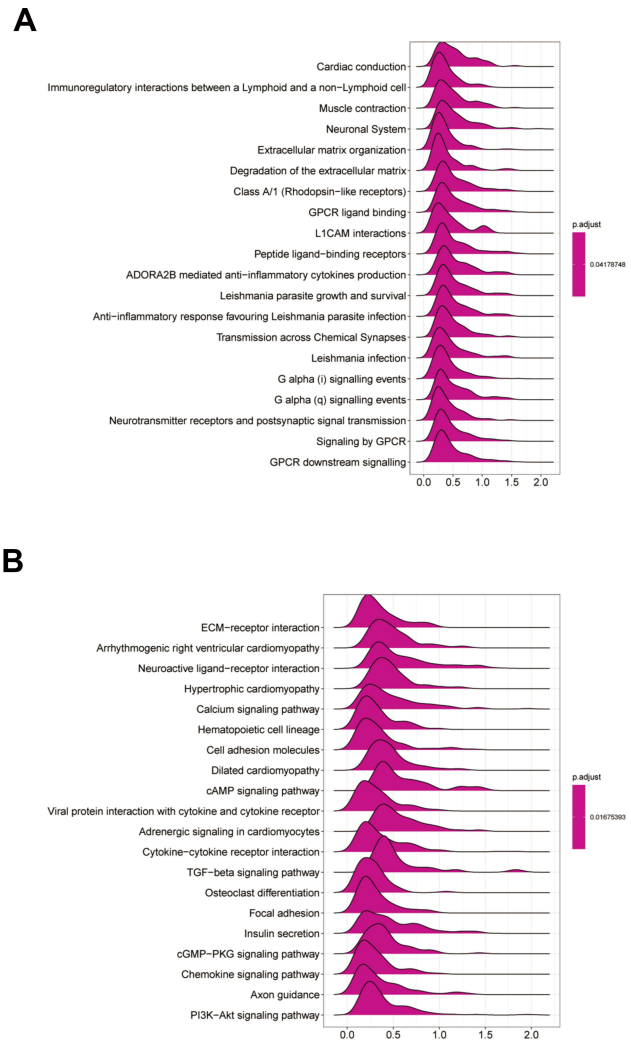

Figure 4 GSEA analysis of THBS1 expression in GC. The top 20 GSEA results of Reactome pathways (**A**) and KEGG pathways (**B**).

Correlation Between *THBS1* Expression and TME in GC

The TCGA-STAD cohort was analyzed by ESTIMATE algorithm, Figure 5 shows that stromal and immune scores were positively correlated with *THBS1* expression, and prognostic analysis combining both stromal and immune scores showed that patients with lower scores had significantly higher survival rates than those with higher scores. In addition, we used by scRNA-seq dataset (STAD_GSE134520) for exploring the relationship of *THBS1* expression in cells associated with TME of gastric cancer. As shown in Figure 6, *THBS1* was differentially expressed in immune cells, stromal cells and malignant cells. *THBS1* expression was highest in myofibroblasts, followed by dendritic cells, fibroblasts and malignant cells, which was consistent with the results of the ESTIMATE algorithm.

Correlation Between *THBS1* Expression and the Level of Immune Infiltration in GC

To investigate the relationship between *THBS1* expression and immune infiltration in GC, *THBS1* expression was most strongly correlated with macrophage (COR=0.682, P=3.35e-53) by TIMER2 database analysis (Figure 7A). The proportion of 22 TIICs in GC was also analyzed by CIBERSORT and a correlation analysis was performed (Figure S1). Correlation of *THBS1* expression with the proportion of immune cells of 22 subtypes was quantified with dividing *THBS1* into high and low expression groups. Figure 7B shows that M2 macrophages, Monocytes and CD4 T cells memory resting were positively correlated with *THBS1*, while T cells follicular helper, Tregs, M1 macrophages and CD8 T cells were negatively correlated with *THBS1*.

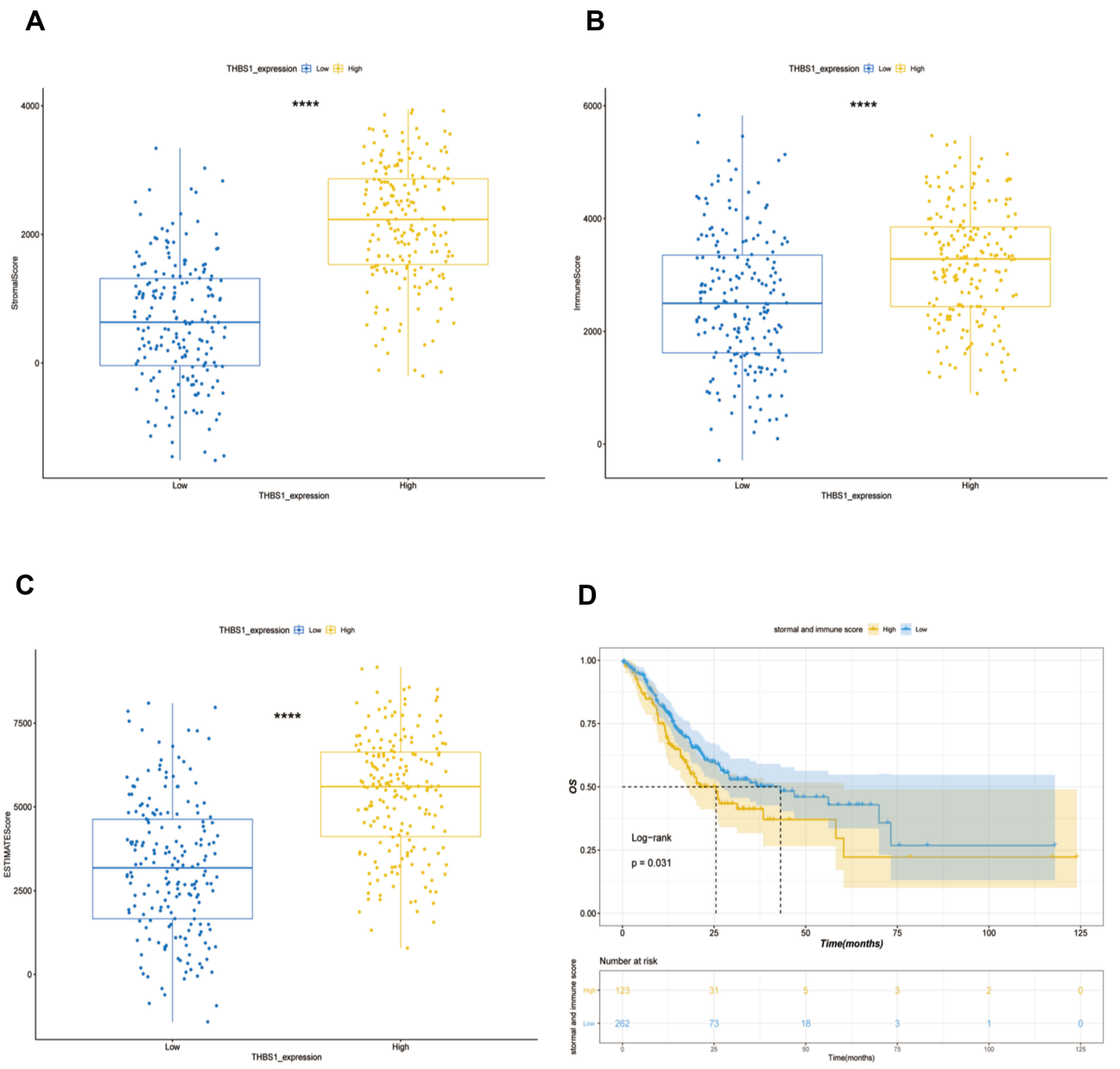

Figure 5 Relationship between *THBS1* and immune and stromal scores in GC. (**A**) High *THBS1* expression was associated with high stromal score in GC. (**B**) High *THBS1* expression was associated with high immune score in GC. (**C**) High *THBS1* expression was associated with high immune and stromal scores in GC. (**D**) High immune score and stromal score are negatively associated with prognosis of GC. ****P<0.0001.

We further investigated the correlation between *THBS1* and the markers of M1 and M2 macrophage, as well as TAMs. *THBS1* expression was weakly related to markers of M1 macrophages but moderately related to markers of M2 macrophages and TAMs. (Figure S2), and *THBS1* may promote polarization of TMAs to M2 type, associated with M2-like TAMs.

We have already found relatively high expression of *THBS1* in fibroblasts in our study. Cancer associated fibroblast (CAFs) can secrete TGFB1 to promote tumor progression,[22] and *THBS1* is considered to be an activator of TGFB1,[23] so we analyzed the correlation between *THBS1* and infiltration level by TIMER2 database, and as shown in the Figure 8 *THBS1* expression was positively correlated with CAFs in GC.

Correlation of *THBS1* with Immune Checkpoint Members

To further investigate the impact of *THBS1* expression in the microenvironment of GC, we investigated the

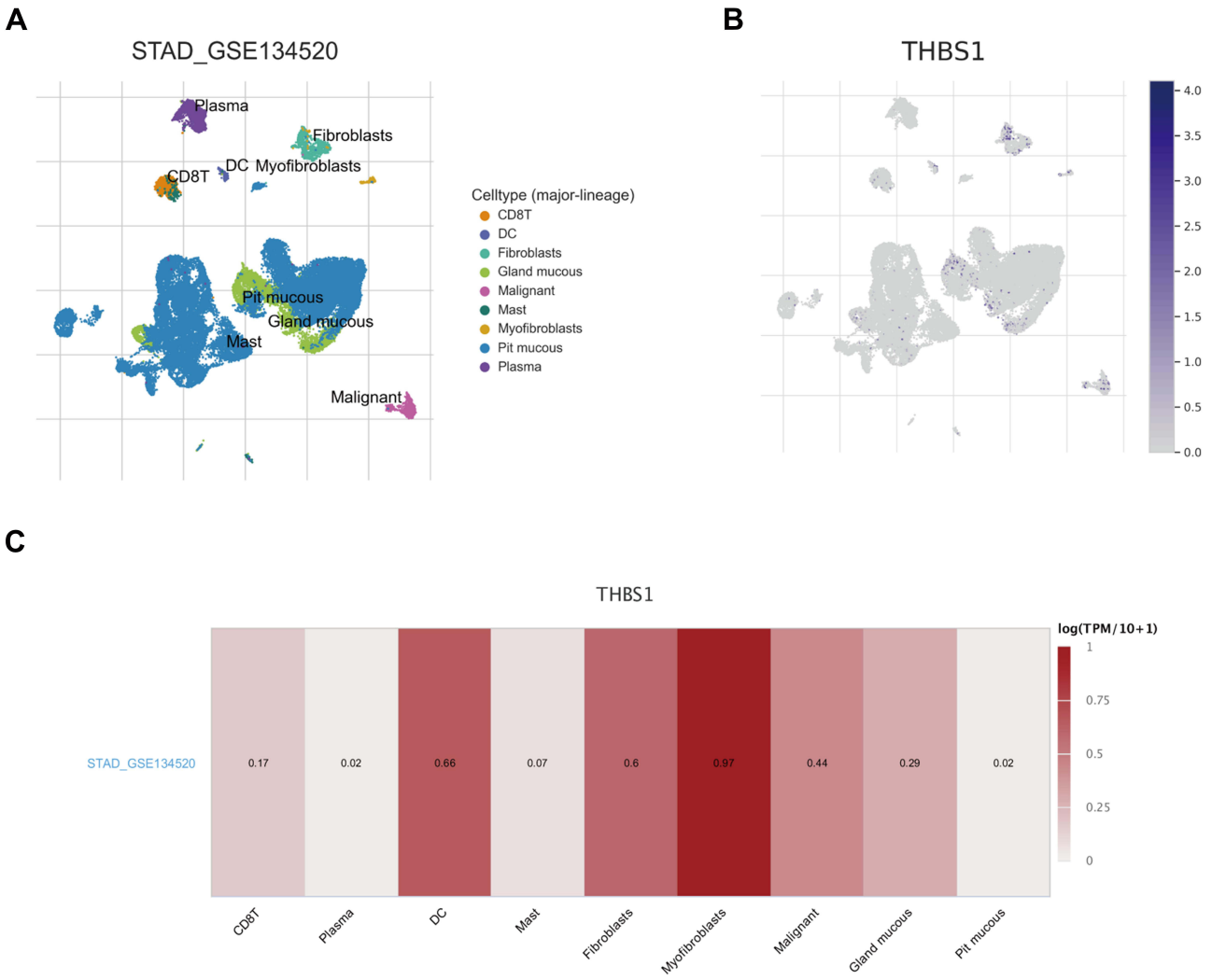

Figure 6 Correlation of *THBS1* with TME in GC. (**A**) Cell types and distribution of STAD_GSE134520. (**B**) Expression and distribution of *THBS1* in each cell. (**C**) Correlation of *THBS1* expression with TME.

correlation between *THBS1* and immunoinhibitors in pan-cancer through the TISIDB database (Figure 9A). In addition, we found a significant correlation between *THBS1* and immune checkpoint members (including PDL1, PDL2, TIM-3, CTLA4, PD1) in TCGA-STAD cohort (Figure 9B).

Correlation Between *THBS1* and Drug Sensitivity

The correlation between THBS1 and antitumor drug sensitivity was explored through the CellMiner database,61 anticancer drugs that showed significant correlation with *THBS1* expression were screened. Among them, 47 drug sensitivities were significantly and negatively correlated with *THBS1* expression. Figure 10 shows the top 9 results, we found that *THBS1* expression was significantly negatively correlated with the sensitivity of Oxaliplatin, Tamoxifen, By-Product of CUDC-305, Everolimus, Ixabepilone, Crizotinib, PX-316 and Nilotinib and positively correlated with the sensitivity of Staurosporine and Everolimus. Detailed results are shown in Table S1.

Discussion

THBS1 is an extracellular matrix protein that has been reported to promote tumor adhesion, invasion and metastasis in many tumors.[24] However, studies on the prognostic value and the potential molecular mechanisms of *THBS1* in the pathogenesis of GC are less available. This study is the first to comprehensively explore the role of *THBS1* expression in GC and the mechanism of immune interaction with tumors.

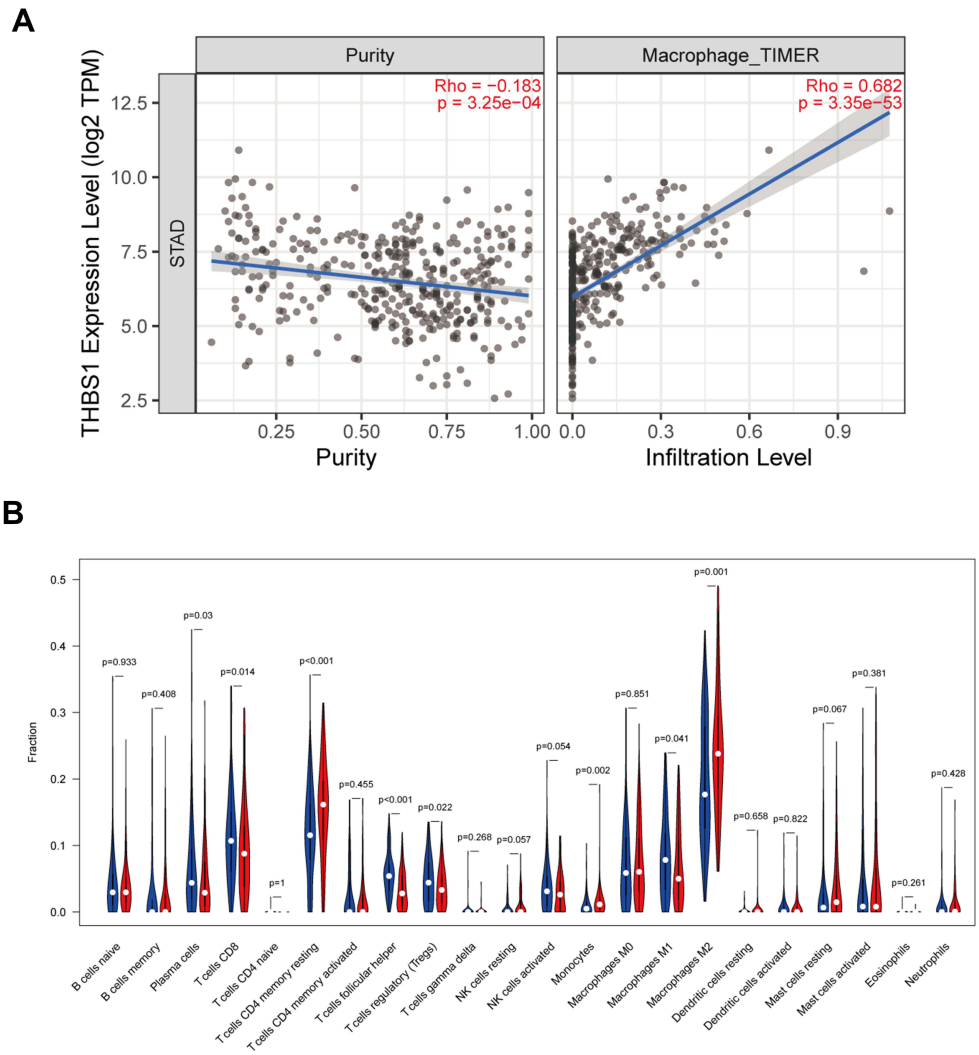

Figure 7 Relationship between *THBS1* and immune infiltration. (**A**) *THBS1* expression relates to immune cell infiltration levels in STAD. (**B**) Relationship between high and low expression of *THBS1* in 22 types of tumor-infiltrating immune cells, the blue violin diagram presented low *THBS1* expression group, and the red violin diagram presented high THBS1 expression group.

In our study, we found that *THBS1* was significantly highly expressed in GC. In addition, survival analysis showed that high THBS1 expression was significantly associated with shorter OS and DFS in GC. The results are consistent with previous studies that *THBS1* promotes liver metastasis in colorectal cancer by promoting epithelial-mesenchymal transition,[25] and high expression in cutaneous T-cell lymphoma promotes tumor invasion and metastasis, leading to poor prognosis.[7] The above suggests that THBS1 is associated with poor prognosis in GC.

To investigate the role of *THBS1* in GC, we performed GSEA analysis of *THBS1*, the enriched pathways in the *THBS1* high expression group that were analyzed by GSEA and related to immunity were TGF-beta signaling pathway and chemokine signaling pathway. Previous studies have shown that high *THBS1* expression in melanoma increases macrophages recruitment[11] and that the effect of *THBS1* on early natural killer cell proliferation is associated with TGF-beta activation.[26] Related to cell adhesion are focal adhesion and cell adhesion molecules. It has been shown that *THBS1* can promote cell adhesion to the stroma of osteosarcoma cells through α4β1 integrins.[27] The above indicates that *THBS1* is associated with tumor adhesion and immune infiltration.

The tumor microenvironment (TME) is a complex network of tumor cells, fibroblasts, endothelial cells, immune cells and extracellular matrix.[28] In the present study, the role of *THBS1* expression in TME of GC was investigated. *THBS1* expression was compared with immune/stromal scores, and high *THBS1* expression was found to correlate

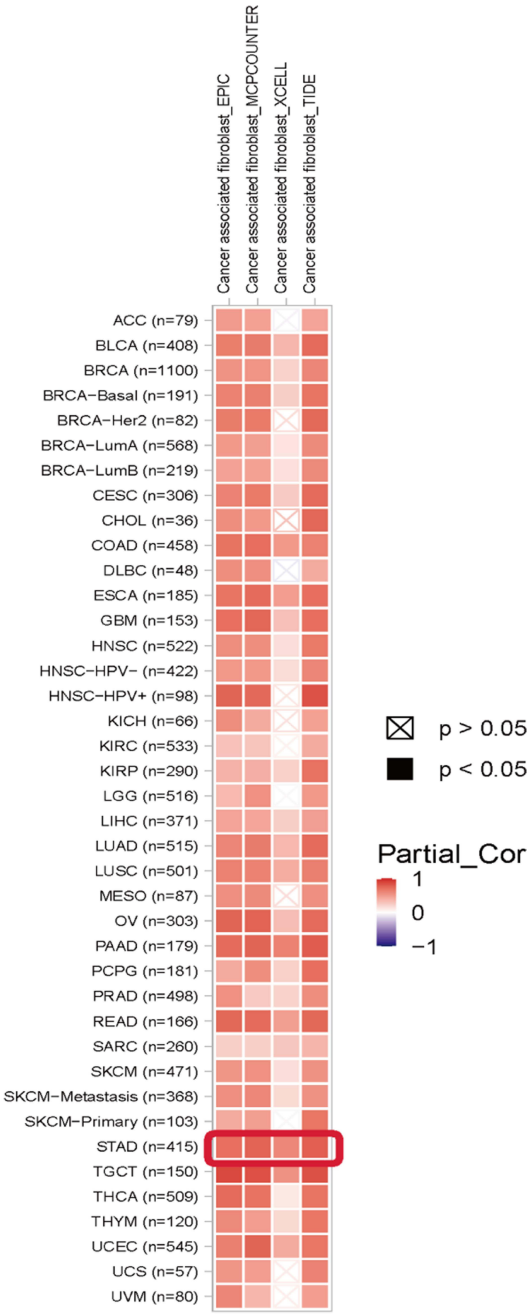

Figure 8 Correlation of *THBS1* expression with CAFs. Red represents positive correlation and blue represents negative correlation. *THBS1* expression is significantly positively correlated with CAFs in pan-cancer.

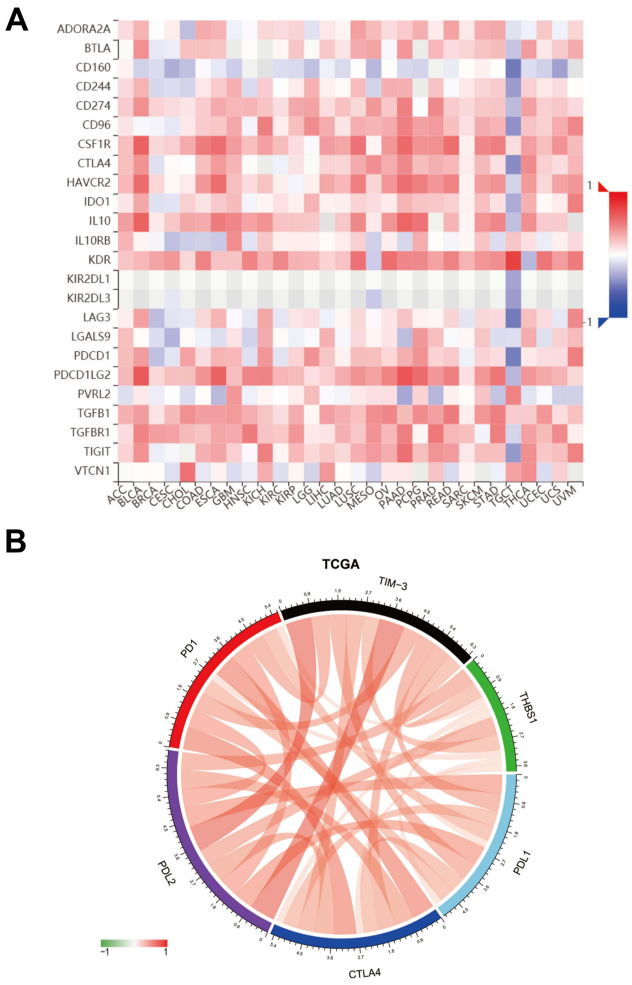

Figure 9 Correlation of *THBS1* with immune checkpoint members. (**A**) *THBS1* expression is correlated with immunoinhibitors in pan-cancer, red represents positive correlation, blue represents negative correlation, the deeper the color, the stronger the correlation. (**B**) *THBS1* expression is correlated with immune checkpoint members in TCGA-STAD, red represents positive correlation, green represents negative correlation.

with high immune/stromal scores, suggesting a correlation between high *THBS1* expression and the level of immune cell infiltration. One study indicated that high immune/stromal scores were significantly associated with poorer GC prognosis, which is in accordance with the findings of the previous study.[29] *THBS1* was significantly correlated with multiple TIICs by TIMER database analysis, with the strongest correlation with Macrophage. Further analysis of the proportion of TIICs in GC by CIBERSORT revealed that *THBS1* was positively correlated with M2 macrophages, Monocytes and Mast cells resting, and with Tregs, CD8 T cells and NK cells activated negatively. The above results suggest that *THBS1* expression correlates with immune infiltration of GC.

Macrophages can be bipolarly differentiated into M1 (classical) activation and M2 (alternate) activation. M1 macrophages are mainly associated with pro-inflammatory, bactericidal and anti-tumor effects, whereas M2 macrophages are mainly associated with tumor progression and immunosuppression.[30] TAMs are a group of TIICs that usually exhibit an M2-like immunosuppressive phenotype in most tumors and tend to promote angiogenesis, promote extravascular invasion and immune escape,

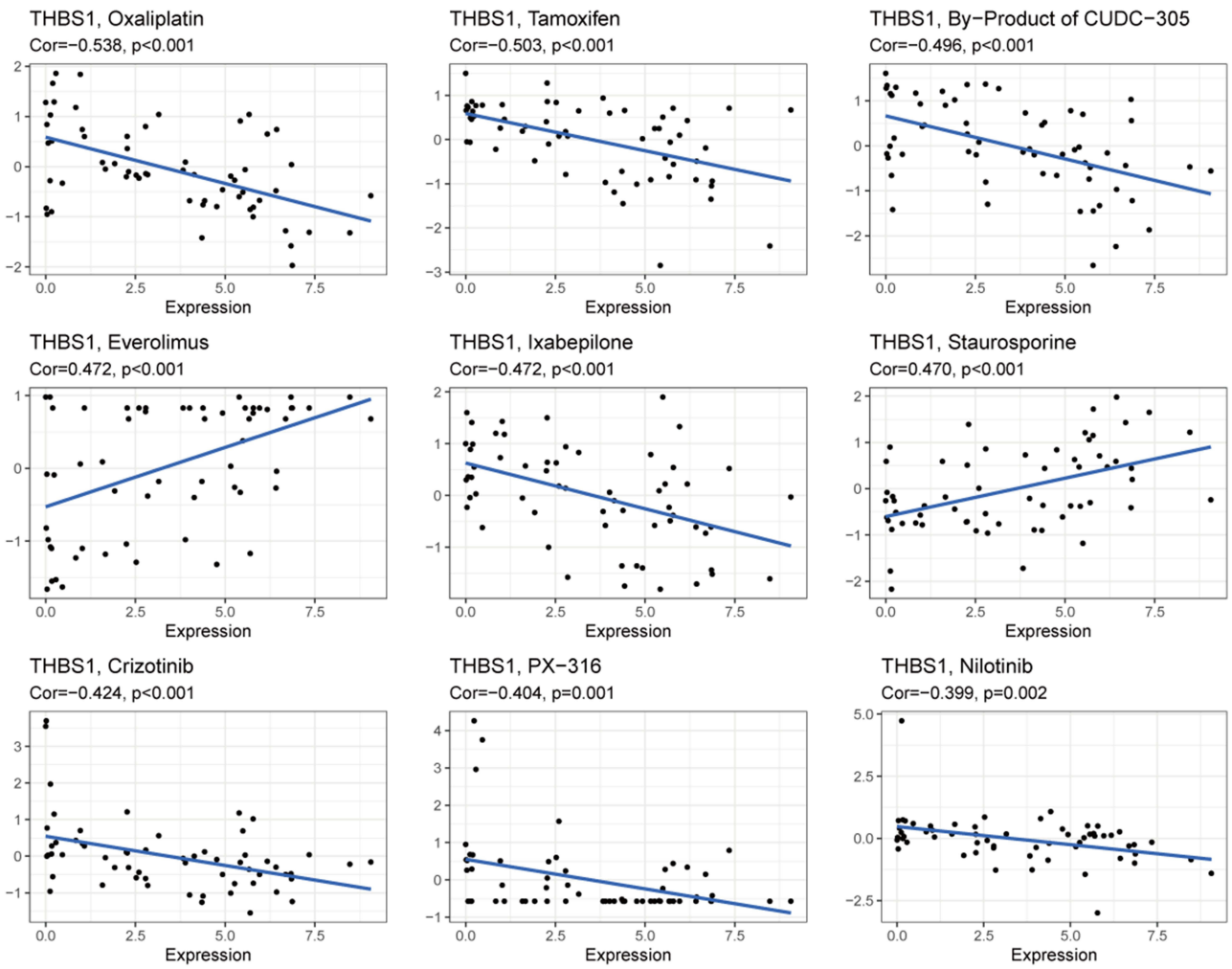

Figure 10 Correlation of THBS1 expression with the sensitivity of anticancer drugs.

ultimately leading to tumor progression and metastasis.[28] The results of CIBERSORT showed that among 22 TIICs, high *THBS1* expression was positively correlated with the proportion of M2 macrophages and negatively correlated with the proportion of M1 macrophages. In addition, the correlation between *THBS1* expression and markers of macrophages was also investigated, and *THBS1* expression was found to be moderately correlated with markers of M2 macrophages and TAMs, and weakly correlated with markers of M1 macrophages. It is suggested that *THBS1* may be involved in the mechanism of M2 macrophage-mediated tumorigenesis and thus differentiation into TAMs. *THBS1* binding to CD36 and CD47 receptors can play different roles in TME, and *THBS1* interacts with CD47 on immune cells, thus inactivating anti-tumor immune surveillance, which may attenuate the anti-tumor effect of M1 polarized macrophages in GC[31] and CD36 could promote the activation of M2 macrophages and also promote the migration and recruitment of TAMs in tumor tissues, thus promoting the progression of tumor.[32] Therefore, we speculate that *THBS1* may be involved in the mechanism of M2 macrophages and TAMs-mediated tumorigenesis through binding to CD36, to be proven by subsequent studies. Since TAMs have immunosuppressive effects in TME,[33] we explored the relationship between THBS1 expression and tumor immunosuppression and observed that *THBS1* expression was positively correlated with immune checkpoint members in GC, suggesting that upregulation of *THBS1* was strongly correlated with immunosuppressive status.

Clinically recommended chemotherapy regimens for gastric cancer include FLOT and FOLFOX regimens, which can effectively prolong the overall survival of advanced GC.[34] Our results from CellMiner database analysis found that *THBS1* was negatively correlated with the sensitivity of most anticancer drugs, including oxaliplatin (COR=−0.54)

and fluorouracil (COR=−0.28). It can be seen that upregulation of *THBS1* may affect the effect of chemotherapy in GC.

In summary, upregulation of *THBS1* expression was positively correlated with immunosuppression in GC and negatively correlated with anticancer drug sensitivity, suggesting that *THBS1* may serve as a potential target for the treatment of GC.

Conclusion

THBS1 was upregulated in GC and was related to an unfavorable prognosis. The overexpression of *THBS1* was significantly associated with M2-like TAMs and CAFs in GC and positively correlated with immunosuppressive genes. In addition, *THBS1* was negatively correlated with the sensitivity of many anticancer drugs, suggesting that *THBS1* is associated with chemoresistance. Our study suggests that *THBS1* may function as a promising target for treating GC.

Acknowledgments

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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