Serpin peptidase inhibitor clade A member 1-overexpression in gastric cancer promotes tumor progression *in vitro* and is associated with poor prognosis

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Abstract. Gastric cancer is the second most common cause of cancer-associated death in Asia. The incidence and mortality rates of gastric cancer have markedly increased in the past few decades. Therefore, the identification of novel gastric cancer biomarkers are needed to determine prognosis. The role of serpin peptidase inhibitor clade A member 1 (SERPINA1) has been studied in several types of cancer; however, little is known about its mechanism in gastric cancer. The present study aimed to evaluate *SERPINA1* as a potential prognostic biomarker in gastric cancer and to identify the possible mechanisms underlying its action. The expression levels of *SERPINA1* in several gastric cancer datasets were assessed, and it was identified that high expression of *SERPINA1* was associated to poor clinical outcomes. Furthermore, using histochemical analysis, western blotting, apoptotic analysis, gap closure and invasion assays in cell lines, it was reported that silencing of *SERPINA1* inhibited the formation of cellular pseudopodia and did not affect apoptosis, but promoted cell cycle S-phase entry. In addition, overexpression of *SERPINA1* increased the migration and invasion of gastric cancer cells, whereas knockdown of *SERPINA1* decreased these functions. Moreover, SERPINA1 overexpression increased the protein levels of SMAD4, which is a key regulator of the transforming growth factor (TGF)-β signaling pathway. Taken together, the present data demonstrated that SERPINA1 promotes gastric cancer progression through TGF-β signaling, and suggested that *SERPINA1* may be a novel prognostic biomarker from tumor tissue biopsy in gastric cancer.

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Introduction

Gastric carcinoma is the fifth most frequently diagnosed cancer and the third leading cause of cancer-associated death worldwide (1). In 2018 alone, 1,033,701 new cases and 782,685 deaths from gastric cancer were expected globally (1). The 5-year overall survival rate of patients with metastatic gastric cancer is only 2%, with a median survival time of 8.6 months (2). Several studies have identified various oncogenes and tumor suppressors that regulate the tumorigenesis of gastric cancer (3,4). For instance, TP53 regulates target genes in response to cellular stress and BRCA2 is involved in DNA repair, which are both major genes that are frequently mutated in gastric cancer (5,6). However, no well-established targets besides human epidermal growth factor receptor 2 (7,8) have been shown to modify the outcomes of patients with gastric cancer. Therefore, it is essential to develop novel targets and therapeutic approaches.

Transforming growth factor (TGF)-β is one of the most extensively expressed cytokines in the tumor microenvironment, and it plays an important role in tumor initiation and progression (9). TGF-β is produced in large amounts by numerous tumor types and is known to be pro-oncogenic (10,11). The activated TGF-β receptor transduces its signal via the phosphorylation of SMAD2/3 and subsequent recruitment of SMAD4 intracellularly. This protein complex then enters the nucleus and initiates the transcription of the mesenchymal markers SNAI1, SNAI2, TWIST1 and ZEB1 (12-14), eventually promoting the migration and invasion of tumor cells (15). It has been reported that high expression of TGF-β1 decreases the overall survival rate of patients with gastric cancer (16).

Serpin peptidase inhibitor clade A member 1 (SERPINA1), a member of the protease inhibitor family of proteins, is primarily synthesized in the liver. It is also produced in certain neoplastic cells, such as those of colon, ovarian and lung cancer (17-19). Tumor cells synthesize and release SERPINA1, which plays a major role in physiological and pathological processes, such as angiogenesis, wound healing, and tumor invasion and metastasis (20). The expression of *SERPINA1* has been reported to be correlated with poor prognoses in terms of metastasis among patients with lung, colon and skin cancer (18,19,21,22). However, details regarding the mechanism

underlying the role of SERPINA1 in the progression and metastasis of gastric cancer remains unknown.

The present study aimed to provide important insights into the mechanism underlying the pathogenesis of gastric cancer and to evaluate *SERPINA1* as a potential prognostic biomarker.

Materials and methods

Database analyses. The ONCOMINE database (https://www.oncomine.org) (23) was used to compare the transcriptional profiles of *SERPINA1* in cancer tissues and adjacent normal tissues [cut-off values, P<0.01 and fold-change (FC)>1.5]. The Gene Expression Profiling Interactive Analysis (GEPIA2) bioinformatics tool (http://gepia2.cancer-pku.cn) (24) was used to assess the mRNA expression levels of *SERPINA1* in gastric cancer tissues and normal tissues deposited in The Cancer Genome Atlas (TCGA) (https://www.cancer.gov/tcga) and Genotype-Tissue Expression (GTEx) (https://www.gtexportal.org/) databases. The TCGA stomach adenocarcinoma (STAD) dataset (25) (408 tumor and 36 normal tissues), colon adenocarcinoma (COAD) dataset (26) (275 tumor and 41 normal tissues), esophageal carcinoma (ESCA) dataset (27) (182 tumor and 13 normal tissues) and pancreatic adenocarcinoma (PAAD) dataset (28) (179 tumor and 4 normal tissues), together with the GTEx database (29) (923 normal tissues), were used to determine the association between the mRNA levels of *SERPINA1*, as well as the overall stage (according to the TNM system) (30) of patients. Survival curves of patients with gastric cancer were generated using the Kaplan-Meier Plotter online tool (https://kmplot.com/) (31). Differentially expressed genes (DEGs) in the TCGA-STAD dataset were analyzed (cut-off values, $|\log_2 FC|>2$ and q-value <0.01). Gene Set Enrichment Analysis (GSEA) was performed using online tools (http://software.broadinstitute.org/gsea) (32). The cBioPortal for Cancer Genomics (https://www.cbioportal.org/) (5,33) was used to identify genes associated with SERPINA1. Biological function networks were generated using Ingenuity Pathway Analysis (IPA) software (https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis) (34). The BioGRID (https://thebiogrid.org/) (35) and Wiki-Pi (https://hagrid.dbmi.pitt.edu/wiki-pi/) (36) databases were used to query the protein-protein interactions of SERPINA1.

Cell culture and transfection. The AGS gastric cancer cell line was purchased from the American Type Culture Collection and authenticated using short tandem repeat analysis (Beyotime Institute of Biotechnology) in 2020. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal calf serum (both Invitrogen; Thermo Fisher Scientific, Inc.) in an incubator at 37˚C with 5% carbon dioxide. For *SERPINA1*-knockdown analyses, AGS cells were transfected with 10 nM SERPINA1 small interfering (si)RNA (siSERPINA1) or non-targeting negative control siRNA (siCONTROL) using RNAiMAX transfection reagent (all Invitrogen; Thermo Fisher Scientific, Inc.). For *SERPINA1*-overexpression experiments, AGS cells were transfected with 1 μg *SERPINA1* overexpression vector pcDNA3.1(+)-SERPINA1 (pSERPINA1) plasmid (GenScript) or 1 μg empty control vector pcDNA3.1(+)-SERPINA1_del (pCONTROL) plasmid (GenScript) and selected using 1,200 μg/ml of G418 for 4 weeks.

Histochemical staining. Cells were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at room temperature for 15 min and permeabilized in 0.1% Triton X-100 in PBS. Filamentous (F)-actin was labeled with Alexa Fluor 555 phalloidin (165 nM; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at room temperature and nuclei were stained with DAPI (1:10; PerkinElmer, Inc.) for 15 min at room temperature. Images were captured using the Mantra quantitative pathology imaging system (v1.03; PerkinElmer, Inc.).

Gap closure assay. An ibidi culture-insert (Ibidi GmBH) was placed in one well of a 6-well cell culture plate. Cells (70 μl/well; $1x10^6$ cells/ml) were seeded into both wells and incubated at 37˚C in 5% carbon dioxide. After 24 h, the insert was removed, creating a 500-μm cell-free gap, and subsequently 2 ml/well serum-free cell culture medium (DMEM) was added. Gap closure was tracked and images were captured using an inverted light microscope with a digital camera under x10 magnification (Olympus Corporation). Quantification of gap closure was performed using ImageJ software (v1.52; National Institutes of Health).

Invasion assay. Matrigel (Corning, Inc.) was diluted with cold H_2O to a final concentration of 0.15 μg/μl, and 50 μl diluted Matrigel was added to the upper Transwell chamber (Corning, Inc.). The chambers were left at room temperature overnight and after Matrigel had completely dried on the membranes, cells (100 μl/well; $5x10^5$ cells/ml in DMEM containing 0.1% BSA; Sigma-Aldrich; Merck KGaA) were seeded into the upper chamber and DMEM medium containing 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) was added to the bottom well of the Transwell chamber (600 μl/well). After 24 h of incubation at 37˚C, the membranes were fixed with 4% PFA in PBS for 15 min and stained with 0.2% crystal violet for 15 min at room temperature, and the cells that had invaded onto the lower surface of the porous membrane were captured and counted in four random squares using a light microscope under x10 magnification with a digital camera (Olympus Corporation) and ImageJ software (v1.52; National Institutes of Health), respectively.

Analysis of apoptosis. Apoptosis was determined using Alexa Fluor 488-labeled Annexin V and the PI apoptosis detection kit (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were seeded in a 6-well plate (2 ml/well; $0.15x10^6$ cells/ml) and cultured at 37˚C for 48 h. Cells were then harvested and resuspended, stained with Annexin V and propidium iodide (PI), and analyzed using flow cytometry (BD LSRFortessa, BD Biosciences) and FlowJo software (v10.6.1; BD Biosciences) within 1 h.

Cell cycle analysis. Cells were harvested, resuspended, and fixed in 70% cold ethanol at 4˚C for 12 h. The cells were then treated with DAPI (BD Biosciences) in PBS for 30 min at room temperature in the dark. The cell cycle was analyzed using the BD LSRFortessa flow cytometer and FlowJo software (v10.6.1; BD Biosciences).

Protein isolation and western blotting. Cultured cells were lysed in a radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.) containing a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Inc.). Lysates were sonicated at 20 kHz for 15 sec on ice and centrifuged at 10,000 x g for 10 min at 4˚C. Whole-lysate proteins (15-25 μg) were loaded in each lane. Gel electrophoresis was performed using a 4-12% gradient polyacrylamide gel (Invitrogen; Thermo Fisher Scientific, Inc.) and the proteins were transferred to polyvinylidene difluoride membranes (Invitrogen; Thermo Fisher Scientific, Inc.) according to standard protocols (37). The membranes were then cut and incubated with SERPINA1 (1:1,500, cat. no. ab207303, Abcam), SMAD4 (1:1,500, cat. no. 46535S) or GAPDH (1:3,000, cat. no. 5174S) (both Cell Signaling Technology, Inc.) antibodies according to the known molecular weights of the proteins. HRP-coupled goat anti-rabbit secondary antibody (cat. no. 7074S, Cell Signaling Technology, Inc.) was used at 1:3,000 dilution. Enhanced chemiluminescence signals were recorded and quantified using the ChemiDoc MP imaging system and Image Lab v5.0 software (both Bio-Rad Laboratories, Inc.).

RNA preparation and reverse transcription-quantitative (RT-q) PCR. Total RNA was isolated from cultured cell lines using the RNeasy Plus Mini kit (Qiagen GmBH). RNA was reverse transcribed into cDNA using the SuperScript IV First-Strand Synthesis System kit (Invitrogen; Thermo Fisher Scientific, Inc.) at 50˚C for 20 min and then 80˚C for 10 min. RT-qPCR was performed using the QuantStudio 7 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the PowerUp SYBR Green Master mix (Invitrogen; Thermo Fisher Scientific, Inc.). The PCR primer sequences were as follows: SERPINA1, Forward: 5'-GAAGAGCGTCCTGGGTCAAC-3' and reverse: 5'-TGGTCAGCACAGCCTTATGC-3'; SMAD4, forward: 5'-CCCATCCCGGACATTACTGG-3' and reverse: 5'-TAGGGCAGCTTGAAGGAACC-3'; PAI-1, forward: 5'-GCAAGGCACCTCTGAGAACT-3' and reverse: 5'-GGG TGAGAAAACCACGTTGC-3'; ACTB, forward: 5'-TGA CATTAAGGAGAAGCTGTGCTA-3' and reverse: 5'-GAG TTGAAGGTAGTTTCGTGGATG-3'. The relative expression of genes was assessed using the $2^{-\Delta\Delta Cq}$ method (38), and the expression of ACTB was used as a reference.

Statistical analyses. Statistical analyses were carried out using GraphPad Prism version 7 (GraphPad Software, Inc.). Data are presented as the mean ± standard deviation and were compared using unpaired Student's t-tests. The association between *SERPINA1* expression and tumor stages in digestive system cancer datasets was analyzed using one-way ANOVA. P<0.05 was considered to indicate a statistically significant difference.

Results

Transcriptional levels of SERPINA1 are elevated in patients with digestive system cancer. To understand the expression patterns of *SERPINA1*, the transcriptional levels of *SERPINA1* in different cancer types were compared with those in normal samples using data from the ONCOMINE database (23). The mRNA expression levels of *SERPINA1* were significantly upregulated in 58 analyses and downregulated in 38 analyses in different cancer types (cut-off values, P<0.01 and FC >1.5) (Fig. 1A). Notably, *SERPINA1* was significantly upregulated in analyses of digestive system cancer types, such as gastric, colorectal, esophageal and pancreatic cancer (Fig. 1A). In the studies conducted by Skrzypczak *et al* (39) and Sabates-Bellver *et al* (40), SERPINA1 was found to be overexpressed in colon cancer compared with normal samples, with FCs of 3.273 and 2.592, respectively (Table SI). Kimchi *et al* (41) and Kim *et al* (42) reported that the mRNA levels of *SERPINA1* were also elevated in esophageal adenocarcinoma, with FCs of 23.160 and 6.244, respectively, compared with those in normal esophageal tissues (Table SI). In a study conducted by Wang *et al* (43), SERPINA1 was found to have an increased FC of 3.319 in patients with gastric cancer compared with that in normal gastric tissues (Table SI). *SERPINA1* overexpression was also found in pancreatic ductal adenocarcinoma, with an FC of 5.122 in a study conducted by Badea *et al* (44), and in pancreatic adenocarcinoma, with an increased FC of 6.752, in the study conducted by Logsdon *et al* (45) (Table SI).

Using GEPIA2 (24), the mRNA expression levels of *SERPINA1* between STAD, ESCA, PAAD and COAD tissues and normal tissues were compared using TCGA and the GTEx databases. The results indicated that the expression levels of *SERPINA1* were significantly higher in tissues of the aforementioned cancer types compared with those in normal tissues (all P<0.05; Fig. 1B). The expression of *SERPINA1* was also analyzed based on clinical tumor stages for the four aforementioned cancer types. The STAD, ESCA and PAAD groups did not show any significant differences; however, in the COAD group, *SERPINA1* expression was significantly associated with clinical stage (P<0.01; Fig. 1C). Overall, the present results suggested that *SERPINA1* upregulation may be involved in digestive system cancer development.

Increased mRNA expression of SERPINA1 is associated with poor prognosis of patients with gastric cancer. The effect of SERPINA1 on survival among patients with gastric cancer was further analyzed by examining the association between high *SERPINA1* mRNA expression and poor prognosis using the Kaplan-Meier Plotter online survival analysis tool (31). The overall survival (OS) of 876 patients with gastric cancer showed that high *SERPINA1* expression was associated with shorter survival time in probe 211429_s_at, 202833_s_at and 211428_at (log-rank P=2.6×10^{-5}, 7.5×10^{-4} and 2.5×10^{-7}, respectively; Fig. 2A). The progression-free survival (PFS) time of 646 patients with gastric cancer also showed the same outcomes in probe 202833_s_at, 211428_at and 211429_s_at (log-rank P=3.5×10^{-4}, 3.0×10^{-4} and 3.5×10^{-5}, respectively; Fig. 2B), as did the post-progression survival time of 499 patients in all probes (Fig. 2C). In the analyses of OS and PFS, the difference obtained for probe 230318_at did not reach statistical significance (P<0.05). However, a similar tendency was observed with the probes mentioned above (log-rank P=0.13 and 0.09, respectively; Fig. 2A and B). Overall, *SERPINA1* is associated with clinical outcome of gastric cancer patients and high *SERPINA1* expression indicates a short lifespan.

SERPINA1 is predicted to be involved in cellular movement. Employing GEPIA2, the DEGs in the gastric

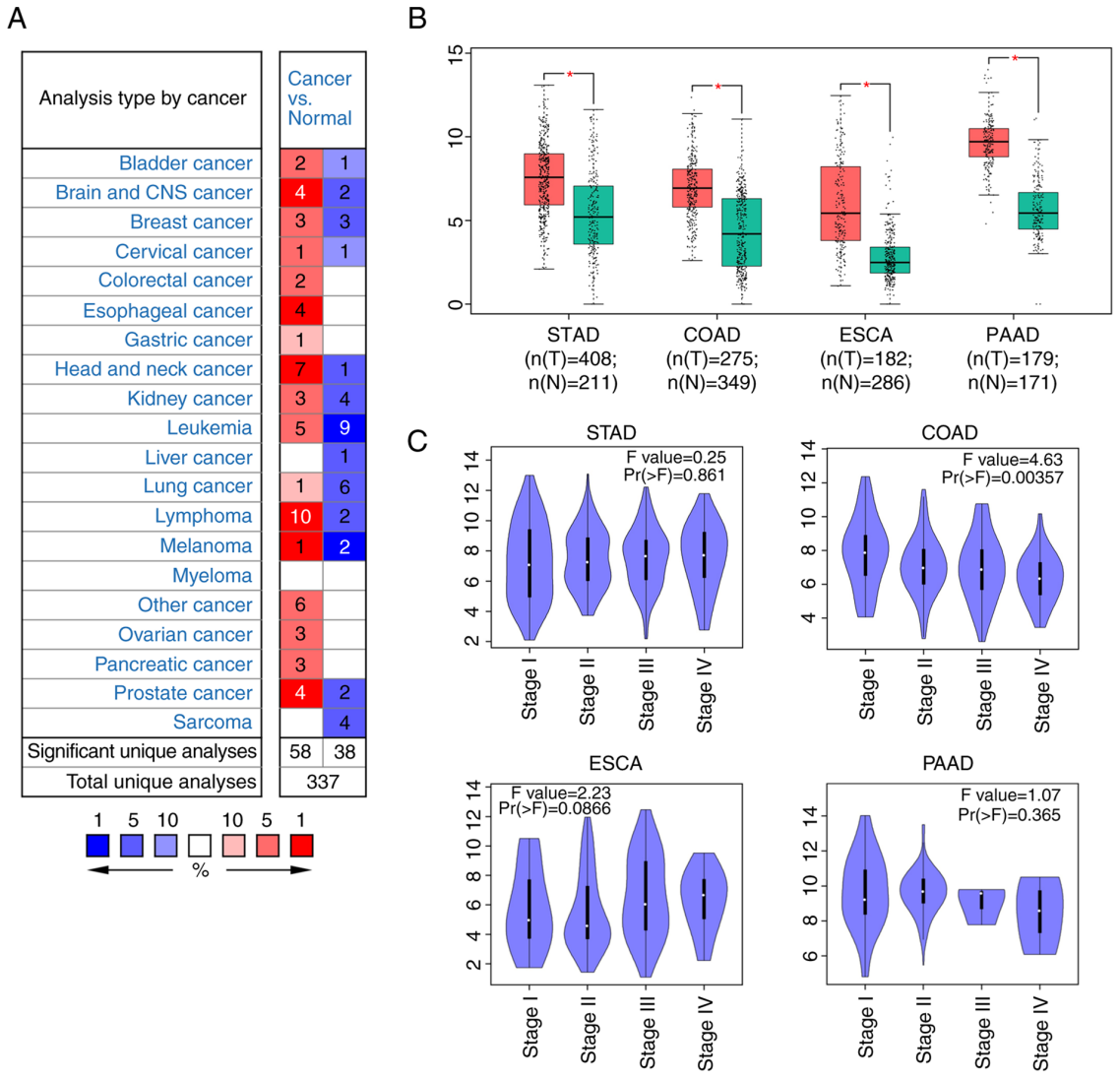

Figure 1. Expression profiles of *SERPINA1* in different cancer types. (A) Summary of *SERPINA1* expression in the ONCOMINE database. The numbers in each cell indicate the ONCOMINE profiles, with significant gene overexpression (red) or underexpression (blue) for each combination. Cell color is determined by the gene rank percentile for the analyses within the cell. (B) The mRNA expression of *SERPINA1* in STAD, COAD, ESCA and PAAD. Box plots derived from gene expression data using Gene Expression Profiling Interactive Analysis comparing expression of tumor tissues and normal tissues. *P<0.05. (C) Association between SERPINA1 expression and tumor stage in STAD, COAD, ESCA and PAAD. In (B) and (C) the Y-axis represents the relative expression levels of *SERPINA1* in terms of $\log_2$(Transcripts Per Million +1). *SERPINA1*, serpin peptidase inhibitor clade A member 1; STAD, stomach adenocarcinoma; COAD, colon adenocarcinoma; ESCA, esophageal carcinoma; PAAD, pancreatic adenocarcinoma.

adenocarcinoma TCGA-STAD dataset were analyzed. *SERPINA1* ($Log_2FC=2.374$, adjusted $P=3.34x10^{-19}$) was identified as one of 843 DEGs (Table SII). A subsequent GSEA (32) was performed that focused on hallmark gene sets representing 50 specific well-defined, large-scale biological processes and displaying coherent expression (46). By analyzing the TCGA-STAD dataset, five hallmark gene sets (G2M checkpoint, E2F targets, mitotic spindle, epithelial mesenchymal transition and inflammatory response) were identified in which DEGs were upregulated, and two hallmark gene sets (Kras signaling down and myogenesis) in which the DEGs were downregulated [all false discovery rate (FDR) q-value <0.05; Fig. 3A]. Furthermore, two of the upregulated gene sets, 'mitotic spindle' [normalized enrichment score (NES)=2.31] (Fig. 3B) and 'epithelial-mesenchymal-transition' (EMT; NES=1.66) (Fig. 3B) showed an association with tumor progression.

Using the enrichment analysis function of cBioPortal for Cancer Genomics (5,33), an online TCGA data analysis platform, it was reported that the expressions of 480 genes were correlated with *SERPINA1* expression (all P<0.001; Table SIII) in the TCGA-STAD dataset. To explore the potential functions of SERPINA1, IPA software (34) was then used to illustrate the biological pathways of these SERPINA1-enriched genes. IPA analysis revealed that the top five cell biological function networks were associated with cellular movement, cellular function and maintenance, cell cycle, cell death and survival and cellular development (Fig. 3C). The dominant biological function was cellular movement, which refers to cell migration and motility.

Silencing of SERPINA1 inhibits the formation of cellular pseudopodia, cell migration and invasion in vitro. To obtain evidence supporting the role of SERPINA1 in cell

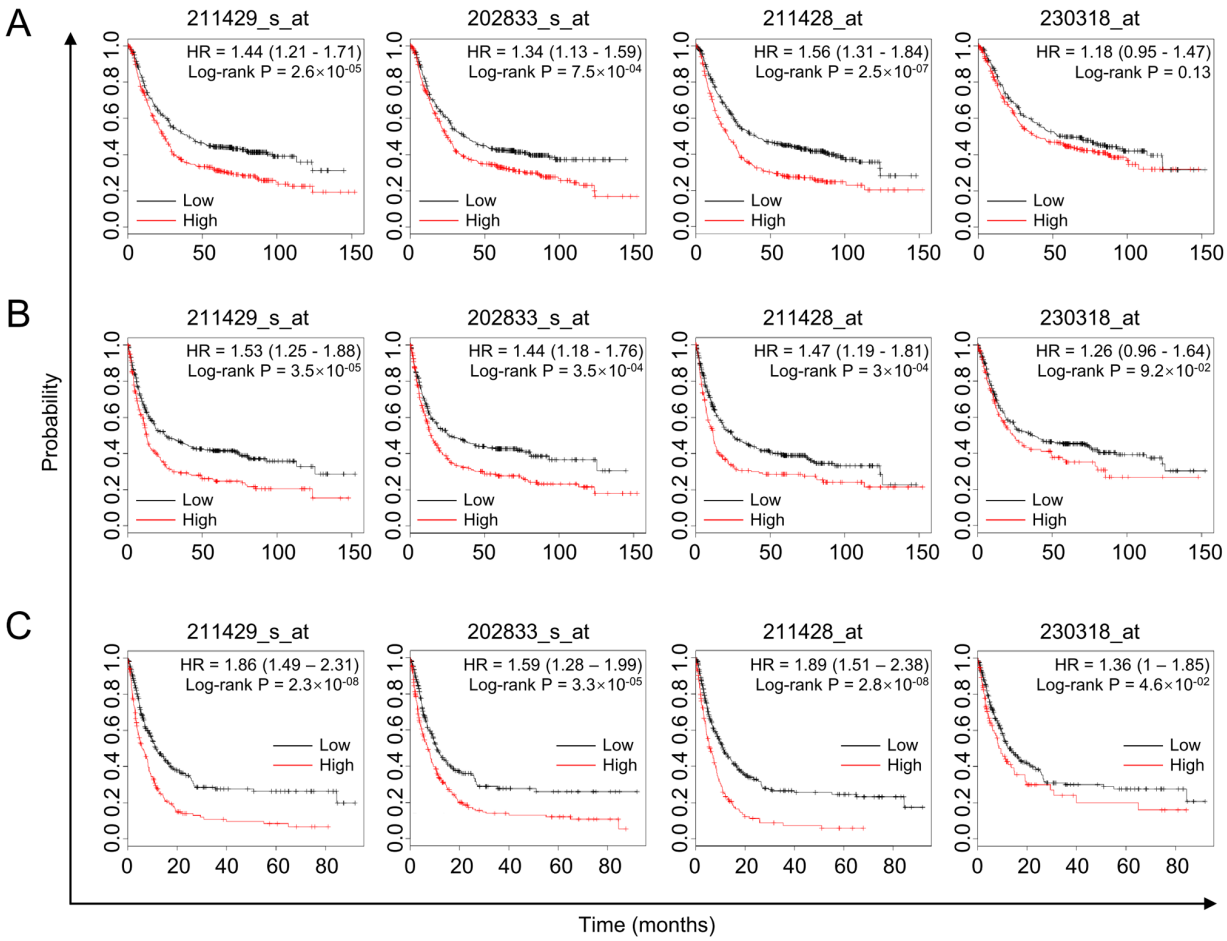

Figure 2. The prognostic value of *SERPINA1* expression in gastric cancer. (A) Kaplan-Meier plots showing overall survival time among patients with gastric cancer (n=876). (B) Survival curves plotted for progression-free survival time (n=646). (C) Survival curves plotted for post-progression survival time of 499 patients with gastric cancer. High *SERPINA1* expression is indicated by the red line, and low *SERPINA1* expression by the black line. *SERPINA1*, serpin peptidase inhibitor clade A member 1; HR, hazard ratio.

movement *in vitro*, the gene expression of *SERPINA1* was knocked down in the AGS gastric cancer cell line using siRNA targeting exon 3 of *SERPINA1* mRNA. After 24 h of 10-nM siRNA transfection, the *SERPINA1* expression levels in siSERPINA1-treated cells were reduced to 10% of that of the control siRNA-treated cells. This reduction persisted for at least 72 h (Fig. 4A). Following this, phalloidin, a high-affinity F-actin probe (47), was used to assess remodeling of the cytoskeleton. The AGS gastric cell line was stained with Alexa Fluor 555-labeled phalloidin after gene silencing of *SERPINA1*, and changes in cell morphology were assessed using light microscopy. The extension of long membranous pseudopodia in AGS cells was decreased in cells treated with 10 nM SERPINA1 siRNA compared to control siRNA-treated cells (Fig. 4B).

To assess whether silencing of *SERPINA1* affected cell migration, a gap closure assay was performed to evaluate the motility of AGS cancer cells. *SERPINA1*-knockdown significantly decreased the cell migration area by 18.89% after 16 h compared with that of control siRNA-transfected cells (Fig. 4C). The invasion of the cells was assessed using a Matrigel assay. *SERPINA1*-knockdown significantly reduced the number of invasive cells from 40.7 cells/field to 26.3 cells/field (Fig. 4D). Overall, these observations further support that *SERPINA1* may be involved in the migration and motility of cancer cells.

Knockdown of SERPINA1 does not impact apoptosis but promotes the S-phase entry of AGS cells. To exclude the possibility that the inhibition of cell migration and invasion was due to an increased rate of apoptosis and/or a state of proliferation arrest among cells, these effects in the AGS cell line were analyzed after *SERPINA1*-knockdown. Dual staining of the SERPINA1-downregulated AGS cells with PI and Annexin V-Alexa Fluor 488 was used to assess apoptosis. Flow cytometry revealed no significant difference between SERPINA1 siRNA-treated AGS cells and control siRNA-treated cells regarding the proportions of live cells, early apoptotic cells and late apoptotic cells (Fig. 5A and B). Cell cycle distribution was also evaluated by flow cytometric analysis using DAPI staining. No significant shifts in the G_1 and G_2 peak positions were observed among AGS cells after SERPINA1 siRNA transfection (Fig. 5C). However, a significantly increased proportion of S phase cells (from 20.2 to 27.4%), decreased G_0/G_1-phase (from 56.2 to 49.6%) and G_2/M-phase (from 17.0 to 14.1%) cells were observed (Fig. 5D) after *SERPINA1*-knockdown. This suggested that gene silencing of *SERPINA1* promoted S phase entry in the AGS cell line.

A

Up-regulated in gastric cancer

Gene set	ES	NES	FDR
G2M checkpoint	0.73	2.55	0.000
E2F targets	0.71	2.44	0.000
Mitotic spindle	0.75	2.31	0.000
Epithelial mesenchymal transition	0.51	1.66	0.019
Inflammatory response	0.55	1.56	0.046

Down-regulated in gastric cancer

Gene set	ES	NES	FDR
Kras signaling down	–0.71	–2.48	0.000
Myogenesis	–0.43	–1.7	0.021

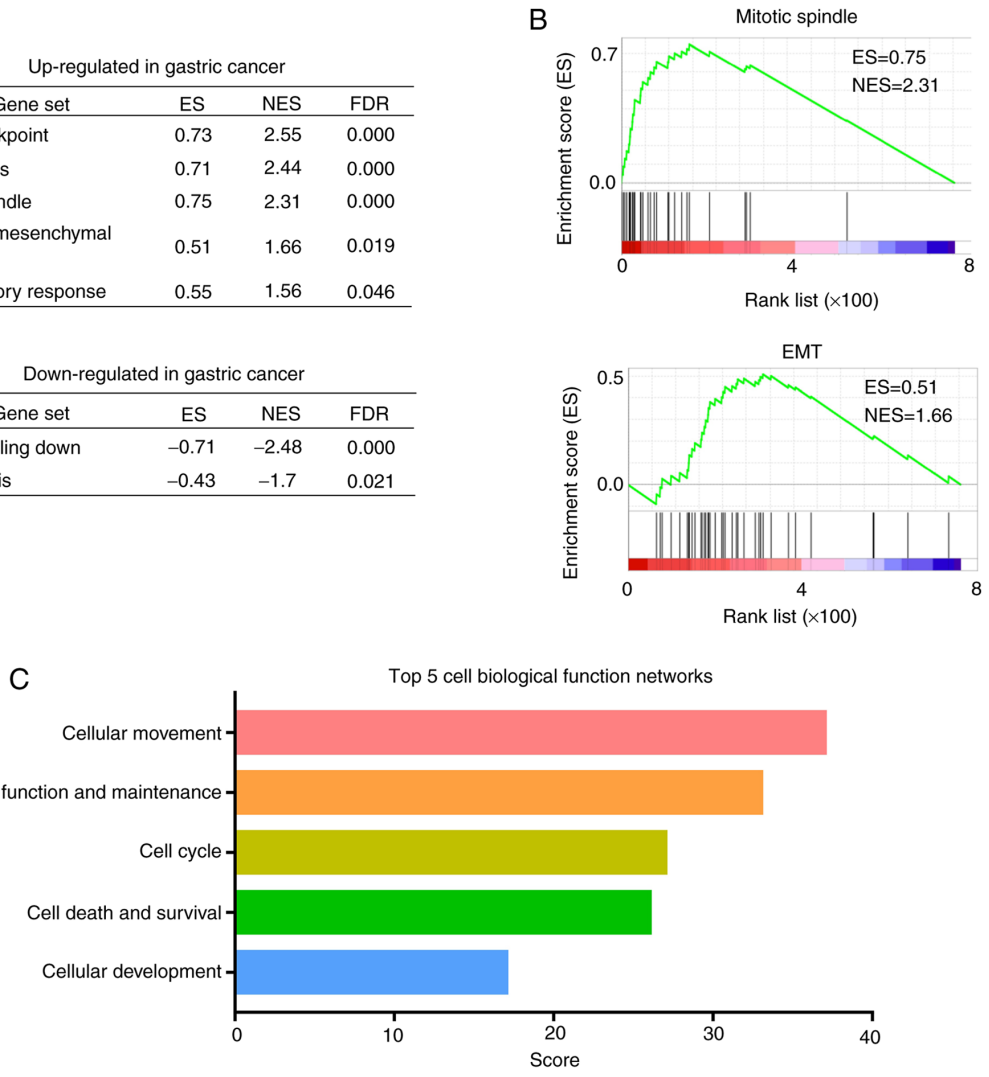

Figure 3. Bioinformatics analysis of The Cancer Genome Atlas-stomach adenocarcinoma dataset. (A) Gene Set Enrichment Analysis of mRNA profiles from tumor tissues vs. normal tissues of patients with gastric cancer (FDR q-value <0.05). (B) Selected enrichment score plots of tumor progression related gene sets. (C) Top five cell biological function networks identified using Ingenuity Pathway Analysis. ES, enrichment score; NES, normalized ES; FDR, false discover rate.

Overexpression of SERPINA1 in gastric cancer cell lines promotes tumor cell migration and invasion, but decreases the number of S phase cells. To confirm the earlier finding that knockdown of *SERPINA1* inhibited cell migration and invasion, a *SERPINA1*-overexpression plasmid (pSERPINA1) and its control plasmid (pCONTROL) were generated. The AGS gastric cancer cell line was transfected with either of these vectors and selected with G418 for 4 weeks. *SERPINA1* overexpression in these cells after transfection was confirmed using RT-qPCR (Fig. 6A). The gap closure assay showed that the average migration area of AGS cells transfected with *SERPINA1* overexpression plasmid was significantly increased by 11.81% compared with that of cells transfected with control plasmid after 16 h (Fig. 6B). The number of invasive AGS cells significantly increased from 41.4 cells/field to 97.3 cells/field (Fig. 6C) after induced *SERPINA1* overexpression. The rate of apoptosis remained the same after *SERPINA1*-upregulation (Fig. 6D). A decreased proportion of S phase cells (from 22.4 to 17.8%) and an increased proportion of G_0/G_1 phase cells (from 50.4 to 54.7%) were observed in AGS cells with *SERPINA1*-overexpression compared with control cells (Fig. 6E). Altogether, these results confirm that increased *SERPINA1* expression promotes gastric cancer cell migration and invasion, but inhibits cell proliferation.

SERPINA1 upregulates SMAD4, a regulator of the TGF-β signaling pathway. To elucidate the mechanism underlying the effects of SERPINA1, protein-protein interaction data of SERPINA1 were queried using the BioGRID (35) and Wiki-Pi (36) databases. Seventy-three interactions involving SERPINA1 were identified in the BioGRID database (Fig. 7A and Table SIV), and 26 interactions were reported in the Wiki-Pi database (Table SV). SMAD4, which plays a role in regulating TGF-β-mediated EMT in breast cancer (48), nasopharyngeal carcinoma (49) and squamous cell carcinoma of the head and neck (50), was found to interact with SERPINA1 in both databases.

To further assess whether SERPINA1 regulated the TGF-β signaling pathway via SMAD4, the expression of SMAD4 protein in both *SERPINA1*-knockdown and

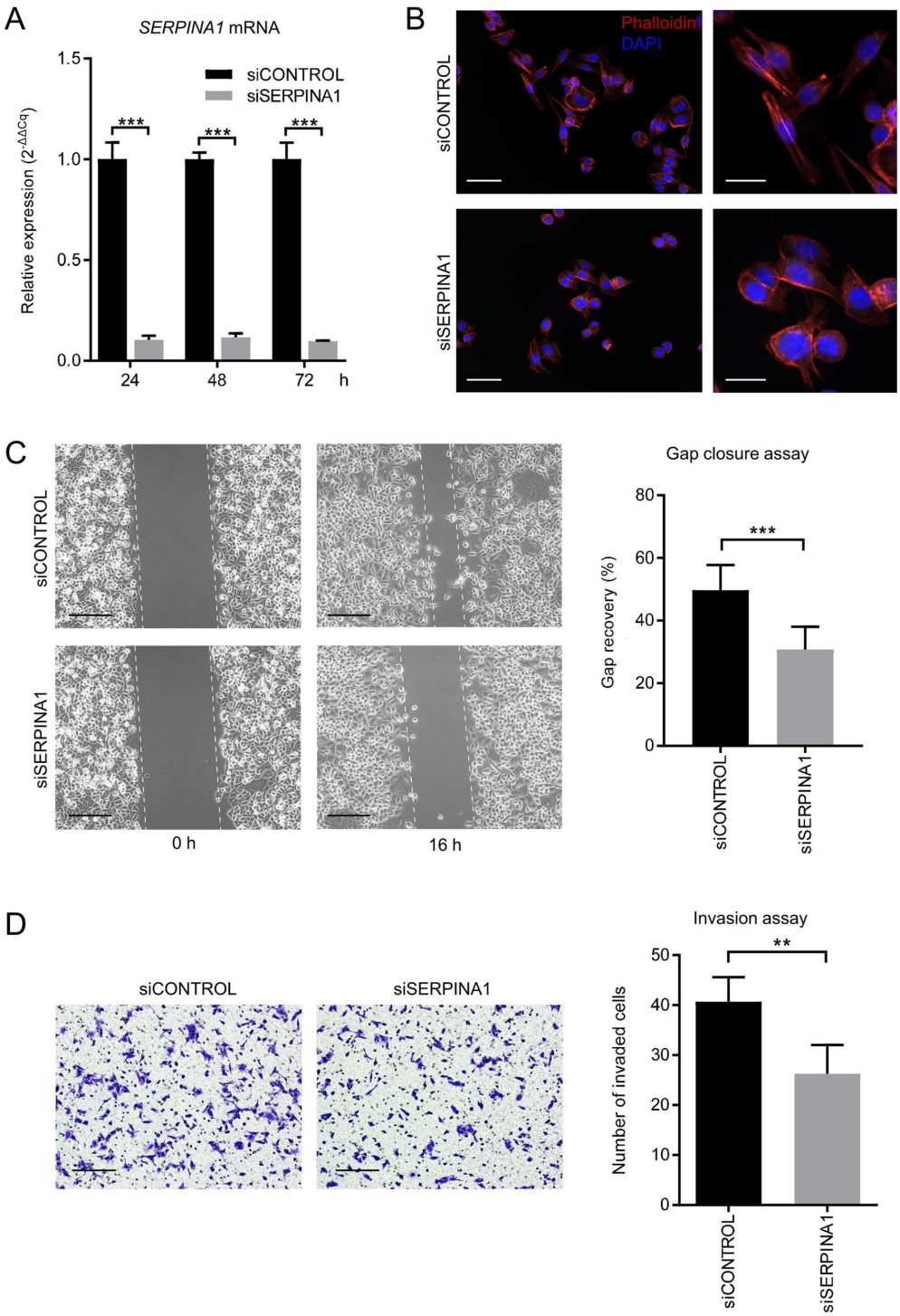

Figure 4. Effect of siRNA interference on expression of SERPINA1 in AGS gastric cancer cells. (A) mRNA expression of *SERPINA1* detected using reverse transcription-quantitative PCR at 24, 48 and 72 h after transfection with negative control siRNA (siCONTROL) or *SERPINA1* siRNA (siSERPINA1). The relative levels of *SERPINA1* mRNA were normalized using β-actin (n=3). (B) The morphology of siRNA-treated AGS cells. Actin fibers (filamentous actin) were visualized using a phalloidin-Alexa Fluor 555 probe (red), and nuclei were detected using DAPI (blue) at 72 h after siRNA transfection. Scale bar, 100 μm (left panels) and 20 μm (right panels). (C) Gap closure assay. The areas of the gaps were measured at 0 and 16 h post gap insert removal and % gap recovery of the initial gap areas were compared (n=8). Scale bar, 200 μm. (D) Invasion assay. The invading cells were stained with 0.2% crystal violet and counted in four random squares. n=4. Scale bar, 200 μm. **P<0.01, ***P<0.001. *SERPINA1*, serpin peptidase inhibitor clade A member 1; si, small interfering.

SERPINA1-overexpression AGS cells was analyzed. Western blotting showed that the SMAD4 protein levels were downregulated after silencing *SERPINA1* (Fig. 7B), whereas overexpression of *SERPINA1* led to an upregulation of SMAD4 protein (Fig. 7C). In addition, changes in the mRNA levels of plasminogen activator inhibitor 1 (a SMAD4-dependent TGF-β signaling target gene) (51) (Fig. 7D) were consistent with changes in SMAD4 protein expression in AGS cells subjected to *SERPINA1*-knockout and overexpression. These findings suggested that SERPINA1 might regulate the TGF-β signaling pathway through interaction with SMAD4.

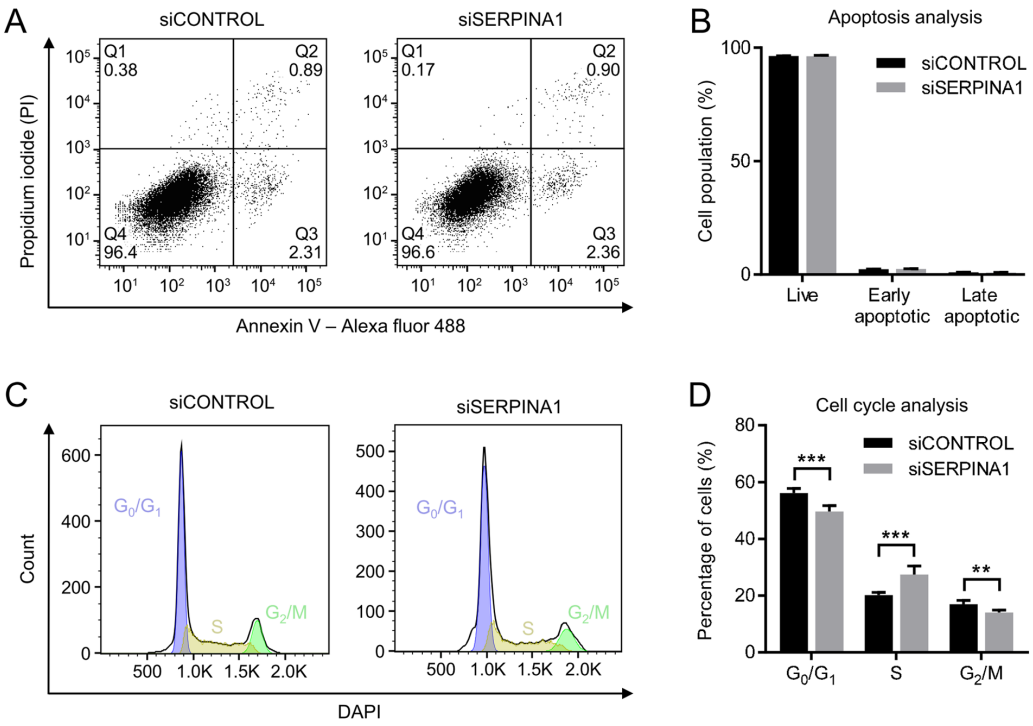

Figure 5. Flow cytometry analysis of *SERPINA1*-knockdown AGS cells. (A) Cell apoptosis analysis was performed using the Annexin V-PI kit. (B) Apoptotic cells were measured at 72 h after siRNA transfection (n=6). (C) Cell cycle analysis was performed with DAPI after 72 h treatment with the indicated siRNAs. (D) Quantification of the cell cycle analysis of AGS cells, n=6. **P<0.01, ***P<0.001. *SERPINA1*, serpin peptidase inhibitor clade A member 1; si, small interfering.

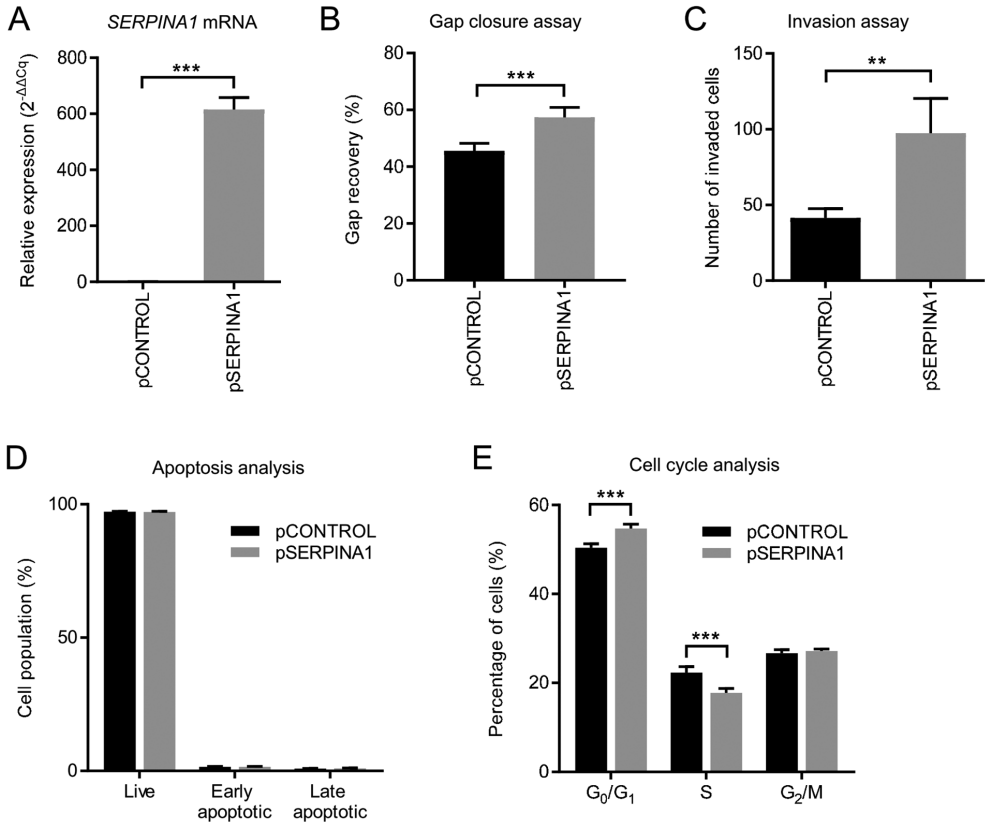

Figure 6. Effect of *SERPINA1* overexpression in gastric cancer cells. (A) mRNA expression of *SERPINA1* in AGS cells after treatment with overexpression vector (pSERPINA1) or control vector (pCONTROL) was detected using reverse transcription-quantitative PCR after 4 weeks of G418 selection. The relative levels of *SERPINA1* mRNA were normalized using β-actin as an internal control. n=3. (B) Gap closure assay. The areas of the gaps were quantified at 0 and 16 h post gap insert removal and % gap recovery of the initial gap areas were compared. n=8. (C) Invasion assay. The invading cells were stained with 0.2% crystal violet, and counted in four random squares. n=4. (D) Apoptotic cells were measured at 72 h after being seeded (n=6). (E) Cell cycle analysis was performed with DAPI after 72 h in culture. n=6. **P<0.01, ***P<0.001. *SERPINA1*, serpin peptidase inhibitor clade A member 1; si, small interfering.

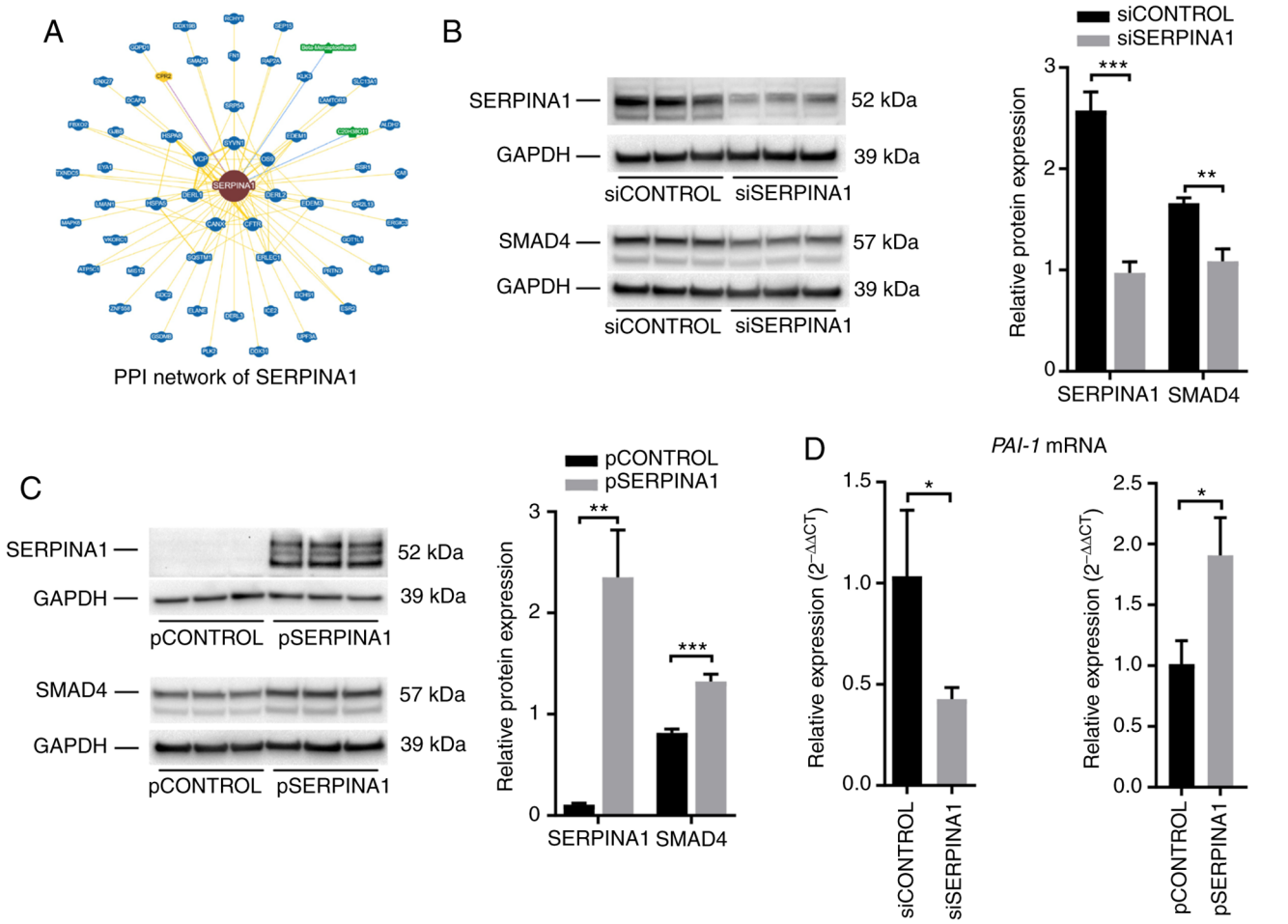

Figure 7. SERPINA1 regulates the TGF-β signaling pathway through SMAD4. (A) PPI network of SERPINA1 was queried from the BioGRID database. Green nodes represent chemicals, blue nodes represent proteins from the same organism and yellow nodes represent proteins from different organisms. Yellow edges represent protein interactions, blue edges represent chemical interactions and purple edges represent both protein and genetic interactions. (B) SERPINA1 and SMAD4 protein expression were evaluated using western blotting with lysates from AGS cells that were treated with a 72-h transfection of the indicated siRNAs (left panel). Quantification of SERPINA1 and SMAD4 protein. Columns indicate the ratio of SERPINA1 or SMAD4 intensity to GAPDH intensity (right panel, n=3). (C) Western blots of SERPINA1 and SMAD4 proteins in SERPINA1 overexpression vector or control vector-transfected AGS cells (left panel). The relative intensities of the bands were normalized using GAPDH (right panel, n=3). (D) The transcription levels of PAI-1 were measured using reverse transcription quantitative-PCR under the indicated conditions. β-actin was used as an internal control (n=3). *P<0.05, **P<0.01, ***P<0.001. *SERPINA1*, serpin peptidase inhibitor clade A member 1; SMAD4, SMAD family member 4; PPI, protein-protein interaction; si, small interfering; PAI-1, plasminogen activator inhibitor 1.

Discussion

In several Western Asian countries, gastric cancer is the most commonly diagnosed cancer and, in Eastern Asia, incidence rates have increased markedly in the past few decades; for example, the crude incidence rate in the Republic of Korea was 78.3 per 100,000 among males and 37.2 among females in 2012, compared with a crude incidence rate of 49.2 among males and 27.3 among females in 1999 (1,52,53). However, the underlying mechanisms driving poor clinical outcomes are not understood. Therefore, the identification of novel biomarkers and therapeutic targets for gastric cancer are essential to improve prognosis determination and treatment.

SERPINA1 is a member of the serpins superfamily of protease inhibitors, which play a crucial physiological role in hormone transport, blood clotting, corticosteroid binding and blood pressure regulation (54). However, serpins have also been found to function in tumorigenesis and cancer metastasis (54). Particularly, *SERPINA1* has been reported to be overexpressed in various malignant tumors. High expression of *SERPINA1* has been observed in prostate (55), lung (55) and colorectal (18) cancer. *SERPINA1* was also reported to be upregulated in serum samples of patients with gastric cancer compared with healthy individuals (56). The present study demonstrated that *SERPINA1* was overexpressed in colorectal, esophageal, gastric and pancreatic cancer, which indicated a strong association between a high *SERPINA1* mRNA levels and digestive system tumorigenesis. In addition, it was reported that overexpression of *SERPINA1* was associated with a shorter lifespan in four gastric cancer datasets. Consistent with the present results, Kwon *et al* (57) also reported an inverse correlation between *SERPINA1* expression and survival time in the Korean population.

Despite its clinical relevance, the functional role of SERPINA1 in tumor cells remains unknown. Using bioinformatics analyses of the TCGA-STAD database, the present study revealed that the expression of SERPINA1 was significantly upregulated compared with normal tissues. Further GSEA

revealed that the 'mitotic spindle' and 'EMT' gene sets were enriched in cancer tissues, which were associated with the function of the cytoskeleton. In addition, IPA analysis demonstrated that the top biological function network of SERPINA1-enriched genes was 'cellular movement'. All these findings suggested that SERPINA1 may be involved in cellular movement, which is mediated by the cytoskeleton (58). To resolve the underlying molecular mechanisms of SERPINA1 in gastric cancer progression, the expression of the *SERPINA1* gene was manipulated *in vitro*. A series of investigations revealed that SERPINA1 regulated cell morphology, migration and invasion and the number of S phase cells, but had no impact on apoptosis in the AGS gastric cell line. Similarly, the regulation of SERPINA1 in cell migration and invasion has also been reported in ovarian (17) and colon cancer (18) cell lines.

Although the mechanisms underlying the role of SERPINA1 in tumor cell migration/invasion and cell cycle have not been fully elucidated, it has been shown that fibronectin is upregulated by SERPINA1 (18). The upregulation of fibronectin promotes cell migration and invasion in colorectal cancer (18). Byon *et al* (59) and Hernanda *et al* (60) observed enhanced cell migration through SMAD4 in breast cancer cells and hepatoma cells, respectively. The present study demonstrated that overexpression of *SERPINA1* upregulated the expression of SMAD4, and subsequently activated the SMAD4-dependent TGF-β signaling pathway. This promoted the migration and invasion of human gastric cancer cells and reduced the proportion of S phase cells. Based on the present data, it was hypothesized that SERPINA1 may protect the intracellular transport of SMAD4 from the cell membrane to the nucleus (61), as SERPINA1 inhibits the activity of protease and may prevent SMAD4 complex degradation (62). However, co-immunoprecipitation experiments are required to confirm the binding of SERPINA1 and SMAD4, and further signal transduction studies may be helpful to elucidate the precise mechanisms underlying the regulation of SMAD4 expression by SERPINA1.

Taken together, the results of the present study illustrated that *SERPINA1* expression is elevated in digestive system cancer tissues and is associated with less favorable clinical prognoses in patients with gastric cancer from database analyses. These data provide evidence that SERPINA1 induces gastric cancer cell migration and invasion, possibly through the TGF-β pathway mediated by SMAD4, which might be a potential mechanism involved in tumor progression. These results suggested that SERPINA1 may be a novel biomarker for tumor metastasis and could be a novel therapeutic target.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LJ and LGH conceived and designed this study and wrote the manuscript. LJ performed the experiments and analyzed the data. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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