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Identified THBS3 as a risk gene for Gastric Cancer: A Summary Data-Based Mendelian Randomization

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Abstract. Gastric cancer (GC) currently ranks as the fourth leading cause of cancer-related deaths worldwide. Genetic variations may influence GC by regulating genes. The aim of this study was to find pleiotropic genes associated with GC risk. Methods: Summary data-based Mendelian Randomization (SMR) is employed to identify genetic variations that are associated with the GC. The Gene interaction network of THBS3 was established through GeneMANIA, and the molecular pathogenesis was elucidated by function and gene set enrichment analysis (GSEA). Furthermore, we conducted bioinformatics analysis using TIMER2.0, TCGA database, and TCIA database to identify the expression signatures, prognosis value, and immune characteristics of the identified gene. SMR analysis of eQTL data from GTEx and CAGE showed that THBS3 showed pleiotropic association with GC risk (CAGE: PSMR = 2.20E-07, PHEIDI = 0.061; GTEx_stomach: PSMR = 1.64E-06, PHEIDI = 0.069). THBS3 and its related genes primarily function through processes such as Extracellular matrix organization, Signaling by PDGF, and Integrin cell surface interactions. The expression of THBS3 is correlated with survival, clinical features, immune cells, and tumor microenvironment. Patients in the low-expression group had better responses to immunotherapy. Our study suggests that THBS3 may play an important role in the occurrence and prognosis of GC and provides a list of prioritized genes for further research on the underlying mechanisms of GC.

Keywords: GC; THBS3; eQTL; GWAS; SMR.

1. Introduction

Gastric cancer (GC) ranks fifth globally in terms of incidence and fourth in terms of cancer-related deaths [1]. Although the incidence of GC has decreased in recent years, it remains a significant global health issue[2]. The emergence of genome-wide association studies (GWASs) provides an important opportunity to identify multiple susceptibility loci associated with the risk of GC [3]. These genetic loci may influence GC by regulating genes. However, the underlying mechanism of this relationship remains largely unclear. Therefore, it is of great significance to explore the expression of genes that are potentially causally correlated with GC risk.

The Summary data-based Mendelian Randomization (SMR) integrates GWAS and eQTL data based on the principles of MR to explore pleiotropic associations between gene expression and outcomes, the heterogeneity in dependent instruments (HEIDI) test is used to assess whether the observed association is due to linkage[4]. The integration of GWAS and eQTL analysis could provide a deeper understanding of the genetic mechanism underlying GC[5].

The objective of this study was to investigate the potential association between genes and the risk of GC using the SMR approach, which integrates GWAS and eQTL data. Bioinformatics analysis was conducted on the identified genes to determine their expression signatures, prognosis value, and immune characteristics.

2. Materials and methods

2.1. Data sources

eQTL data: for blood tissue, the CAGE eQTL summarized data (n=2765) was used for the study afterward[6]. For stomach tissue, the GTEx eQTL summarized data (V8) was utilized for subsequent research (n=324)[7]. The eQTL data were public and could be available from SMR | Yang Lab (westlake.edu.cn).

GWAS data: the GWAS data for GC were provided by the FinnGen consortium, which included a total of 1,227 cases and 259,583 controls [8]. The GWAS data can be downloaded at Access results | FinnGen.

2.2. Summary Data-Based Mendelian Randomization (SMR)

Zhu et al. [4]proposed a SMR method to evaluate the likely causal relationship between gene expression and traits, which was undertaken with *cis*-eQTL as the IVs. We performed SMR analysis based on the default setting of SMR ($P_{eQTL} < 5\times10^{-8}$, minor allele frequency [MAF] > 0.01, excluding SNPs in very strong linkage disequilibrium [LD, $r^2 > 0.9$] and in low LD or not in LD [$r^2 < 0.05$] with the top associated eQTL). $P_{HEIDI} > 0.05$, which means a gene having "pleiotropy" effects (no heterogeneity)[4].

R version 4.2.1 and SMR software for data cleaning and statistical/bioinformatical analysis.

2.3. Bioinformatical analysis of THBS3 expression

The gene interaction network of THBS3 was established through GeneMANIA (http://genemania.org/) to discover the genes most associated with THBS3. Metascape (https://metascape.org/) was utilized to perform the enrichment analysis. Gene set enrichment analysis (GSEA) was performed based on the overall trend of THBS3 expression levels in GC gene annotation files.

GC gene expression patterns (32 normal / 375 tumor) and clinical data (survival time, survival status, age, sex, grade, stage, and TMN stage) were acquired from The Cancer Genome Atlas (TCGA) database (GDC (cancer.gov)). The pan-cancer analysis of THBS3 was performed through the TIMER2.0 (timer.cistrome.org). Difference analysis and survival analysis were utilized to assess the relationship between THBS3 expression and survival. Clinical correlation analyses were conducted to determine if there were variations in *THBS3* expression among different groups. The GC patients were divided into high- and low-expression groups based on *THBS3* expression levels. The association between the tumor microenvironment (TME) score and the high- and low-expression groups was analyzed. The ESTIMATE algorithm was used to evaluate the stromal and immune scores of each patient, while the CIBERSORT algorithm was employed to determine the content of immune cells in each GC sample. Additionally, the Cancer Immunome Atlas (TCIA, tcia. at) was utilized to predict the patients' susceptibility to immunotherapy.

3. Results

3.1 Study design and data information

The overall design of the study is described in Figure 1. Detailed information on CAGE and GTEx_stomach eQTL data and GC GWAS data is shown in Table 1.

Table 1. The detailed information of the GWAS data and eQTL data.

Data	Total participants or cases/controls	project	website
GWAS	1227/259583	FinnGen	Access results | FinnGen
eQTL_blood	2765	CAGE	SMR | Yang Lab (westlake.edu.cn)
eQTL_stomach	324	GTEx	

GWAS: genome-wide association studies, QTL: quantitative trait loci, CAGE: the Consortium for the Architecture of Gene Expression, GTEx: The Genotype-Tissue project Expression

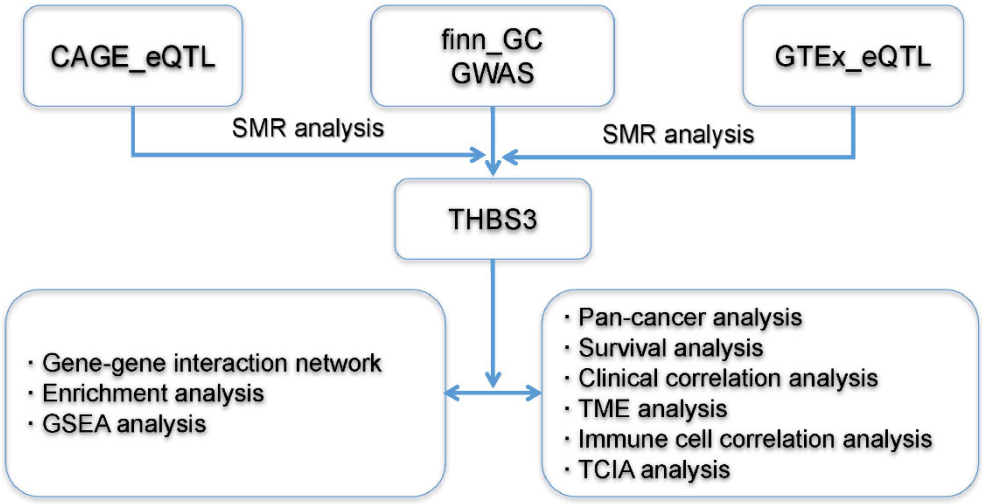

Fig. 1. The overall design of the study. eQTL: expression quantitative trait loci; GC: gastric cancer; SMR: summary data-based Mendelian randomization; GWAS: genome-wide association study; TME: the tumor microenvironment; TCIA: the Cancer Immunome Atlas.

3.2 SMR results

The study identified multiple genes that had a pleiotropic association with GC. Using the *cis*-eQTL data from CAGE, we identified a total of 83 probes (63 genes), one probes were statistically significant after FDR correction. For GTEx_stomach, we identified 44 probes (44 genes), three probes were statistically significant after FDR correction. The results showed that *THBS3* (CAGE: ILMN_1804663, P_{SMR} = 2.20E-07, P_{HEIDI} = 0.061; GTEx_stomach: ENSG00000169231, P_{SMR} = 1.64E-06, P_{HEIDI} = 0.069) had a pleiotropic association with GC in the SMR analysis of two eQTL data (Table 2 and Fig. 2).

Table 2. The top ten probes were identified in the SMR analysis.

Prode_ID	Chr	Gene/Top_SNP	A1/A2	Freq	B_{SMR}	SE_{SMR}	P_{SMR}	P_{HEIDI}	FDR
GTEx_stomach									
ENSG00000167653	8	PSCA/rs2976388	A/G	0.438	0.362	0.061	3.74E-09	0.312	1.51E-05
ENSG00000169231	**1**	**THBS3/rs760077**	**A/T**	**0.397**	**0.795**	**0.166**	**1.64E-06**	**0.069**	0.003
ENSG00000160886	8	LY6K/rs2976388	A/G	0.437	0.597	0.138	1.50E-05	0.137	0.015
CAGE_blood									
ILMN_1804663	**1**	**THBS3/rs760077**	**A/T**	**0.397**	**0.564**	**0.109**	**2.20E-07**	**0.061**	1.87E-03

Chr: chromosome; P_{GWAS}: the *P* value for the top associated *cis*-eQTL in the GWAS analysis; P_{Eqtl}: the *P* value of the top associated *cis*-eQTL in the eQTL analysis; B_{SMR}: the estimated effect size in SMR analysis; SE_{SMR}:

the corresponding standard error; P_{SMR}: the *P* value for SMR analysis; P_{HEIDI}: the *P* value for the HEIDI test; N_{HEIDI} is the number of SNPs involved in the HEIDI test; HEIDI: heterogeneity in dependent instruments; SNP: single-nucleotide polymorphism; SMR: summary data-based Mendelian randomization; QTL: quantitative trait loci. Bold font means it was found in SMR analysis results of more than two eQTL datasets.

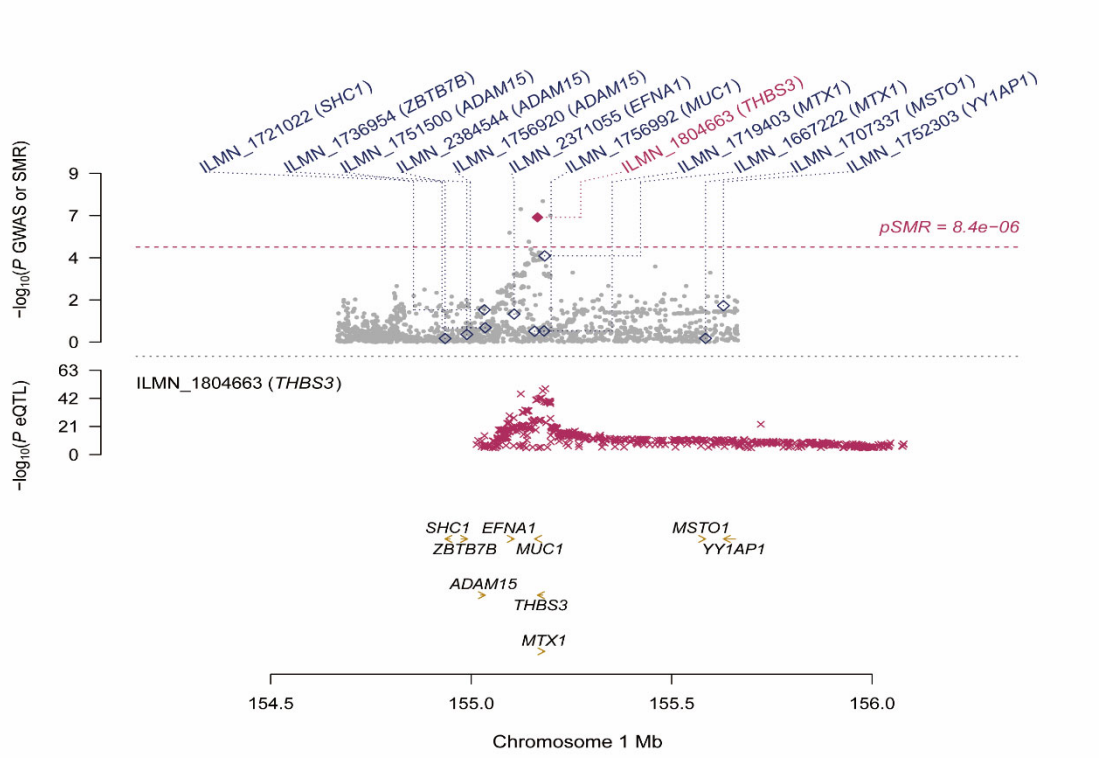

Fig. 2. SMR analysis results in the THBS3 region. The top plot, gray dots indicate the P values of SNPs in GWAS of GC, and the rhombuses indicate the P-values of the SMR test probes. Middle plot, eQTL results for the probe ILMN_1804663 tagging THBS3. Bottom plot, location of genes labeled by the probes. GWAS: genome-wide association study, GC: gastric cancer, SMR: summary data-based Mendelian randomization, HEIDI: heterogeneity in dependent instruments, eQTL: expression quantitative trait loci.

3.3 Enrichment analysis for THBS3 and related genes

We constructed the gene interaction network of *THBS3* through the GeneMANIA website (Fig.3A), *THBS3* was the intersection gene surrounded by 20 nodes of significantly related genes: *THBS4*, *PDGFA*, *PDGFB*, *THBS2*, *FURIN*, *COMP*, *CD36*, *THBS1*, *CD47*, *MRC2*, *APC*, *LTBP4*, *IQSEC2*, *COL5A1*, *P3H3*, *SETDB1*, *SDC3*, *LAMB2*, *COL16A1*, *VASH1*. The results of functional enrichment analysis using Metascape showed that among the top 12 clusters of representative enrichment terms (one per cluster), there were 8 items of GO biological process, as well as 3 items of Reactome Gene Sets and 1 item of WikiPathways (Fig.3B, Supplementary Table S1). To further investigate the relationship between these terms, a subset of enriched terms was selected and created a network plot (Fig.3C). GSEA analysis revealed that calcium signaling pathway, focal adhesion, and neuroactive ligand receptor interaction were up-regulated, while the oxidative phosphorylation and ribosome were down-regulated (Fig.3D).

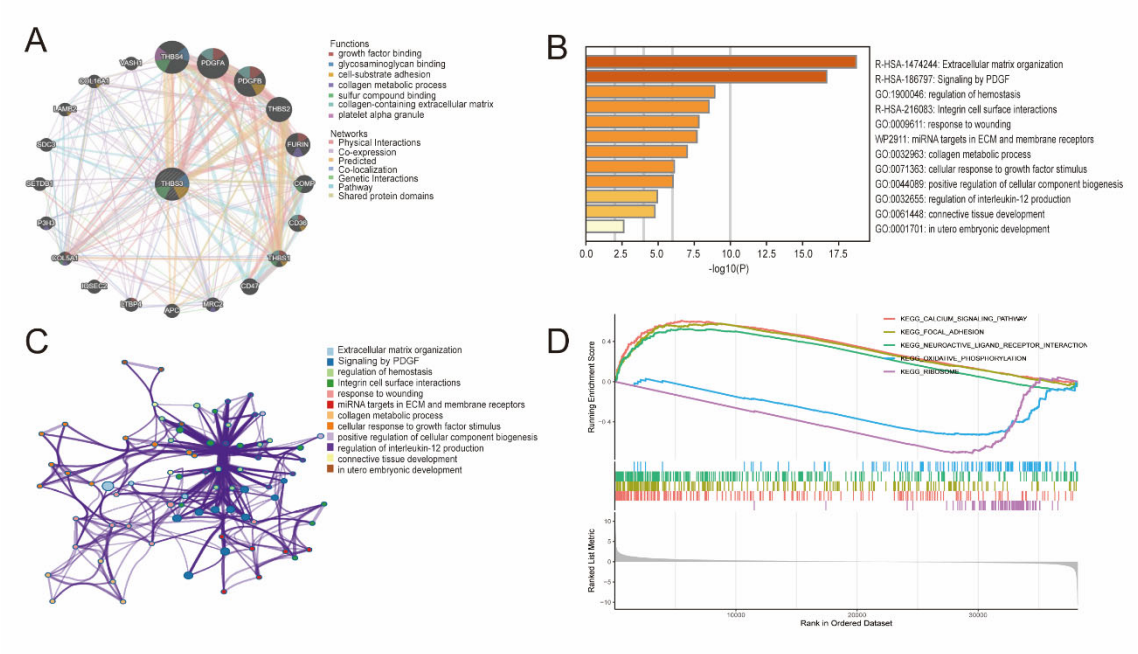

Fig. 3. Enrichment analysis for THBS3 and related genes. (A) The gene–gene interaction network for THBS3. (B) Results of enrichment analysis, colored by P values. (C) Enriched terms network plot. (D) The result of GSEA enrichment analysis.

3.4. Analysis of THBS3 expression

Pan-cancer analysis showed that *THBS3* expression was different in multiple tumors (Fig. 4A, Supplementary Table S2). The results of the differential analysis in GC cohered with those of the pan-cancer analysis. (Fig. 4B). The Kaplan-Meier curve indicated that patients with low *THBS3* expression had a more positive overall survival rate in comparison to patients with high *THBS3* expression (log-rank test, $P = 0.037$; Fig. 4C). We did a correlation analysis in clinical, Chi-square test results showed that there was no obvious difference in *THBS3* expression at age, sex, N stage, and M stage. There are variations in the expression level of *THBS3* across different grades, stages, and T stages. (G2 vs G3: $P = 0.03$; Stage I vs Stage II: $P = 0.0075$, T1 vs T3: $P = 0.032$; T1 vs T4: $P = 0.047$). The results of CIBERSORT revealed a positive correlation between the expression of *THBS3* and Macrophages M2, Mast cells resting, T cells CD4 memory resting, T cells regulatory (Tregs), Monocytes, and a negative correlation with Mast cells activated, Macrophages M1, T cells CD4 memory activated, and Plasma cells (Fig. 4D). In addition, we compared TME score between the high and low expression groups, and the results showed TME scores in the high expression group was higher (Fig.4E). Though the TCIA database, the susceptibility of patients to immunotherapy was predicted: the group with low-expression demonstrated a greater immunophenoscore (IPS) compared to the group with high-expression, which implies that the patients in low-expression group might exhibit higher responsiveness to immune checkpoint inhibitors (Fig. 5A-B).

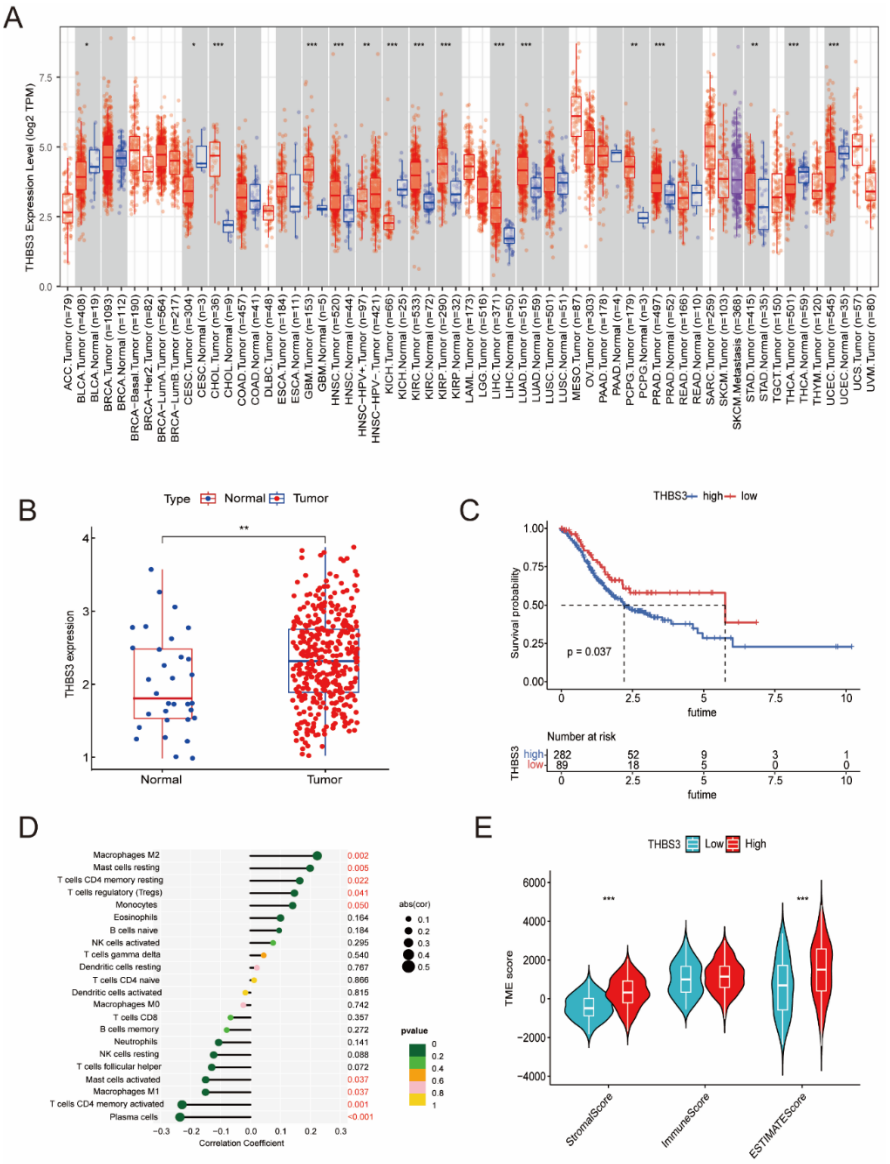

Fig. 4. Analysis of THBS3 expression. (A) The expression of THBS3 in the TIMER2.0 database. (B) Expression distributions of THBS3 between normal and tumor tissue in GC. (C) Kaplan-Meier survival curve of THBS3. (D) Lollipop plot of THBS3 correlation with immune cells. (E) TME scores of GC patients in the low- and high-expression group. GC: gastric cancer; TME: the tumor microenvironment.

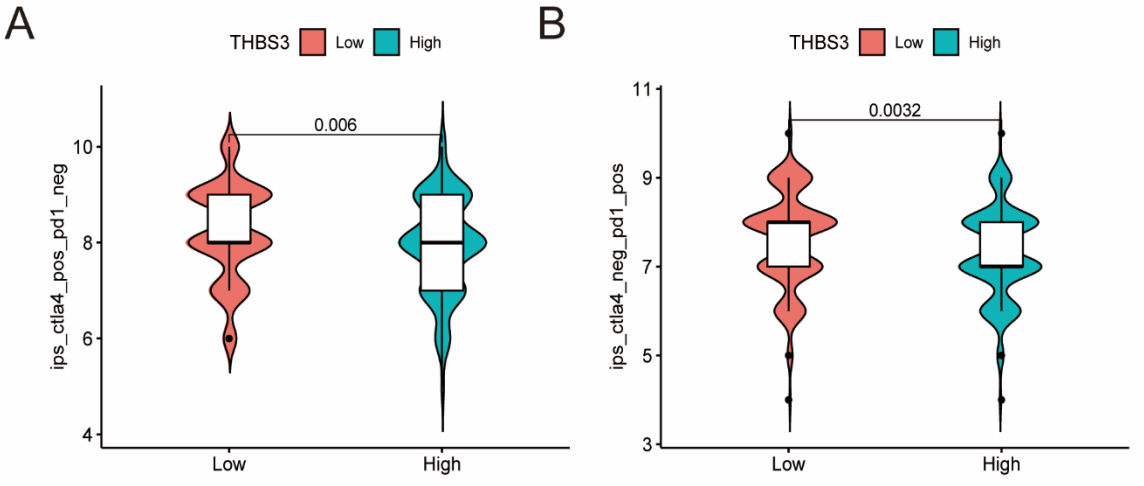

Fig. 5. The prediction of the susceptibility of patients to immunotherapy. IPS of anti-CTLA-4 antibody (A) and anti-PD-1/PD-L1 antibody (B) response in low and high expression groups

4. Discussion

Thrombospondins (THBSs) have a profound influence on the advancement and progression of tumors, such as cellular migration, proliferation, tumor expansion and metastases, angiogenesis, as well as inflammation[9, 10]. THBSs take part in a wide range of biological processes in humans and are differentially expressed in various types of tumors, like GC[11], colorectal[12], clear cell renal cell carcinoma[9], breast cancer[13], and osteosarcoma[14]. Thrombospondin 3 (THBS3) is one of the members of THBSs and participates in cell-cell and cell-matrix interactions and makes a connection to the ECM receptor interaction pathway[15]. Studies have shown that the expression of ECM is up-regulated in prostate cancer tissue and influences tumor invasion and spread of tumors in GC[16, 17], indicating that THBS3 may be associated with the invasion and spread of tumors. The normal functioning of organs, particularly the heart, heavily relies on the crucial link between cells and matrix[18]. The overexpression of THBS3 may disrupt the connection between cells and the matrix, potentially resulting in the development of diseases.

The growth and development of tumors are closely tied to angiogenesis. *THBS3*, due to its strong ability to promote angiogenesis, can act as a contributing factor in tumor progression[19]. Previous studies have indicated that metastatic tissue biopsy samples exhibit elevated levels of THBS3 expression[14], which can sustain the angiogenesis ability triggered by THBS3. Therefore, investigating the expression of THBS3 in GC could be valuable in the development of anti-angiogenic treatment strategies for GC. THBS3 has been relatively little studied in GC and the findings have been inconsistent. Yi Lu, et al.[20] showed that data from different databases had inconsistent results. It is reported that THBS3 is related to the prognosis of GC patients[21]. In our study, results from TIMER2.0 data and TCGA data analysis showed that THBS3 was highly expressed in tumors. Data from more databases may be needed to verify the accuracy of the result.

The cancer development and occurrence are closely related to the tumor microenvironment (TME), which is constituted by various stromal cells, immune cells, and extracellular components[22]. The study showed that THBS3 expression was correlated with a poor prognosis, but positively correlated with both matrix and immune scores and multiple immune cell infiltrations. There could be several reasons. First, The ESTIMATE algorithm may not cover all aspects of the tumor microenvironment. ESTIMATE is a package that aims to forecast tumor purity and identify the existence of stromal and immune cells within tumor tissues based on gene expression data[23]. In addition, studies have shown that immune cells have dual roles[24], on the one hand, they can exhibit an anti-tumor effect; on the other hand, under the influence of the tumor microenvironment, they can undergo a transformation into a pro-tumor phenotype, enabling tumor escape and facilitating tumor progression. Previous studies have demonstrated that M2 macrophages and regulatory T cells are predominantly associated with a poor prognosis in tumors and, it has been found that mast cells also contribute to the promotion of GC[25-28]. Macrophages of the M2 phenotype exhibit immunosuppressive properties and promote cancer progression through the release of pro-oncogenic factors such as vascular endothelial growth factor (VEGF) [29]. Chen et al. demonstrated that Chitinase-3-like protein 1 is segregated by M2 macrophages facilitates the migration of GC and breast cancer cells whether in vitro or in vivo[26]. The expression of THBS3 is positively correlated with M2 macrophages, and both can facilitate the formation of new blood vessels, which accelerates the progression of cancer and leads to poor prognosis.

Multiple studies have demonstrated that mast cells are responsible for producing various pro-angiogenic factors (such as VEGF-A, VEGF-B, and FGF-2), as well as lymphangiogenic factors (VEGF-C and -D)[27]. Although mast cells have been found to play a cancer-promoting role in GC, numerous studies have also indicated that the presence of resting mast cells is associated with poorer

health[30, 31]. Research has suggested that resting mast cells may contribute to biological events through the release of various cytokines[32], but the specific mechanisms are still not fully understood.

Regulatory T (Treg) cells could protect autoimmunity in terms of maintaining self-tolerance, and exhibit an immunosuppressive function within the TME by expressing various inhibitory molecules, which ultimately impairs effective tumor immunity.[25] Tregs could create an immunosuppressive TME through increasing IL-10 expression and decreasing interferon γ (IFN-γ) production and hinder the proliferation and activity of CD8+ T cells through cell-to-cell interaction, which allows GC tumor cells to evade the immune response against tumors, ultimately leading to the development and advancement of GC[33, 34]. Moreover, M2 macrophages could recruit Tregs and promote angiogenesis by secreting chemokines CCL17 and CCL22[25, 28], which may be the reason why THBS3 is positively correlated with Treg cells and affects the progression of GC.

Unfortunately, the study has some limitations that demand further improvement. The analysis was performed using only blood and stomach eQTL data, and more other omics studies are needed to explore GC risk susceptibility genes. The study included only European populations and our findings may not be generalizable to other populations, therefore, more research is needed to verify these findings. Further biological evidence should be provided, which provides further evidence for the potential mechanism of action of *THBS3* expression regulation in GC. The accuracy of the results may be compromised due to the limitations of the SMR method and the presence of high LD.

5. Conclusion

To sum up, the comprehensive study indicates that *THBS3* is a novel GC risk gene whose expression level may be associated with GC risk. Other potential genes that may be associated with GC susceptibility/prognosis were also identified. The outcomes of the research have offered novel insights into comprehending the underlying mechanisms of GC and have presented a catalog of prioritized genes for subsequent functional investigations of GC.

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