

Cancer-associated Fibroblast-derived Spondin-2 Promotes Motility of Gastric Cancer Cells

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Abstract. *Background/Aim: Peritoneal dissemination (PD) occurs frequently in gastric cancer (GC) and is fatal. The interactions between tumor cells and stromal cells are critical for cancer progression. Our aim was to identify a novel PD-associated gene derived from stromal cells in GC. Materials and Methods: Among the candidate PD-associated genes identified in our previous study, we focused on spondin-2 (SPON2), an extracellular matrix-secreted protein. Clinicopathological and prognostic analyses of SPON2 mRNA expression were performed using GC datasets. Localization of SPON2 expression was assessed by immunohistochemistry. In vitro migration assay and immunofluorescence staining were also conducted using GC cell lines. Results: SPON2 was expressed in and secreted from cancer-associated fibroblasts in GC. High expression of SPON2 in tumor tissues was correlated with PD, tumor size and poor prognosis in GC. The motility of GC cells was increased by treatment with a SPON2 recombinant protein in vitro. Conclusion: Cancer-associated fibroblast-derived SPON2 may promote PD, in part, by facilitating GC cell motility and serve as a predictive marker for PD in GC.*

Gastric cancer (GC) is a common malignancy and a leading cause of cancer-related death worldwide, especially in East Asia (1). The malignant potential of GC is characterized by peritoneal dissemination (PD), defined as disseminating tumor cells throughout the peritoneal cavity. In a clinical study, PD is diagnosed in 14% of GC patients at the initial examination, and the survival time of such patients is only 1 year, although multimodal therapies have improved the clinical outcome of GC patients with PD (2-7). Therefore, identification of biomarkers and therapeutic targets for PD is warranted to improve the prognosis of GC patients.

Recent *in silico* analyses using large-scale public datasets such as The Cancer Genome Atlas (TCGA) have contributed to identifying candidate driver genes responsible for metastasis or poor prognosis. We previously identified *DDR2*, *ARLAC* and *FGFR1* as PD-related driver genes of GC cells (8-10). However, recent cancer research has shed light on the interactions between cancer cells and various stromal cells, including fibroblasts, endothelial cells and immune cells in the tumor microenvironment (TME) (11-15).

The TME is believed to promote cancer cell invasion, followed by metastasis. We recently reported that *KIF15*, which is expressed in host inflammatory monocytes, facilitates the proliferation of tumor cells in hepatocellular carcinoma (16). Thus, we hypothesized that stromal cells in the TME contribute to PD of cancer cells in GC. In this study, we demonstrated that spondin-2 (SPON2), an extracellular matrix-secreted protein, is a novel PD-associated gene derived from stromal cells in the TME and determined its clinical and biological significance in GC.

Materials and Methods

TCGA dataset. We obtained mRNA expression and clinical data from 406 GC patients from the Broad Institute's Firehose

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(http://gdac.broadinstitute.org/runs/stddata__2015_06_01/data/STAD/20150601/). The mRNA expression data (FPKM, raw count) from 406 tumor tissues and 31 normal tissues were subjected to quantile normalization. Clinical data were available from 375 patients.

GSE15459 dataset. GSE15459 dataset was downloaded from the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>). This dataset consists of mRNA expression and clinical data from 198 GC patients in Singapore but contains no mRNA expression data from normal tissues. The mRNA expression data were subjected to quantile normalization. Because PD data, as well as clinical data, were available from 174 of these patients, we used the data from these patients to identify PD-associated genes. Of the 174 patients, there were 32 with PD (peritoneal nodules or positive cytology) and 142 without PD.

Beppu dataset. Cancer tissues were obtained from 170 GC patients who underwent gastrectomy at the Kyushu University Beppu Hospital and affiliated hospitals from 1995 to 2009 after providing written informed consent. Of these patients, 47 were excluded because of either loss to follow-up or poor sample quality. Thus, we enrolled 123 GC patients in our survival analysis. The median follow-up period of these patients were 586 days. Paired tumor and normal tissues were obtained from 98 GC patients. All data associated with the samples including age, sex, tumor size, depth of tumor invasion and lymph node metastasis were obtained from clinical records. We treated the patients according to the Japanese Gastric Cancer Treatment Guidelines. No patients received neoadjuvant systemic therapy.

The tumor and normal tissues were placed in RNAlater (Takara Bio Inc, Shiga, JAPAN), frozen in liquid nitrogen and stored at -80°C . This study was approved by the Ethics and Indications Committee of Kyushu University (#2020-302).

Cell culture. The human GC cell lines AGS, KE-39, MKN1, MKN7, MKN28, MKN45, MKN74 and NUGC3 and the human fibroblast cell line KMST6 were purchased from the Japanese Collection of Research Bioresources Cell Bank, National Institutes of Biomedical Innovation, Health and Nutrition (Osaka, Japan). The human GC cell lines NUGC4, SH10TC and KLN205 were purchased from RIKEN BioResource Center (Tsukuba, Japan). The human leukemia cell lines Jurkat, Raji and THP1 were purchased from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). Two human GC-associated fibroblast (CAF) lines (CAF-100 and CAF-102) derived from surgical gastric tissue were established at Osaka City University as described previously (17).

AGS, KE-39, MKN1, MKN7, MKN28, MKN45, MKN74, NUGC3, NUGC4, SH10TC, KLN205, Jurkat, Raji and THP1 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium, and KMST6 cells were maintained in Dulbecco's Modified Eagle's Medium. All media contained 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin sulfate. CAF-100 and CAF-102 were maintained in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin sulfate and 10% glucose. All cells were cultured in a humidified 5% CO_2 incubator at 37°C .

Total RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from

cell lines and tissues using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. 8 μg total RNA was reverse transcribed using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qPCR analysis of SPON2 and 18S was performed using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Indianapolis, IN, USA) as described previously (18). The mRNA level of SPON2 was normalized to that of 18S, as an internal control, and expressed relative to the level of the cDNA from human universal reference total RNA (Takara Bio Inc, Shiga, Japan).

Immunohistochemical analysis. Immunohistochemical analysis of SPON2 and αSMA was performed using formalin-fixed paraffin-embedded surgical sections obtained from patients with GC, as described previously (19). A primary rabbit polyclonal antibody against SPON2 (HPA040170; Sigma Aldrich, St. Louis, MO, USA) was used at a dilution of 1:100, and a mouse monoclonal antibody against αSMA (MA5-11544, Thermo Fisher Scientific, Waltham, MA, USA) was used at a dilution of 1:100. Histological analysis was performed independently by an experienced research pathologist (TT) at Kyushu University Beppu Hospital.

Western blot analysis. Western blot analysis was performed as previously described (8). Cell lysates were mixed with a $10\times$ volume of 10% trichloroacetic acid in acetone, incubated for 1 h at -20°C , and centrifuged at 14,000 rpm for 5 min at 4°C . Precipitates were washed in ice-cold acetone, air-dried at room temperature, and dissolved in lysis buffer. The following specific antibodies were used: primary rabbit polyclonal antibody against SPON2 (HPA040170; Sigma Aldrich) at a dilution of 1:250 and primary mouse polyclonal antibody against β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:10,000. The protein expression of SPON2 was normalized to that of β -actin.

Gene set enrichment analysis (GSEA). The associations between SPON2 expression and previously defined gene sets were analyzed by GSEA using GC expression profiles from TCGA dataset. Biologically defined gene sets were obtained from the Molecular Signatures Database v5.2 (<http://software.broadinstitute.org/gsea/msigdb/index.jsp>).

SPON2 recombinant protein (rSPON2). SPON2 recombinant protein was purchased from Sigma-Aldrich (HPA043890).

Wound healing migration assay. For the wound healing migration assay, 3×10^5 cells were plated per well of a 6-well plate and treated with rSPON2 and control ddH₂O in triplicate. After the cells reached 80-90% confluence, vertical scratches were generated in the cell layer using a 1,000 μl pipette tip. Photographs of the wound area were taken at 0, 96 and 196 h after scratch formation to calculate the cell migration rate.

Immunofluorescence analysis. The actin stain 488 phalloidin (Cytoskeleton, Denver, CO, USA) was used to stain actin filaments in AGS and KE39 cells treated with rSPON2, and fluorescent images were observed using a fluorescence microscope (BZ-X700, Keyence, Osaka, JAPAN). We counted the number of cells containing lamellipodia.

Statistical analysis. GC was staged in all patients according to the 7th edition of the Union for International Cancer Control TNM

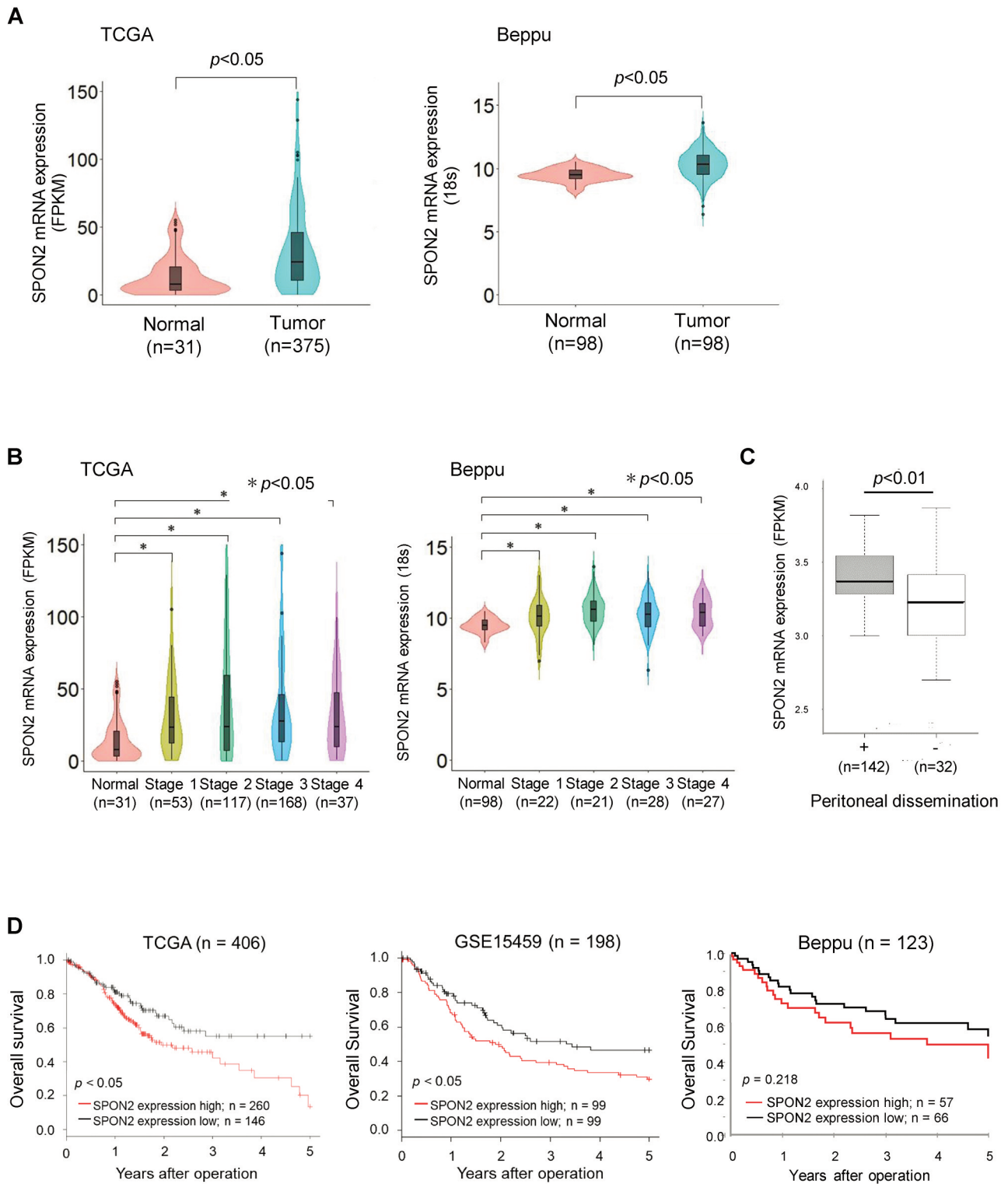


Figure 1. Spodin2 was identified as a PD-associated candidate gene in gastric cancer. (A) SPON2 mRNA expression in tumor and normal tissues from gastric cancer patients. Left: TCGA dataset. Right: Beppu dataset. (B) SPON2 mRNA expression in tumor tissues in gastric cancer patients according to TNM stage. Left: TCGA dataset. Right: Beppu dataset. (C) Comparison of SPON2 mRNA expression in GC patients between with and without PD in GSE15459 dataset. (D) Kaplan–Meier curves of overall survival in gastric cancer patients according to SPON2 mRNA expression. Left: TCGA dataset. Middle: GSE15459 dataset. Right: Beppu dataset.

classification. For the clinical analyses, the patients were divided into two groups based on the SPON2 expression level using the minimum *p*-value approach to identify the optimal SPON2 expression value for stratifying the patients to evaluate survival (20). Associations between the variables were assessed using Student's *t*-test or Fisher's exact test. The degree of linearity was estimated using Pearson's correlation coefficient. OS was estimated using the Kaplan–Meier method, and survival curves were compared using the log-rank test. A two-sided *p*<0.05 was considered significant. Data analyses were performed using R software v3.1.1 (The R Foundation) and JMP Pro 15 software (SAS Institute, Cary, NC, USA).

Results

SPON2 is a candidate PD-associated gene in GC. In our previous study, we identified candidate GC PD-associated genes that satisfied the following three criteria: 1) overexpression in GC tissues with PD compared to GC tissues without PD in the GSE15459 dataset, 2) overexpression in GC tissues compared to normal tissues in TCGA datasets, and 3) high gene expression identified as a poor prognostic factor in GC patients according to a TCGA dataset (9). *SPON2* was among these candidate genes. *SPON2* encodes an extracellular matrix-secreted protein that has multiple biological functions in processes including neuronal development and innate immune response (21). *SPON2* was reported to promote invasion, which is important for PD (22).

Figure 1 shows the verification of *SPON2* as a PD-associated gene in GC. The expression of *SPON2* mRNA was significantly higher in GC tissues than in normal tissues in TCGA and Beppu datasets (Student's *t*-test, *p*<0.05) (Figure 1A). However, there was no statistical difference in *SPON2* mRNA expression according to TNM stage in those datasets (Figure 1B). Notably, the expression of *SPON2* mRNA was higher in GC tissues with PD than in those without PD in the GSE15459 dataset (*p*<0.01) (Figure 1C).

The prognostic significance of *SPON2* mRNA expression in these three GC datasets is shown in Figure 1D. The trends indicated that high *SPON2* expression in GC tissues was associated with a poorer prognosis. A statistically significant difference in OS according to *SPON2* expression was noted in TCGA and GSE15459 datasets (log-rank test, *p*<0.05).

SPON2 is expressed mainly in CAFs in GC. We measured *SPON2* mRNA expression by RT-qPCR in various cancer cell lines, including fibroblasts, lymphoblasts, muscle cells and malignant cells (fibroblast lines: CAF-100, CAF102 and KMST6; GC cell lines: MKN1, MKN7, MKN28, MKN45, MKN74, NUGC3, NUGC4, AGS, SH10TC and KLN205; leukemia and lymphoma cell lines: Jurkat, Raji and THP-1). Interestingly, *SPON2* mRNA expression was highest in the two CAF cell lines among all cell lines evaluated (Figure 2A, Student's *t*-test, *p*<0.05).

Table I. *SPON2* mRNA expression and clinicopathological factors in Beppu dataset (n=123).

Factors	High <i>SPON2</i> mRNA expression (n=57)	Low <i>SPON2</i> mRNA expression (n=66)	<i>p</i> -Value
	Number (%)	Number (%)	
Age (years)			0.852
<65	21 (36.8)	23 (34.8)	
≥5	36 (63.2)	43 (65.2)	
Gender			0.708
Male	36 (63.2)	44 (66.7)	
Female	21 (36.8)	22 (33.3)	
Histology			0.857
Intestinal type	26 (45.6)	32 (48.5)	
Diffuse type	31 (54.4)	34 (51.5)	
Tumor size (cm)			0.016**
≤3	7 (12.3)	21 (31.8)	
<3	49 (86.0)	44 (66.7)	
Unknown	1 (1.7)	1 (1.5)	
Depth of invasion (muscularis propria invasion)			0.053
Absent	8 (14.0)	19 (28.8)	
Present	49 (86.0)	47 (71.2)	
Lymphatic involvement			1.000
Absent	21 (36.8)	25 (37.9)	
Present	36 (63.2)	41 (62.1)	
Vascular involvement			0.844
Absent	41 (71.9)	46 (69.7)	
Present	16 (28.1)	20 (30.3)	
Lymph node metastasis			0.338
Absent	16 (28.1)	25 (37.9)	
Present	41 (71.9)	41 (62.1)	
Peritoneal dissemination			0.282
Absent	48 (84.2)	60 (90.9)	
Present	9 (15.8)	6 (9.1)	
Distant metastasis*			0.120
Absent	41 (71.9)	56 (84.8)	
Present	16 (28.1)	10 (15.2)	

*including peritoneal dissemination; **statistically significant.

Next, we analyzed the correlations between the mRNA level of *SPON2* and those of CAF markers (smooth muscle alpha-actin 2, fibroblast activation protein alpha, periostin, desmin, vimentin, tenascin C, platelet-derived growth factor subunit B and S100 calcium binding protein A4) using a TCGA dataset (12). *SPON2* mRNA expression was positively correlated with the mRNA expression of all CAF markers (Figure 2B, Spearman's correlation coefficient, R=0.24-0.68, *p*<0.01).

Of note, immunohistochemical analysis indicated that *SPON2* was expressed mainly in αSMA-positive fibroblasts, namely CAFs, in GC tissue (12) (Figure 2C). Furthermore, secreted *SPON2* protein was detected in the supernatants of the CAF cultures (Figure 2D). Taken together, our results

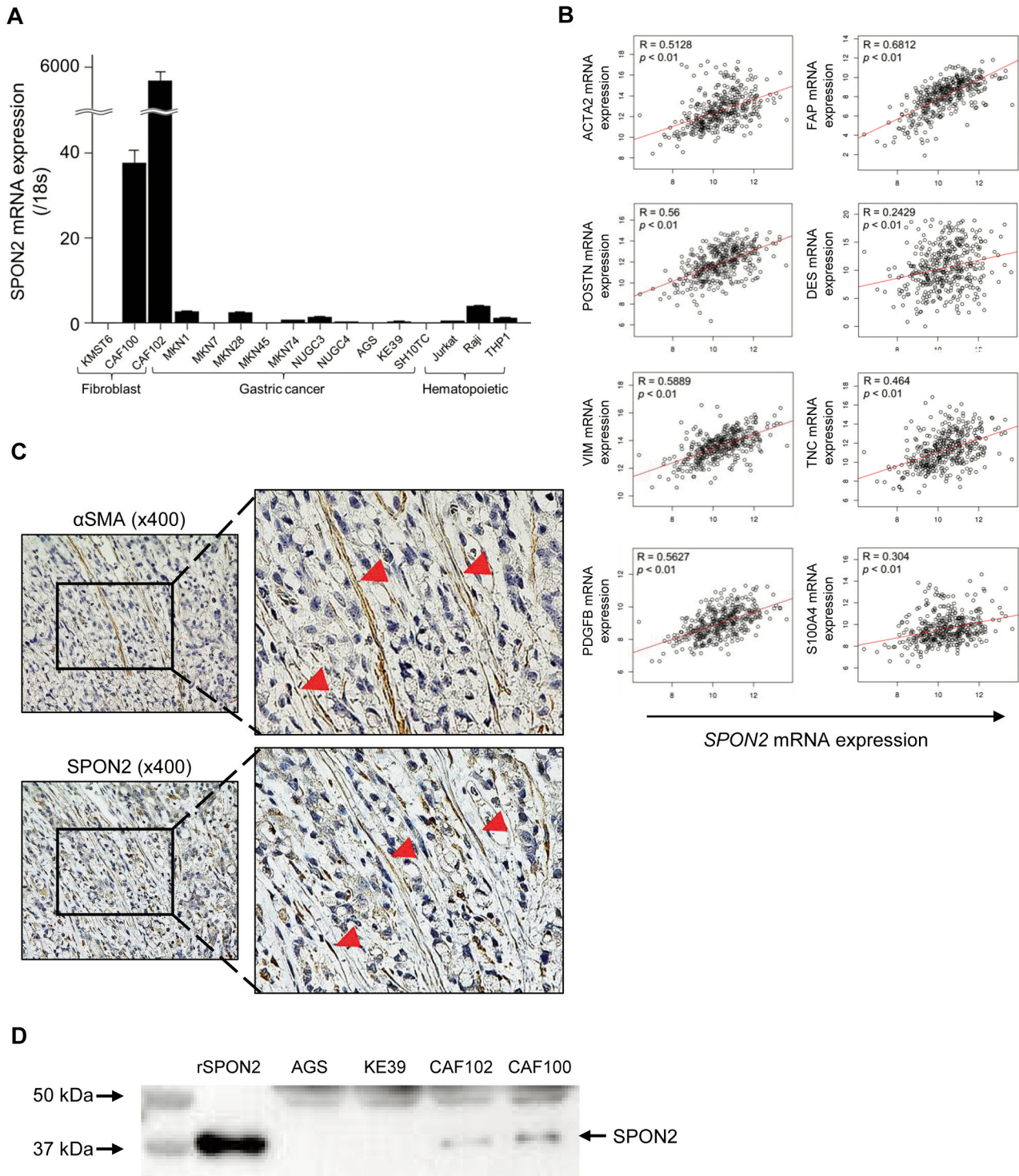


Figure 2. *SPON2* is expressed in cancer-associated fibroblasts in gastric cancer tissues. (A) Comparison of *SPON2* mRNA expression among fibroblast, gastric cancer and hematopoietic cell lines. *SPON2* mRNA was highly expressed in cancer-associated fibroblasts. (B) Correlations between mRNA levels of *SPON2* and those of cancer-associated fibroblast markers in gastric cancer patients from the TCGA dataset. The cancer-associated fibroblasts markers were smooth muscle alpha-actin 2, fibroblast activation protein alpha, periostin, desmin, vimentin, tenascin C, platelet-derived growth factor subunit B and S100 calcium binding protein A4. (C) Immunohistochemical staining of *SPON2* in representative gastric cancer tissues. Original magnification, $\times 200$ and $\times 400$. (D) *SPON2* protein expression in the supernatant of cancer-associated fibroblast lines by western blotting.

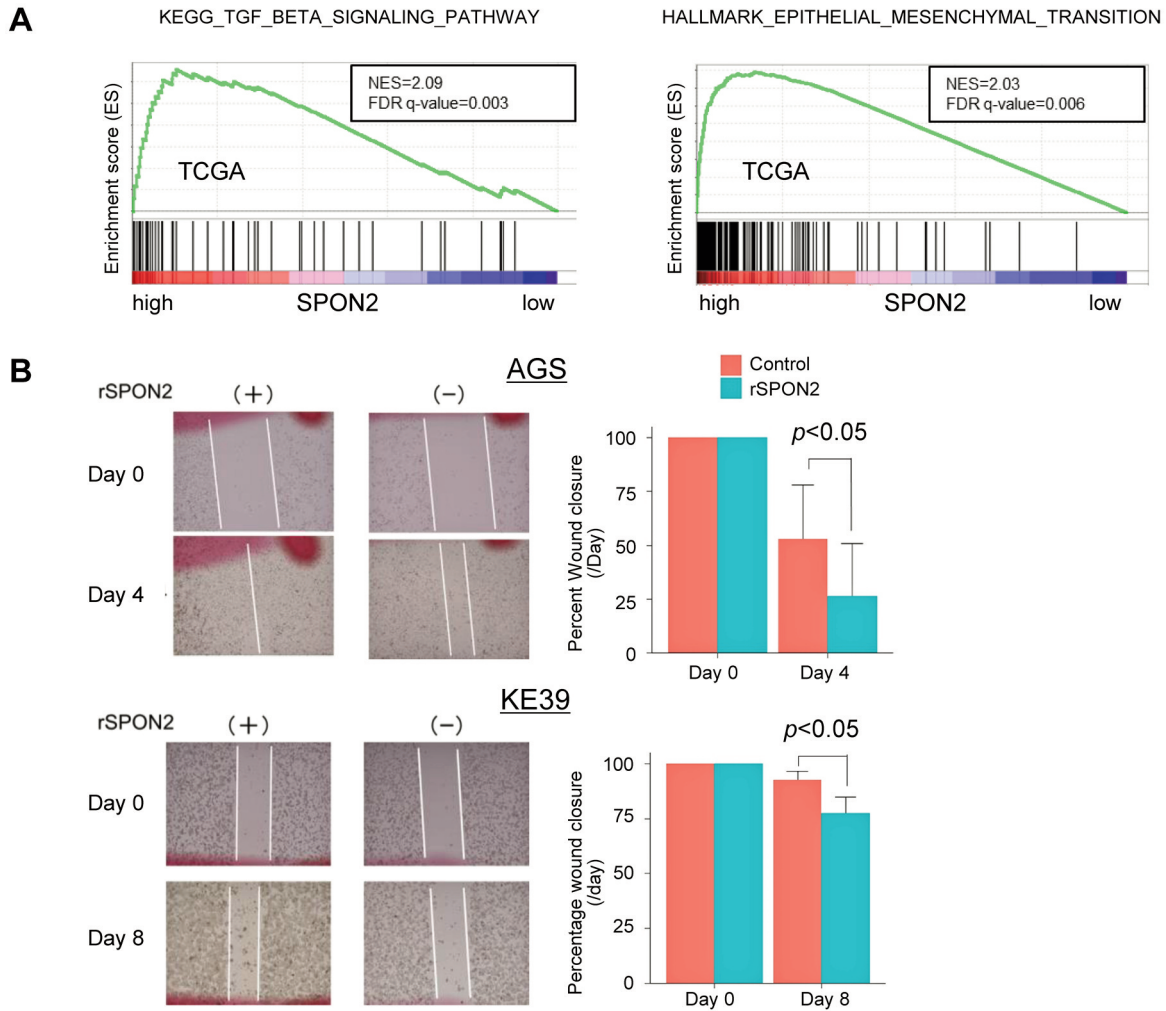


Figure 3. Continued

provide evidence that SPON2 is expressed in and secreted from CAFs in GC tissues.

SPON2 mRNA expression has clinicopathological significance in GC patients. As shown in Table I, high SPON2 mRNA expression in GC tissues was positively associated with tumor size (Fisher’s exact test, $p < 0.05$). Although the rate of PD positivity was higher in GC tissues with high SPON2 expression than in those with low expression (15.8% vs. 9.1%), the difference was not significant, which may be explained by the small number of GC patients with PD in Beppu dataset.

SPON2 promotes the motility of GC cells in vitro. To investigate the effect of SPON2 expression on PD, we performed GSEA using TCGA datasets. GSEA showed that high SPON2 expression was positively correlated with

transforming growth factor β (TGF- β) and epithelial-mesenchymal transition (EMT) gene sets (Figure 3A). An increased level of TGF- β in the TME can induce tissue-resident fibroblasts to acquire a CAF phenotype, such as a high migration ability, which is associated with up-regulation of α -SMA (23). EMT is a critical switch for tumor cell invasiveness, leading to PD (24). Therefore, we focused on the role of SPON2 in migration in GC and performed *in vitro* experiments to elucidate these mechanisms. We performed a wound healing migration assay using rSPON2 in AGS and KE39 cells. rSPON2 enhanced the migration capacity of both AGS and KE39 cells (Student’s *t*-test, $p < 0.05$, Figure 3B).

A line of evidence suggests that SPON2 expression induces actin cytoskeleton remodeling, such as pseudopodium formation, a process that is important for the motility of tumor cells (25). To examine the role of SPON2 expression in GC

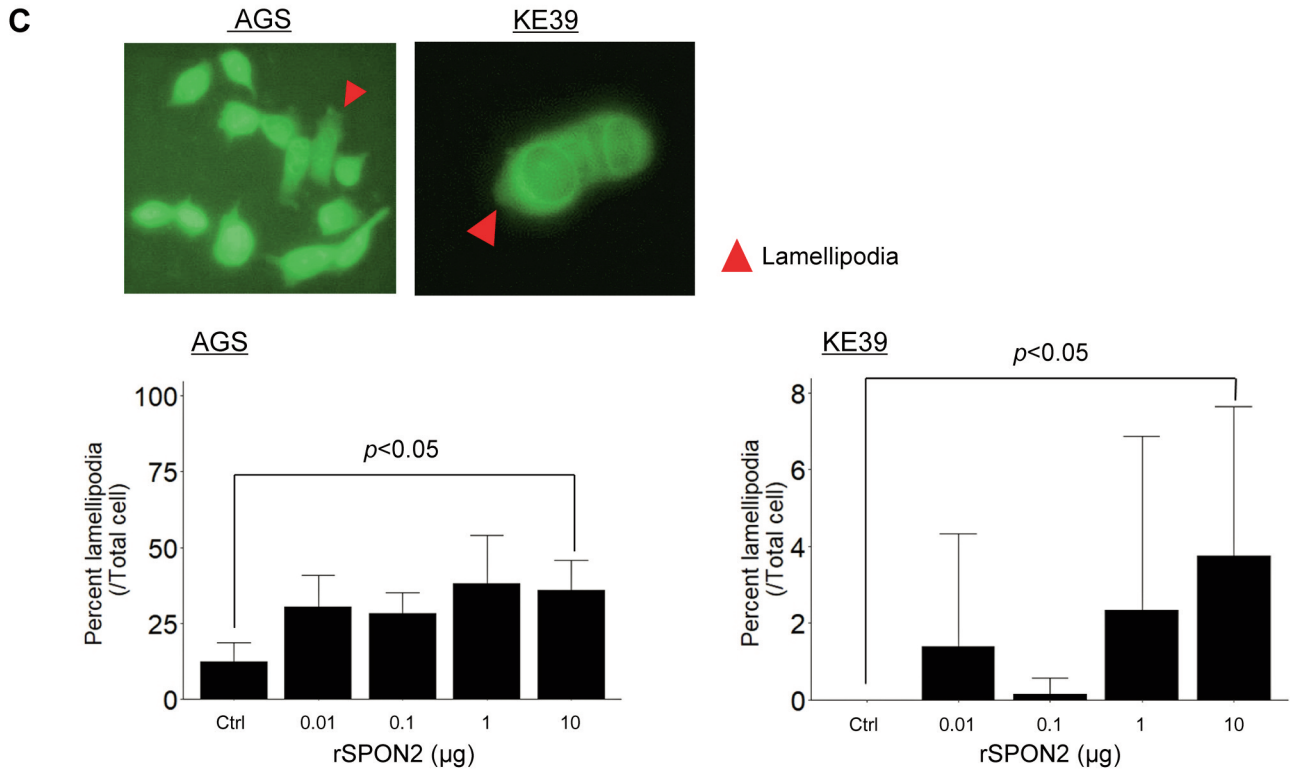


Figure 3. *SPON2* can promote the motility of gastric cancer cells. (A) Gene set enrichment analysis (GSEA) of gastric cancer cases using the TCGA dataset. (B) Wound healing migration assay using gastric cancer cells treated with *SPON2* recombinant protein (rSPON2). rSPON2 enhanced the migration capacity of both AGS and KE39 cells (Student's *t*-test, $p < 0.05$). (C) Immunofluorescence staining of actin filaments with phalloidin of gastric cancer cells treated with *SPON2* recombinant protein (rSPON2). Arrow: lamellipodia. The number of gastric cancer cells with formation of lamellipodia was higher in AGS and KE39 cells treated with rSPON2 than in the control cells (Student's *t*-test, $p < 0.05$).

cells in actin cytoskeleton remodeling, immunofluorescence staining of actin filaments was performed in AGS and KE39 cells. The number of cells with formation of lamellipodia, which are thin, sheet-like membrane protrusions found along the leading edge of motile cells (26), was significantly higher in AGS and KE39 cells treated with rSPON2 than in the control cells (Student's *t*-test, $p < 0.05$, Figure 3C).

Discussion

Herein, we identified *SPON2* as a possible PD-associated gene, and we showed that *SPON2* is expressed in CAFs in the TME by, in part, promoting the migration of GC cells. Thus, the expression of *SPON2* mRNA in tumor tissues may be a biomarker predicting a poor prognosis of GC patients. CAFs contribute to cancer progression by expressing various factors (*e.g.*, cytokines, chemokines and growth factors such as TGF- β , Interleukin-22, vascular endothelial growth factor and hepatocyte growth factor) that regulate the deposition of extracellular matrix, metabolic reprogramming and tumor cell proliferation, thereby promoting EMT, angiogenesis and

chemotherapy resistance in various malignancies including GC (13, 27, 28). Remarkably, we found that *SPON2* was expressed in and secreted from CAFs, and that *SPON2* expression was positively associated with the TGF- β signaling pathway and EMT according to the GSEA, suggesting that *SPON2* affects tumor progression in GC.

SPON2 (also known as mindin) is a member of the mindin-F-spondin family of extracellular matrix proteins. *Drosophila melanogaster* M-spondin, zebrafish mindin 1 and 2 and rat F-spondin and mindin are included in this family (21). A previous study reported that *SPON2*- $\alpha 4\beta 1$ integrin signaling activated RhoA and Rac1, increased reorganization of F-actin and facilitated recruitment of M1-macrophage (25), suggesting that *SPON2* enhances tumor cell motility by activating RhoA and Rac1 signaling. Herein, we showed that *SPON2* enhanced the migration potential and lamellipodia formation of GC cells *in vitro*. The clinicopathological analysis indicated that high *SPON2* expression in GC tissues is positively correlated with tumor size. Furthermore, high expression of *SPON2* was associated with a poor prognosis in GC patients. Several lines of evidence also suggest that high expression of *SPON2* in

tumor cells is associated with a poor prognosis in malignancies including colorectal cancer, ovarian cancer and lung cancer (22, 29-31). These findings support our hypothesis that SPON2 facilitates GC progression by promoting GC cell motility.

The development of PD is initiated by the penetration of tumor cells through the gastric wall. Tumor cells need to migrate and invade for successful detachment from the primary tumor and spread into the peritoneal cavity (7, 32). SPON2 secreted from CAFs may facilitate PD possibly by enhancing the motility of GC cells. However, it remains unknown how the expression of SPON2 is regulated in CAFs. Further investigation into how SPON2 facilitates PD and the factors that regulate SPON2 expression in CAFs may lead to a good understanding of the association between SPON2 and PD. *In vitro* co-culture analyses may provide a basis for addressing these challenges as demonstrated elsewhere (33).

In summary, we provide clinical and biological evidence that CAF-derived SPON2 has the potential to promote GC cell motility and potentially PD development, and thus may serve as a biomarker predicting a poor prognosis. There is increasing evidence that CAFs play a critical role in tumor progression and are promising therapeutic targets for malignancies (34). CAF-derived *SPON2* might be an attractive therapeutic target to suppress PD in GC.

Conflicts of Interest

The Authors have no conflicts of interest.

Authors' Contributions

SK and TM wrote the manuscript. SK and TM conducted the experiments. SK, TM, QH, TT, MY, YT, AK, HO, SI, EO, MM and KM designed the study and interpreted the data. SK and TM prepared the figures and conducted the statistical analysis.

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