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Original Paper

Elevated THBS2, COL1A2, and SPP1 Expression Levels as Predictors of Gastric Cancer Prognosis

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Key Words
Gastric cancer • Tumor invasion • Microarray • *THBS2* • *COL1A2* • *SPP1*

Abstract
Background/Aims: Gastric cancer (GC) is an important health problem. Classification based on molecular subtypes may help to determine the prognosis of patients with GC. Tumor invasion and metastasis are important factors affecting the prognosis of cancer. We aimed to identify genes related to tumor invasion and metastasis, which may serve as indicators of good GC prognosis. ***Methods:*** Tumor tissues and adjacent normal tissues were collected from 105 patients with primary GC who were treated by undergoing radical surgery. Samples were used for tissue microarray analysis. Identified genes with altered expression were further analyzed using the *Gene Ontology (Go)* and *Kyoto Encyclopedia of Genes and Genomes (KEGG)* databases. The expression levels of *THBS2*, *COL1A2* and *SPP1* were analyzed by RT-PCR, western blot and immunohistochemistry. The overall survival curves of patients with high and low expression of each gene of interest were plotted and compared. ***Results:*** Forty-three genes were identified. *THBS2*, *COL1A2* and *SPP1* were selected for further analysis. Altered expression levels of *THBS2*, *COL1A2* and *SPP1* in tumor tissues were confirmed. Patients with low *THBS2* expression had a better prognosis; the expression of *COL1A2* and *SPP1* might not affect the prognosis of patients with GC. ***Conclusion:*** THBS2, but not *COL1A2* and *SPP1*, may serve as an indicator of GC prognosis.

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Introduction

As the fourth most common cancer, gastric cancer (GC) is an important health problem worldwide. An estimated 24,590 new cancer cases and 10,720 GC-related deaths were reported in the United States in 2015 [1]. Globally, approximately 951,600 new stomach cancer cases and 723,100 deaths occurred in 2012 [2]. Developing countries in eastern Asia, eastern Europe and South America suffer from a higher incidence and mortality rate [3]. Moreover, GC accounts for approximately 20% of the global burden of disability-adjusted life-years, taking third place after lung and liver cancers [4]. GC is life-threatening due to its high incidence, mortality rate and impaired quality of life [3].

Chronic *Helicobacter pylori* infection is regarded as one of the primary causes of GC. Owing to the reduction in chronic *H. pylori* infection with improved sanitation and antibiotics, the incidence and mortality rate of GC have decreased in recent years [5]. Epstein-Barr virus is also suspected as a GC-related pathogen. In addition to bacterial and viral causes, approximately 10% of GCs occur with a family history of the disease [6]. Mutations of certain genes and heritable susceptibility to pathogens may play an important role in GC development. Identification of relevant genes in the pathogenesis and progression of GC may elucidate the mechanisms of GC development and help us in clinical practice.

Common symptoms of early stage gastric cancer include nausea, anorexia, dyspepsia, weight loss, and abdominal pain, and these symptoms can easily be confused with other digestive diseases [7]. Thus, patients with GC are often diagnosed at an advanced stage. Once diagnosed, customized treatments based on the molecular subtypes of gastric cancer may help achieve optimized prognosis. For example, patients with a high level of chromosomal instability are more likely to benefit from cisplatin-based neoadjuvant chemotherapy [8]. HER2, an important membrane receptor that regulates cell proliferation and differentiation, is also an indicator of poor prognosis for gastric cancer [9]. However, one study suggested that HER2 might not be a predictor of gastric cancer prognosis [10]. CEA and CA19-9 have also been proposed as prognostic indicators [11, 12]; however, the sensitivity and specificity of CEA and CA19-9 were unsatisfactory. Thus, identifying molecular subtypes that can help indicate the prognosis of gastric cancer is greatly needed.

Tumor invasion and tumor metastasis are important factors affecting the prognosis of cancer. Invasion and metastasis rely on complex mechanisms involving multiple genes and pathways. For example, factors including Twist, Zeb1, Snail, Slug, and BMI1 are implicated in the process of epithelial-mesenchymal transition (EMT), which is essential for the initiation of tumor invasion and metastasis [13, 14]. The large number of potentially involved genes emphasizes the importance of studying multiple gene alterations on a global scale. Microarrays enable us to study the expression status of multiple genes at the same time. In addition, clustering analysis helps us to identify significant pathways and genes for further investigation. In the current study, we used microarray and clustering analyses to identify genes related to tumor invasion and metastasis that may serve as indicators of gastric cancer prognosis.

Material and Methods

Subjects and samples

Samples were collected from 105 patients with primary gastric carcinoma who were treated by radical operation at Wenzhou Seventh People's Hospital from January 2014 to May 2016. For enrolled patients, no chemotherapy, radiotherapy or other drug therapy for cancer had been administered before the surgery. Informed consent was signed by all participants. The study was performed in accordance with the ethical guidelines of the Declaration of Helsinki and approved by the ethics committee of Wenzhou Seventh People's Hospital.

The age of enrolled patients ranged from 37 to 80 years old. Overall, 72 males and 33 females were involved in the study. According to the World Health Organization (WHO) pathologic classification [15], 42

cases were well-differentiated adenocarcinoma, and 63 cases were poorly differentiated adenocarcinoma (undifferentiated carcinoma included). According to the TNM staging system, 19 cases were at stage I, 31 were at stage II, 34 were at stage III, and 16 cases were at stage IV. Tumor tissues were collected during surgery. Pair-matched normal gastric tissues (more than 5 cm away from the tumor) were also collected as controls. All tissues were confirmed pathologically and embedded in paraffin.

Microarray and clustering analysis

Gene expression profiling of 105 paired samples was performed on cDNA microarrays. An Affymetrix Array Platform (Affymetrix, Santa Clara, CA) was used according to the manufacturer's instructions. Briefly, total RNA from paraffin-embedded samples was extracted and transcribed into cDNA. The cDNA was amplified in the presence of biotinylated nucleotides, and the labeled cRNA was fragmented and then hybridized against Affymetrix U133 +2.0. The arrays were scanned using a GeneChip Scanner 3000 and analyzed with the clustering program MeV4.7.1. Identified genes with altered expression were further analyzed using the *Gene Ontology (GO)* and *Kyoto Encyclopedia of Genes* and *Genomes (KEGG)* databases.

Real-time RT-PCR

Real-time RT-PCR was performed to analyze the expression of *THBS2*, *COL1A2* and *SPP1* mRNA based on results from functional analysis. RNA extracted from the last step was transcribed into cDNA with MultiScribe™ Reverse Transcriptase (Applied Biosystems, USA). The quantitative measurement of *THBS2*, *COL1A2* and *SPP1* mRNA levels was performed using an iCycler iQ (Bio Rad, USA). Levels of *THBS2*, *COL1A2* and *SPP1* expression were calculated by the $2^{-\Delta\Delta Ct}$ method, and β-actin was used as the reference. Primers used in the experiment are listed in Table 1.

Western blot analysis

Protein expression levels of *THBS2*, *COL1A2* and *SPP1* were further analyzed by western blot. Briefly, 105 pairs of paraffin-embedded gastric tissues were homogenized, and proteins were purified. BCA assay was used to determine the concentration of the extracted proteins. Loading Buffer (5×) was added to the samples, and samples were boiled at 100°C for 5 min. Then, 30 μg of protein was loaded for electrophoretic separation (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were incubated with 5% fat milk at room temperature for 2 h. Antibodies against *THBS2* (sc-12313, Santa Cruz Biotechnology, CA; 1:1000), *COL1A2* (ab34710, Abcam, UK; 1:1000), *SPP1* (mab10076, Merck Millipore, USA; 1:1000) and β-actin (ab8226, Abcam, UK; 1:800) were subsequently added. Membranes were incubated at 4°C overnight. The next day, the corresponding horseradish peroxidase-labeled secondary antibodies (Vector Laboratories, CA) were added after washing, and the samples were incubated at 37°C for 2 h. The PVDF membranes were then incubated with WesternBright™ ECL Reagent (Advansta, USA) for 10 s and exposed in a dark room. The X-ray film was then developed and fixed.

Immunohistochemistry

Immunohistochemistry was also performed to examine the expression of THBS2, COL1A2 and SPP1. In the current study, immunohistochemical staining was performed with the Dako EnVision System (Dako, USA) according to the manufacturer's instructions. Tissues were deparaffinized and rehydrated. Primary antibodies against THBS2 (sc-12313, Santa Cruz Biotechnology, CA, 1:1000), COL1A2 (ab34710, Abcam, UK; 1:1000) and SPP1 (mab10076, Merck Millipore, USA; 1:1000) were added to the samples and incubated for 10 min. Negative controls were obtained by omission of the primary antibodies, and gastric carcinoma tissues were used as internal controls. Samples were then incubated with HRP-conjugated secondary antibodies (Vector Laboratories, CA) for 10 min followed by incubation with DAB working solution for 15 min.

Table 1. Primer sequences for the 3 genes of interest

cDNA	Forward primer (5'-3')	Reverse primer (5'-3')
THBS2	TTATGGCGTTGCATCCAGGT	GTGGTGCAGAGGAGATGTGT
COL1A2	ATCTTTTTCTGCTTGGCGGT	CAGGGTACTGAATCACGGCA
SPP1	TGGGAATAGCTTTGGGAAGTGG	CCGATGTCCAAAGGTGCAAT

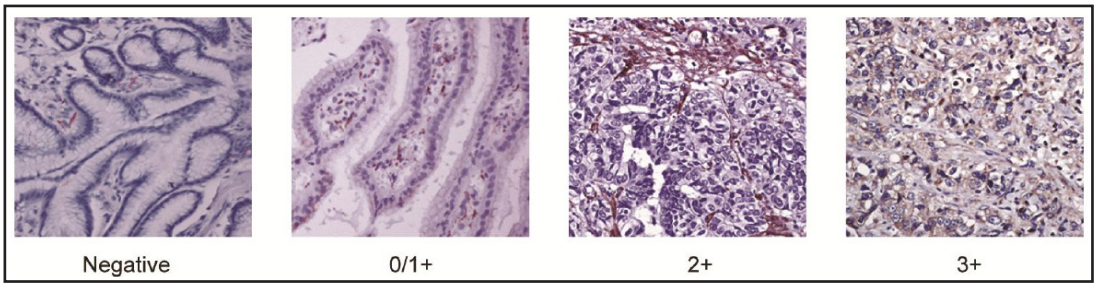

Fig. 1. Examples of immunohistochemical expression of THBS2. Omission of the primary antibodies was considered the negative control. Nearly total absence of immunoreactivity for THBS2 received a score of 0/1+. Moderate expression (less than 50% of the strongest staining) of HTBS2 received a score of 2+. Diffuse immunoreactivity (more than 50% of the strongest staining) received a score of 3+. The grading standards for COL1A2 and SPP1 were similar.

Results of immunohistochemical staining were evaluated in ten fields per section at 40 × 10 magnification of two slides from each specimen to classify samples according to the expression intensities of *THBS2*, *COL1A2* and *SPP1* staining. Two experienced pathologists from Wenzhou Seventh People's Hospital examined the results individually. These pathologists were blinded to the patients' clinical information. As illustrated in Fig. 1, three groups were formed based on categorization of the expression intensity: 0/1+ for negative or weakly positive expression, 2+ for moderate (less than 50% of the strongest) positive expression, and 3+ for strongly (more than 50% of the strongest) positive expression.

Statistical analysis

We used SPSS Statistics 21.0 (IBM, Armonk, NY) software for statistical analysis with one-way ANOVA; $P<0.05$ was considered statistically significant. The overall survival curve was plotted based on the Kaplan-Meier method using GraphPad Prism 6.0. The Wilcoxon method was then applied to compare the differences between the Kaplan-Meier curves.

Results

Hierarchical clustering analysis

In the present study, we identified 43 genes that were significantly upregulated in gastric tumor tissues compared to adjacent normal tissues as illustrated in Fig. 2 (fold change > 4, $P < 0.001$). Clustering analysis based on *GO* indicated that most of the 43 genes were related to digestion, extracellular region, extracellular region part, and extracellular space (Fig. 3A). Similarly, the *KEGG* enrichment analysis results suggested that the identified genes were closely related to ECM-receptor interaction, nitrogen metabolism, collecting duct acid secretion, and gastric acid secretion (Fig. 3B). *THBS2*, *COL1A2* and *SPP1* were identified as important players in ECM-receptor interaction pathways (Fig. 3C).

Expression of THBS2, COL1A2, and SPP1 in tumor and adjacent normal tissues

We applied qRT-PCR to investigate the mRNA expression of *THBS2, COL1A2* and *SPP1* in gastric tumor tissues and corresponding adjacent normal tissues. As presented in Fig. 4A-4C, the expression of *THBS2*, *COL1A2*, and *SPP1* was significantly elevated in tumor tissues compared to normal tissues ($P < 0.0001$). Furthermore, the results of the western blot analysis indicated that the expression of THBS2, COL1A2 and SPP1 was also significantly higher in tumor tissues (Fig. 3D).

The results of immunohistochemistry were consistent with those of RT-PCR and western blot analysis; the statistics are shown in Fig. 5. The results show that 11 (10.5%) of the gastric cancers had a THBS2 expression score of 0/1+, 35 (33.3%) had a score of 2+, and 59 (56.2%) had a score of 3+. Similarly, 14 (13.3%) of the gastric cancers had a Col1A2 expression score of 0/1+, 44 (41.9%) had a score of 2+, and 47 (44.8%) had a score of 3+.

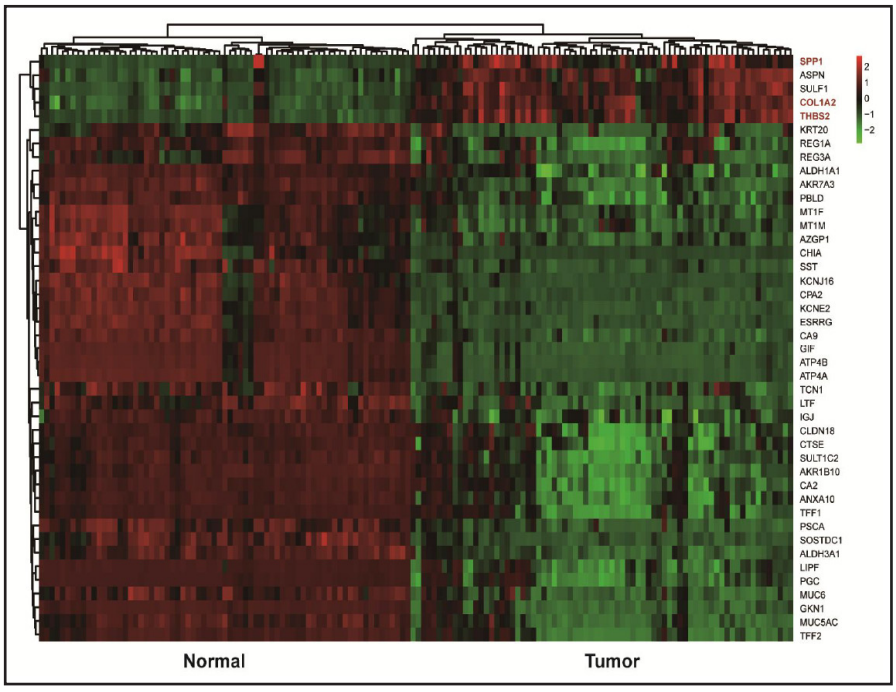

Fig. 2. Unsupervised two-dimensional cluster analysis of mRNA expression in samples from 105 patients. Red suggests high mRNA expression, whereas green suggests low mRNA expression; the genes of interest are highlighted in red.

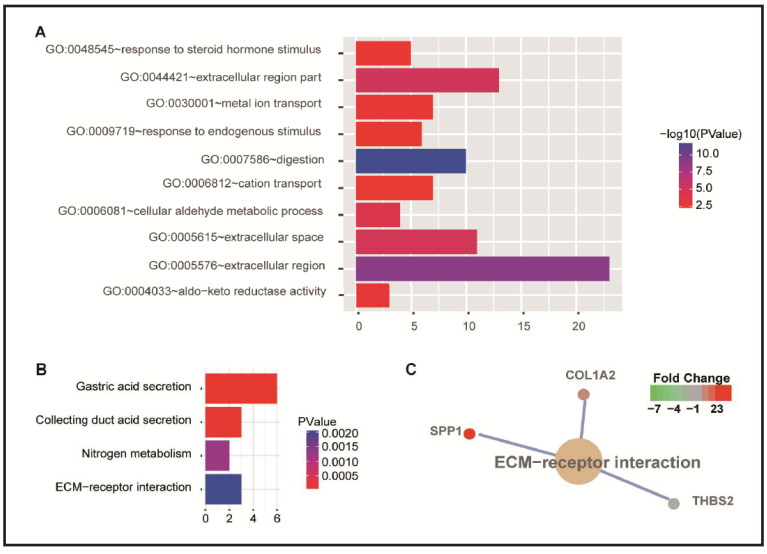

Fig. 3. (A) Gene Ontology analysis according to differential gene screening. (B) KEGG analysis according to differential gene screening. (C) The signaling pathways that involve *THBS2*, *SPP1* and *COL1A2*.

SPP1 was classified as weakly expressed in 7 cases (6.7%), moderately expressed in 29 cases (27.6%) and strongly expressed in 59 cases (56.2%). The expression of THBS2, COL1A2 and SPP1 (in 97, 88 and 92 cases, respectively) in the adjacent tissues had scores of 0/1+. Therefore, the expression of THBS2, COL1A2 and SPP1 was also higher in tumor tissues.

Expression of THBS2, COL1A2, and SPP1 and gastric cancer prognosis

Based on the relative expression of *THBS2*, *COL1A2* and *SPP1*, patients were categorized into either high- or low-expression groups. Subsequently, we generated a Kaplan-Meier plot and compared the overall survival time between each group (Fig. 6). Interestingly, we observed that patients with low *THBS2* expression were associated with a significantly longer survival time (P = 0.00815), whereas the expression levels of *COL1A2* and *SPP1* did not affect the prognosis of patients with gastric cancer (P = 0.05374; P = 0.40115).

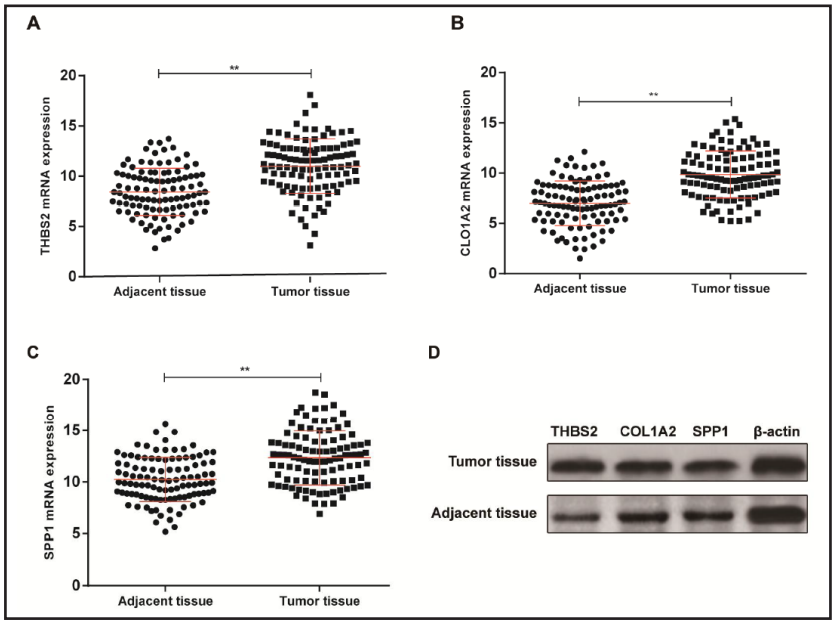

Fig. 4. Real-time quantitative PCR analysis of the mRNA expression levels of *THBS2* (A), *COL1A2* (B) and *SPP1* (C) in tumorous tissues and normal mucosae. The red lines suggest the averages and *suggests a *P* value that is smaller than 0.05. D Western blot results of the three proteins in tumor tissues and adjacent tissues.

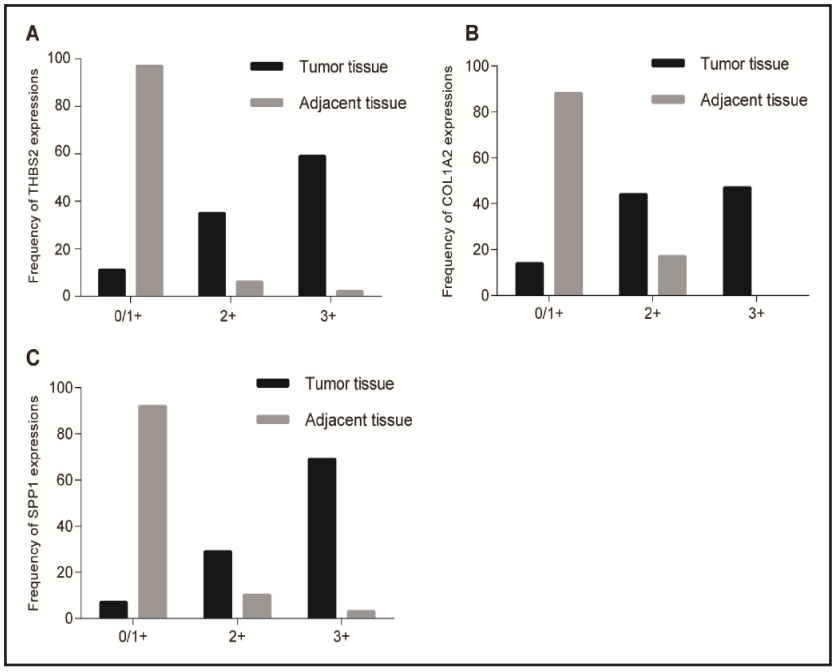

Fig. 5. Numbers of samples with different immunoreactivity that is associated with the expression of THBS2 (A), COL1A2 (B) and SPP1 (C) in gastric tumors and adjacent tissues. The degree of immunoreactivity was classified into three groups: 0/1+, 2+ and 3+ as described previously.

Discussion

In the present study, we identified 43 genes that were significantly over-expressed in 105 tumor samples compared to the corresponding adjacent normal tissues. *THBS2*, *COL1A2* and *SPP1*, which play an important role in ECM-receptor interaction pathways, were selected for further analysis. Elevated expression of *THBS2*, *COL1A2* and *SPP1* in tumor tissues was then confirmed by RT-PCR, western blot and immunohistochemistry. To investigate the association of target gene expression and gastric cancer prognosis, patients were categorized into high- and low-expression groups based on their relative expression levels of *THBS2*, *COL1A2* or *SPP1*. We observed that high *THBS2* expression in patients was related to a better prognosis, whereas the expression levels of *COL1A2* and *SPP1* did not affect the prognosis of patients with gastric cancer.

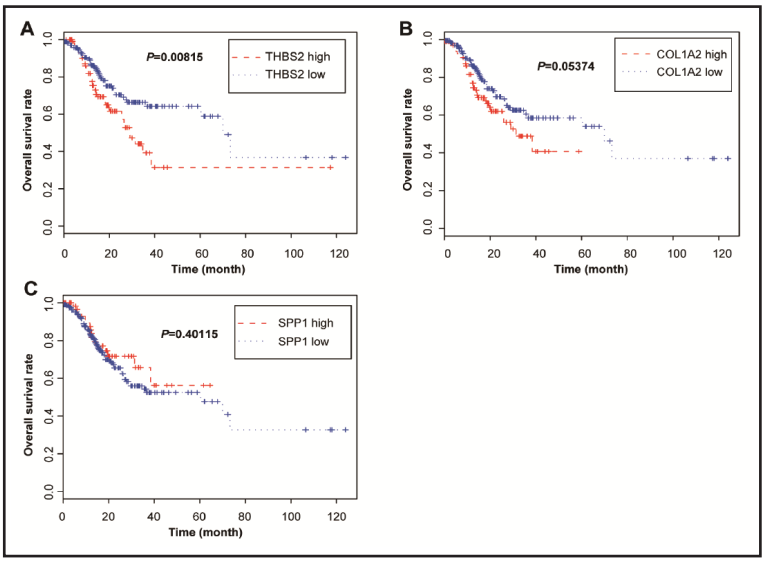

Fig. 6. Kaplan-Meier survival curve with the Wilcoxon test for the overall survival rate of 250 patients with gastric cancer stratified by *THBS2* (Fig. 5A), *COL1A2* (Fig. 5B) and *SPP1* (Fig. 5C) expression. Scores were obtained according to the immunohistochemical results; samples with less than 50% of the strongest intensity were regarded as weakly positive, and samples with more than 50% of the strongest intensity were regarded as strongly positive.

Many efforts have been made to identify molecular subtypes that can help indicate the prognosis of gastric cancer [16, 17]. Our research is distinguished from previous studies in that our sample size was relatively large. Additionally, our selection focused on genes and molecules that may influence the invasion and metastasis of tumors.

Thrombospondin 2 (*THBS2*) belongs to a family of matricellular Ca^{2+}-binding glycoproteins secreted by stromal fibroblasts, endothelial cells and immune cells. *THBS2* can mediate cell-to-cell and cell-to-matrix interactions and is a potent inhibitor of angiogenesis and tumor growth [18]. In this study, we observed that *THBS2* was overexpressed in gastric tumor tissues. The result was in accordance with previous studies, which stressed the critical role of the ECM-receptor interaction pathway in gastric cancer progression [19]. We also proposed THBS2 as a prognostic indicator of gastric cancer, with high THBS2 expression related to poor prognosis. However, Sun et al. reported that aberrant expression of *THBS2* could be a potential predictor of gastric cancer prognosis, i.e., the downregulation of *THBS2* indicated poor prognosis [20]. While Sun et al. used 14 pairs of gastric tumor tissues and adjacent normal tissues in their study, we used a larger number of tissues, which makes our results more convincing. Other than gastric cancer, *THBS2* has also been identified as a prognostic indicator for patients with urothelial carcinomas of the upper urinary tract and bladder [21]. Consistent with our study, in urothelial cancer, *THBS2* overexpression was linked to poor prognosis, namely, disease-specific survival and metastasis-free survival. Nonetheless, we identified THBS2 as a biomarker of good gastric cancer prognosis.

COL1A2 (collagen type I alpha 2 chain) encodes the α2 (I) chain of type I collagen. Type I collagen is the most abundant collagen molecule and is composed of α1 (I) chain and α2 (I) chain. The structural integrity of type I collagen is critical for tissue homeostasis. However, unbalanced expression of the α1 (I) chain and α2 (I) chain impairs the function of type I collagen. We observed in our results that the expression of *COL1A2* was significantly elevated in gastric tumor tissues. In previous research, elevated *COL1A2* expression has also been found in many different types of cancers, including breast cancer [22], head and neck cancer [4], and colorectal cancer [23]. We failed to observe the prognostic value of *COL1A2* in the current analysis, whereas Misawa et al. reported that the *COL1A2* gene might serve as an important biomarker in head and neck squamous cell carcinoma [4]. Controversy regarding the role of *COL1A2* in various cancers exists, and we did not identify *COL1A2* as a prognostic indicator of gastric cancer in this study.

The protein encoded by *SPP1* (secreted phosphoprotein 1, also called osteopontin) helps osteoclasts to bind to the mineralized bone matrix, which has been demonstrated to be used to assist in clinical diagnosis of Parkinson's disease [24]. This protein also plays an

important role in tumorigenesis, invasion and metastasis [25]. Upregulated *SPP1* expression had been confirmed in various types of cancers [26, 27]. A study based on gastric cancer cell lines indicated that the elevated expression of *SPP1* is a critical determinant of poor prognosis and that OPN expression could help to predict the risk of hematogenous metastasis [28]. In addition, in a recent study, *SPP1* was observed to be a novel prognostic marker for clear cell renal cell carcinoma [29]. Previous research concerning esophageal squamous cell carcinoma has also suggested that the elevated expression of SPP1 in tumor tissues was associated with poor prognosis [30].

In conclusion, in the current study, we identified 43 genes with altered expression in gastric cancer by analyzing 105 pairs of tumor tissues and corresponding adjacent normal tissues. *THBS2*, *COL1A2* and *SPP1* were selected as target genes that are overexpressed in gastric tumors. We observed that patients with low *THBS2* expression had a better prognosis, whereas *COL1A2* and *SPP1* expression levels might not affect the prognosis of patients with gastric cancer.

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Disclosure Statement

The authors have no conflicts of interest.

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