

# Inhibition of sphingosine-1-phosphate phosphatase 1 promotes cancer cells migration in gastric cancer: Clinical implications

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**Abstract.** Sphingosine-1-phosphate (S1P) plays an important role in regulating many biological processes. Sphingosine-1-phosphate phosphatase 1 (*SGPPI*) can dephosphorylate S1P into sphingosine and tip the balance of sphingosine-S1P. Increased levels of sphingosine leads to a decrease in the ability of cell invasion as well as an increase in the ability of cell apoptosis. However, little is known regarding the effects of *SGPPI* in gastric cancer. The present study examined the function of *SGPPI* on gastric cancer cell lines as well as its clinical relevance in gastric cancer progression. Using immunohistochemistry and RT-qPCR techniques, the clinical significance of *SGPPI* expression was analyzed in 288 paraffin-embedded gastric tissue specimens and 219 fresh gastric tissues, respectively. Transgenes encoding ribozymes to specifically target human *SGPPI* (pEF-*SGPPI*) was constructed. Human gastric cancer cell lines (AGS and HGC27) were transfected with pEF-*SGPPI* transgene and examined by functional analysis. *SGPPI* was downregulated in gastric cancer tissues, compared with adjacent normal gastric tissues ( $p=0.034$ ). *SGPPI* mRNA levels in gastric cancer tissues were significantly decreased when compared with their adjacent non-cancerous tissues ( $p<0.001$ ). Weakly expressed *SGPPI* was positively correlated with the lymph node metastasis ( $p=0.005$ ) and distant metastasis ( $p=0.031$ ). Kaplan-Meier survival curves revealed that patients with

*SGPPI* positive expression had a significant increase in overall survival (OS) ( $p=0.034$ ) and progression-free survival (PFS) ( $p=0.041$ ). Multivariate analysis indicated the expression of *SGPPI* was an independent prognostic factor in gastric cancer patients ( $p=0.041$ ). *In vitro* experiments showed that knockdown of *SGPPI* resulted in an increase in the invasion (2-fold) and migration (5-fold) of AGS and HGC27. The two gastric cancer cells transfected with pEF-*SGPPI* exhibited a slower rate of growth with less adhesion. Thus, our findings provided evidence that *SGPPI* may serve as a prognostic biomarker for patients with advanced gastric cancers.

## Introduction

Gastric cancer is the fifth most common cancer and the third leading cause of cancer mortality worldwide (1). Surgery alone is no longer acceptable as a standard treatment for resectable gastric cancer (2). Optimal locoregional treatment for gastric cancer can be achieved by a combination of radical surgery with individualized neoadjuvant or adjuvant treatment, with modern 3D radiotherapy and optimum target therapy (3). Many clinical trials showed that although chemotherapy and target therapy are effective in short-term treatment for advanced gastric cancer (4-6), these treatments do not improve overall survival (OS) rate (<1 year). Thus, sensitive validated biomarkers for early detection of the tumor and a more accurate prediction of disease outcome as well as patient response to treatments can significantly improve efficacy of the treatments and greatly decrease the mortality of gastric cancer.

Sphingosine-1-phosphate (S1P) has been identified to play an important regulatory role in proliferation, inflammation, vasculogenesis and anti-apoptosis (7). Sphingosine-1-phosphate phosphatase 1 (SGPPI), which is intracellularly localized on endoplasmic reticulum (ER), is responsible for converting S1P to sphingosine (8). *SGPPI* is located in the region 14q23.2 of the chromosome (9). Furthermore, overexpression of SGPPI may elevate ceramide levels and induce apoptosis, whereas knockdown of *SGPPI* enhanced resistance to TNF- $\alpha$ , the chemotherapeutic agent daunorubicin (8) and radiotherapy (10).

In the present study, we aimed to examine the possible role of SGPPI in the progression of gastric cancer and determine whether SGPPI may serve as a prognostic biomarker.

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## Materials and methods

**Materials.** Human gastric cancer cell lines, AGS and HGC27 were obtained from the European Collection of Cell Cultures, (ECACC; Salisbury, UK). Reagents and kits were obtained from Promega Corporation (Madison, WI, USA) and Gibco Invitrogen Corporation (Paisley, Scotland, UK). Anti-SGPP1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), TRI reagent (Sigma-Aldrich, Poole, UK), Universal Z Probe (TCS Biologicals Ltd., Oxford, UK), and the DC Protein Assay kit (Bio-Rad, Hemel Hempstead, UK) were also used in the present study.

**Gastric tissues.** Gastric adenocarcinoma or Siewert type III gastroesophageal junction (GEJ) adenocarcinoma tissues (282 of paraffin-embedded tissues and 218 of fresh-frozen tissues), along with matched normal tissue from the same patients, were collected immediately after surgical resection at the Beijing Cancer Hospital and were stored in the Tissue Bank of Peking University Oncology School. Clinicopathological factors, including age, gender, histological type, Lauren type, tumor location, vascular invasion, TNM stage and lymph node metastasis, were recorded and stored in the patients' database. All protocols were reviewed and approved by the Local Ethics Committee. Informed consent was obtained from the patients before therapy.

The gastric tissues used in the present study were obtained from gastric cancer patients with cT2-4N0M0 or cT1-4N1-3M0 treated between January, 2003 and December, 2011. Gastrectomy with D2 lymphadenectomy was performed and no treatment was conducted before the surgery. Primary tumor site, grade, depth of tumor invasion, status of lymph node metastasis, distant metastasis and TNM stage were recorded in histopathology reports. Pathological stage was determined according to the seventh edition of the TNM staging system recommended by the International Union against Cancer.

**Immunohistochemistry (IHC).** Sections (4 mm) obtained from formalin-fixed, paraffin-embedded tissues were mounted on poly-L-lysine-coated slides and then deparaffinised in xylene and rehydrated through alcohol to distilled water. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min at room temperature. After pressure cooking the slides in 10 mmol/l EDTA (pH 8.0) for 3 min, the sections were incubated with 5% goat serum, then incubated overnight at 4°C with SGPP1 antibody (1:200; Santa Cruz Biotechnology, Inc.) and without the primary antibody as a negative control. Primary antibodies were detected using a two-step EnVision system (Dako, Glostrup, Denmark). Horseradish peroxidase and diaminobenzedene hydrochloride were the enzyme and chromogen used, respectively. Staining score was independently assessed by two pathologists. The percentage of positive cells and the intensity of cytoplasmic staining were analyzed. Thus all final scoring estimations were stratified as follows: -, 0% of stained cells; +, <20% weakly to moderately stained cells; ++, 10-20% intensively stained cells and 20-50% weakly stained cells; and +++, 20-50% positive cells with moderate-to-marked staining or >50% positive cells. There was a low level of discrepancy (<5% cases) among the pathologists in terms of scoring, but a consensus was reached after joint review.

**RNA isolation and RT-PCR.** Total RNA was isolated from the homogenized gastric tissues and cell lines using the Total RNA Isolation reagent (TRI reagent; Sigma-Aldrich). Synthesis of cDNA and subsequent PCR were performed as previously described (11). SGPP1 primers used were: sense, 5'-GGGCAACGAACCTCTTCTAC-3' and antisense, 5'-TCC AGGTGTCAAGAGTGAA-3'.

**Quantitative RT-PCR.** The level of SGPP1 transcripts was quantitatively analyzed with the iCycler iQ5 system with qPCR Master Mix (both from Bio-Rad) as previously described (12). SGPP1 primers designed using the Beacon Design software (Premier Biosoft, Palo Alto, CA, USA) were as follows: sense, 5'-ATGGACAAGCATCCCTTCC-3' and antisense, 5'-ACTGAACCTGACCGTACACTCTGTCAGGG AAATACCAA3-3'. The underlined sequence in the reverse primers was the additional Z sequence, which is complementary to the Universal Z Probe (TCS Biologicals Ltd., Oxford, UK). Internal standard GAPDH primer sequences used were: sense, 5'-CTGAGTACGTCGTGGAGTCC-3' and antisense, 5'-ACTGAACCTGACCGTACAGAGATGATGACCCTT TTG-3'. To exclude the effect of tissue heterogeneity, the SGPP1 quantification was normalized against the corresponding CK19 (an epithelial marker) of each individual sample. The primers used for CK19 were: sense, 5'-CAGGTC CGAGGTTACTGAC-3' and antisense, 5'-ACTGAACCTGAC CGTACACCGTTTCTGCCAGTGTGTCTTC-3'.

**Construction of SGPP1 ribozyme transgenes and transfection.** Anti-SGPP1 ribozyme transgenes were used to knock down the expression of SGPP1 in the AGS and HGC27 gastric cancer cells and were generated using the methods previously described (13). Briefly, an anti-SGPP1 hammerhead ribozyme was designed based on the secondary structure of SGPP1 mRNA and generated using the Zuker's RNA mFold program. The ribozymes that specifically target DAP3 were generated using the touchdown PCR with the appropriate primers (sense, 5'-CTGCAGTTCAACCACTTCTCCCAGAGCTGATGAGT CCGTGAGGA-3' and antisense, 5'-ACTAGTAGAGAAAGC ACTGAGAAAGGGAGTTTCGTCCTCACGGACT-3'). The amplified ribozymes were cloned into the pEF6/V5-His TOPO TA plasmid vector (Invitrogen, Paisley, UK) in accordance with the protocol provided. Ribozyme transgenes and control plasmids were transfected into HGC27 and AGS cells individually using an Easyjet Plus electroporator (EquiBio, Kent, UK). After up to 5 days of selection with blasticidin, the transfectants were verified for knockdown of SGPP1.

**Western blotting.** HGC27 and AGS cells were plated into small flasks at a density of 25x10<sup>5</sup> cells/well. Proteins were isolated from cells by lysis buffer. Protein concentrations were determined using the DC Protein Assay kit (Bio-Rad) and an ELx800 spectrophotometer (Bio-Tek, Bedfordshire, UK). Western blot analysis was performed after SDS-PAGE and transferred onto membranes. The proteins were probed with the anti-SGPP1-antibody (1:500) and anti-GAPDH-antibody (1:1,000) (Santa-Cruz Biotechnology, Inc.) as an internal control, followed by a peroxidase-conjugated secondary antibody (1:1,000). Protein bands were visualized and photographed using a UVITech imager (UVITech, Inc., Cambridge, UK).

Table I. Association of SGPP1 expression with clinicopathological characteristics in gastric cancer patients.

Clinicopathological characteristics	SGPP1 expression		P-value
	Negative (%)	Positive (%)	
Gender			0.357
Male	138 (71.1)	56 (28.9)	
Female	65 (76.5)	20 (23.5)	
Age (years)			0.616
≤60	100 (71.4)	40 (28.6)	
>60	103 (74.1)	36 (25.9)	
Tumor location			0.016
Upper 1/3	37 (61.7)	23 (38.3)	
Middle 1/3	45 (67.2)	22 (32.8)	
Low 1/3	112 (78.3)	31 (21.7)	
Total	9 (100.0)	0 (0.0)	
Cardia and non-cardia			0.007
Non-cardiac cancer	175 (76.1)	55 (23.9)	
Cardiac cancer	28 (57.1)	21 (42.9)	
Tumor size (cm)			0.855
≤4.0	112 (73.2)	41 (26.8)	
>4.0	91 (72.2)	35 (27.8)	
Lauren type			0.560
Intestinal type	40 (70.2)	17 (29.8)	
Diffuse	116 (75.3)	38 (24.7)	
Mixed type	47 (69.1)	21 (30.9)	
Differentiation			0.216
Well-moderate	90 (69.2)	40 (30.8)	
Poor	113 (75.8)	36 (24.2)	
Well	7 (58.3)	5 (41.7)	0.474
Moderate	83 (70.3)	35 (29.7)	
Poor	101 (76.5)	31 (23.5)	
Signet	12 (70.6)	5 (29.4)	
Histology			0.464
Adenocarcinoma	169 (71.9)	66 (28.1)	
Other types <sup>a</sup>	34 (77.3)	10 (22.7)	
Lymphovascular invasion			0.486
Absent	100 (70.9)	41 (29.1)	
Present	103 (74.6)	35 (25.4)	
Depth of invasion			0.385
T <sub>1</sub>	14 (77.8)	4 (22.2)	
T <sub>2</sub>	25 (67.6)	12 (32.4)	
T <sub>3</sub>	7 (100.0)	0 (0.0)	
T <sub>4</sub>	157 (72.4)	60 (27.6)	
T <sub>1</sub> +T <sub>2</sub>	39 (42.5)	16 (57.5)	0.731
T <sub>3</sub> +T <sub>4</sub>	164 (26.0)	60 (74.0)	
T <sub>1</sub>	14 (77.8)	4 (22.2)	0.787
T <sub>2</sub> +T <sub>3</sub> +T <sub>4</sub>	189 (72.4)	72 (27.6)	
Lymph node metastasis			0.005
N <sub>0</sub>	46 (71.9)	18 (28.1)	
N <sub>1</sub>	28 (53.8)	24 (46.2)	
N <sub>2</sub>	35 (76.1)	11 (23.9)	
N <sub>3</sub>	94 (80.3)	23 (19.7)	
No	46 (71.9)	18 (28.1)	0.856
Yes	157 (73.0)	58 (27.0)	

Table I. Continued. Association of SGPP1 expression with clinicopathological features in gastric cancer patients.

Clinicopathological features	SGPP1 expression		P-value
	Negative (%)	Positive (%)	
Distant metastasis			0.031
No	177 (70.8)	73 (29.2)	
Yes	26 (89.7)	3 (10.3)	
TNM stage			0.171
I	26 (74.3)	9 (25.7)	
II	28 (68.3)	13 (31.7)	
III	123 (70.7)	51 (29.3)	
IV	26 (89.7)	3 (10.3)	
I	26 (74.3)	9 (25.7)	0.828
II-IV	177 (72.5)	67 (27.5)	
I+II	54 (71.1)	22 (28.9)	0.695
III+IV	149 (73.4)	54 (26.6)	

<sup>a</sup>Other types: Signet-ring cell carcinoma and mucinous adenocarcinoma. SGPP1, sphingosine-1-phosphate phosphatase 1.

*Cell growth assay.* Crystal violet assay was conducted as previously described (11). Cells were added into 96-well plates at  $2 \times 10^3$  cells/well. The cells were fixed in 4% paraformaldehyde at room temperature for 10 min after 1, 3 and 5 days. After being washed, the plates were stained with 0.5% crystal violet solution. The plates were washed with tap water and air-dried. Acetic acid (10%) was added to each well for extraction of dye. Growth rates under normal conditions and under treatment were assessed. Absorbance of the staining was determined by a spectrophotometer at 540 nm (ELx800; Bio-Tek).

*Cell matrix adhesion assay.* The cell matrix adhesion assay was performed as previously described (14). Cells were added to a 96-well plate precoated with Matrigel (5 mg/well) ( $2 \times 10^4$  cells/well). After 40 min of incubation, non-adherent cells were washed off using BSS buffer. The remaining cells were fixed with 4% paraformaldehyde, stained with 0.5% crystal violet solution and counted.

*Wound-healing assay.* The motility of gastric cancer cells was analyzed using a wound-healing assay as previously described (15). The cells were grown until they reached confluence. A scrape in the cell monolayer was made in one direction with a fine gauge needle. The wounded cell monolayers were washed with PBS to remove cell debris. The migration of the invading cell front was recorded on a time lapse video recorder and analyzed using Optimas 6.0 motion analysis (Meyer Instruments, Houston, TX, USA).

*Invasion assay.* The *in vitro* Matrigel invasion assay was performed as previously described (15). Transwell inserts with 8-mm pore size were coated with 50  $\mu$ g of Matrigel (Collaborative Research Products, Bedford, MA, USA) and air-dried. Following rehydration,  $4 \times 10^4$  cells were added

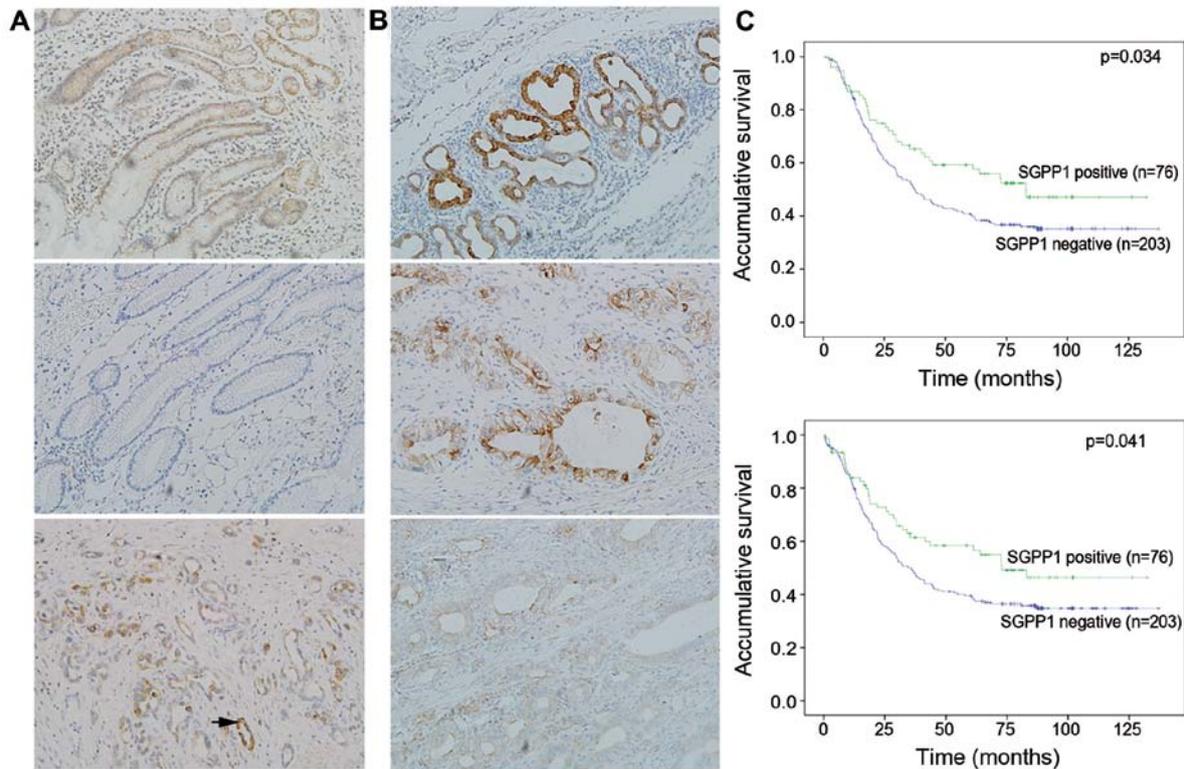


Figure 1. (A) Representative images of immunohistochemical staining of SGPP1 on gastric tissues including the positive control (top panel), the negative control (middle panel) and the location of cytoplasm (bottom panel, with arrow pointing to the staining of SGPP1 in the cytoplasm of cancer cells). (B) Representative images of immunohistochemical staining of SGPP1 on gastric tumor tissues including the strong (top panel), moderate (middle panel) and weak (bottom panel) expression. (C) Kaplan-Meier survival analysis indicated that the relationship between the protein levels of *SGPP1* and overall survival (top) and the relationship between the protein levels of *SGPP1* and progression-free survival (bottom). SGPP1, sphingosine-1-phosphate phosphatase 1.

to each well. After 3 days of incubation, the cells that had migrated through the matrix to the other side of the insert were fixed, stained and counted.

**Statistical analysis.** Statistical analysis was performed using the SPSS software (SPSS standard version 13.0; SPSS Inc.). The relationship between *SGPP1* expression and tumor grade, TNM staging and nodal status was assessed using the Mann-Whitney U test and Kruskal-Wallis test. Survival was analyzed using the Kaplan-Meier survival analysis. Results are presented as mean  $\pm$  SEM.  $P < 0.05$  was considered statistically significant.

## Results

**Immunohistochemical staining of SGPP1 in human gastric specimens.** In total, 282 paraffin-embedded specimens of gastric cancer patients from Beijing Cancer Hospital were included in the IHC study. This cohort comprised 196 males (69.5%) and 86 females (31.5%), with a median age of 69 (22-87) years. The patients underwent surgery without any prior treatment. The median follow-up time was 41.13 (1.00-137.17) months. The result was that, 36 patients had recurrence, 130 patients succumbed to gastric cancer, 15 patients succumbed to other causes (heart attack and cerebral haemorrhage), 11 were lost in follow-up and 107 remained alive and progression-free.

Negative staining results were considered as low expression and '+', '++', '+++' as high expression. *SGPP1* was mainly

detected in cytoplasm and the staining was obviously stronger in normal tissue compared with tumor tissues ( $p < 0.001$ ). A low expression of *SGPP1* was positively associated with lymph node metastasis ( $p = 0.005$ ) and distant metastasis ( $p = 0.031$ ) (Table I). Kaplan-Meier survival curves revealed that patients with a high *SGPP1* expression had a significant increase in OS and progression-free survival (PFS) in gastric cancer patients ( $p = 0.034, 0.041$ ; Fig. 1). Multivariate analysis indicated the expression of *SGPP1* was an independent prognostic factor in gastric cancer patients ( $p = 0.041$ ; Table II).

**Quantification of SGPP1 mRNA expression in human gastric cancer.** mRNA derived from the 219 gastric cancer patient tissues from the Beijing Cancer Hospital were subjected to a *SGPP1* gene-specific qPCR study. This cohort comprised 144 males (65.8%) and 75 females (34.2%). The patients underwent the surgery without any prior treatment. The result was that, 86 patients were alive, 130 patients succumbed to gastric cancer, 9 patients had metastasis and 117 remained disease-free.

We compared the transcript levels of *SGPP1* in gastric cancer tissues with adjacent normal tissues of patients. The transcript level of *SGPP1* was significantly different in the T stage ( $p = 0.009$ ) and TNM stage ( $p = 0.0255$ ). Statistical analysis revealed significant links between the different clinical outcomes ( $p = 0.0379$ ) and different transcript levels. A markedly decreased transcript of *SGPP1* was observed in tumor tissues compared with the normal background tissues ( $p < 0.0001$ ; Table III).

Table II. Patient survival associated with clinicopathological characteristics in gastric cancer.

Clinicopathological characteristics	Univariate analysis			Multivariate analysis		
	RR	95% CI	P-value	RR	95% CI	P-value
Gender						
Male vs. female	1.211	0.858-1.710	0.276			
Age (years)						
≤60 vs. >60	0.787	0.579-1.068	0.125			
Tumor location						
Upper 1/3	0.380	0.184-0.784	0.009			
Middle 1/3	0.212	0.101-0.446	0.230			
Low 1/3	0.257	0.128-0.514	0.118			
Multiple site	0.000					
Tumor size (cm)						
>4.0 vs. ≤4.0	0.661	0.487-0.897	0.008			
Lauren type						
Intestinal vs. diffuse/mixed	1.042	0.718-1.514	0.828			
Differentiation						
Well-moderate vs. poor	1.270	0.935-1.726	0.126	1.411	1.017-1.957	0.039
Histology						
Adenocarcinoma vs. other types <sup>a</sup>	1.264	0.851-1.877	0.246			
Lymphovascular invasion						
Absent vs. present	2.072	1.519-2.825	<0.001	1.449	1.046-2.007	0.026
Depth of invasion						
T <sub>1</sub>	0.000		0.938			
T <sub>2</sub>	0.160	0.160-0.544	0.000			
T <sub>3</sub>	0.153	0.153-2.499	0.500			
T <sub>4</sub>	1		0.001			
T <sub>1</sub> +T <sub>2</sub> vs. T <sub>3</sub> +T <sub>4</sub>	5.375	2.911-9.924	<0.001	2.627	1.372-5.033	0.004
Lymph node metastasis						
N <sub>0</sub>	0.118	0.066-0.211	<0.001			
N <sub>1</sub>	0.241	0.148-0.391	<0.001			
N <sub>2</sub>	0.673	0.431-0.942	0.024			
N <sub>3</sub>	1.000		<0.001			
No vs. yes	5.391	3.055-9.513	<0.001	3.628	1.990-6.614	<0.001
Distant metastasis						
No vs. yes	0.168	0.110-0.256	<0.001	0.277	0.179-0.428	<0.001
SGPPI						
Negative vs. positive	0.673	0.465-0.973	0.036	0.671	0.458-0.985	0.041

<sup>a</sup>Other types: Signet-ring cell carcinoma and mucinous adenocarcinoma. CI, confidence interval; RR, relative risk; SGPPI, sphingosine-1-phosphate phosphatase 1.

*Knockdown effect of SGPPI on the functions of gastric cancer cells.* The expression of *SGPPI* in HGC27 and AGS cells transfected with corresponding anti-*SGPPI* ribozyme transgenes was examined using conventional PCR. The results showed a significantly lower expression level of *SGPPI* compared with the control group (2- to 3-fold) (Fig. 2A), and the same knockdown effect was observed in qPCR and western blot analysis (2- to 3-fold) (Fig. 2B and C).

*Migration and invasion assay.* To determine whether downregulating the expression of *SGPPI* affected the biological behavior of gastric cancer cell lines, we performed a wound-healing assay of the cells. The AGS and HGC27 cells transfected with anti-*SGPPI* ribozyme exhibited a significant increase in the ability of migration of tumor cells compared with the control cells. A significant difference was identified for the two cells ( $p < 0.05$ , 2- to 5-fold; Fig. 3A).

Table III. Correlation of the mRNA expression of SGPP1 and clinical parameters.

Category	No.	Median	Q1	Q3	P-value
T/Nb					<0.001
Normal	183	2.50	0	56	
Tumor	322	0.10	0	21	
Gender					0.7876
Male	229	0.10	0	16	
Female	93	0.10	0	29	
Location					
Cardia	66	0.30	0	53	
Fundus	21	2.90	0	52	0.3310
Corpus	61	0.10	0	27	0.4796
Pylorus	130	0.00	0	16	0.1696
Differentiation					
Diff-H	1	0.00	N/A	N/A	
Diff-HM	6	0.03	0	1.69	
Diff-M	62	0.03	0	6	0.3812
Diff-ML	81	0.08	0	28	0.2121
Diff-L	137	0.10	0	29	0.2510
T stage					
T1	16	0.01	0	0.64	
T2	25	0.00	0	0	0.3706
T3	31	6.20	0	83	0.0586
T4	232	0.08	0	26	0.2526
T1+T2	41	0.00	0	0	0.009
T3+T4	273	0.10	0	28	
N stage					
N0	71	0.00	0	5	
N1	48	0.00	0	8	0.8221
N2	64	0.00	0	24	0.1375
N3	133	0.00	0	45	0.0244
N0	133	0.00	0	45	0.0519
N1+N2+N3	245	0.00	0	29	
M stage			0		0.7139
M0	280	0.10	0	13	
M1	41	0.00	0	93	
TNM stage			0		
I	25	0.00	0	0.60	
II	59	0.02	0	10	0.5474
III	220	0.20		30	0.0516
IV	9	1.00	0	258	0.0334
I+II	84	0.00	0	4	0.0255
III+IV	229	0.00	0	32	
Vascular invasion					0.6462
No invasion	152	0.00	0	23	
Invasion	155	0.10	0	19	
Clinical outcome			0		0.0379
Alive	134	0.00	0	4	
Dead	185	0.40	0	45	

SGPP1, sphingosine-1-phosphate phosphatase 1.

The effect of *SGPP1* knockdown on cell lines on migration was investigated using an *in vitro* invasion assay. The decrease in the expression of *SGPP1* was significantly correlated with an increase in cell invasion in AGS and HGC27 cell lines ( $p < 0.05$ , 3-fold; Fig. 3B).

*Adhesion and growth assay.* An *in vitro* matrix adhesion assay was used to investigate the effect of *SGPP1* knockdown on cell lines on the adhesion ability of gastric cancer cell lines. The HGC27 and AGS cell lines transfected with *SGPP1* ribozyme significantly inhibited matrix adhesion ability compared with the control cell line ( $p < 0.05$ , 2- to 5-fold; Fig. 4A).

Furthermore, to determine whether the expression of *SGPP1* affected the growth of gastric cancer cells, we carried out a growth assay. The results showed that the HGC27 cell line transfected with *SGPP1* ribozyme markedly inhibited growth on day 5 ( $p < 0.05$ ) and there was no significant difference on day 3, whereas this effect was not observed in the AGS cell line transfected with *SGPP1* ribosome (Fig. 4B).

## Discussion

Gastric cancer has become the fifth most common cancer worldwide over the past decades. There are ~480,000 new patients developing this cancer in China each year (1). At the same time, gastric cancer, whose estimated mortality rates ranks the highest in China, has also become the third leading cause of cancer mortality worldwide. Although the incidence rates of gastric cancer have gradually decreased (16), ~80% of patients with gastric cancer in China are at an advanced stage (17).

Despite new developments in the treatment for advanced gastric cancer patients, such as adjuvant chemotherapy (18), new adjuvant chemotherapy and concurrent chemoradiotherapy (19) plus surgery or surgery, the 5-year survival rate of patients has only been marginally improved (20). The main reason for this lack of significant improvement is mostly due to the fact that diagnosis is frequently made after progression to later stages, at which point current therapeutic strategies exert limited effectiveness. Furthermore, the major challenge to cancer treatment is the recurrence of diseases due to therapeutic resistance. In many patients, microscopic or macroscopic metastases have already occurred by the time the *in situ* lesion is detected. Therefore, early detection and more accurate methods in predicting disease outcomes, may enable physicians to make informed decisions regarding the potential necessity of early intervention. Consequently, early intervention would significantly decrease the mortality of gastric cancer and greatly improve the 5-year survival rate.

SIP is a bioactive sphingolipid that is important in regulating diverse biological processes (21). SIP is a component of cell membranes with high amphotericity, which enables SIP to possess sufficient aqueous solubility to move between membranes (22-24) as a bioactive signalling molecule. SIP is known to be involved in the regulation of diverse biological behavior. Particularly, SIP has emerged as an important signalling mediator participating in the regulation of various cell processes and diseases, including cancer, wound healing, atherosclerosis and immunity. SIP functions through either a family of five G protein-coupled membrane receptors known as *SIPRI-5* (25,26) or intracellular targets,

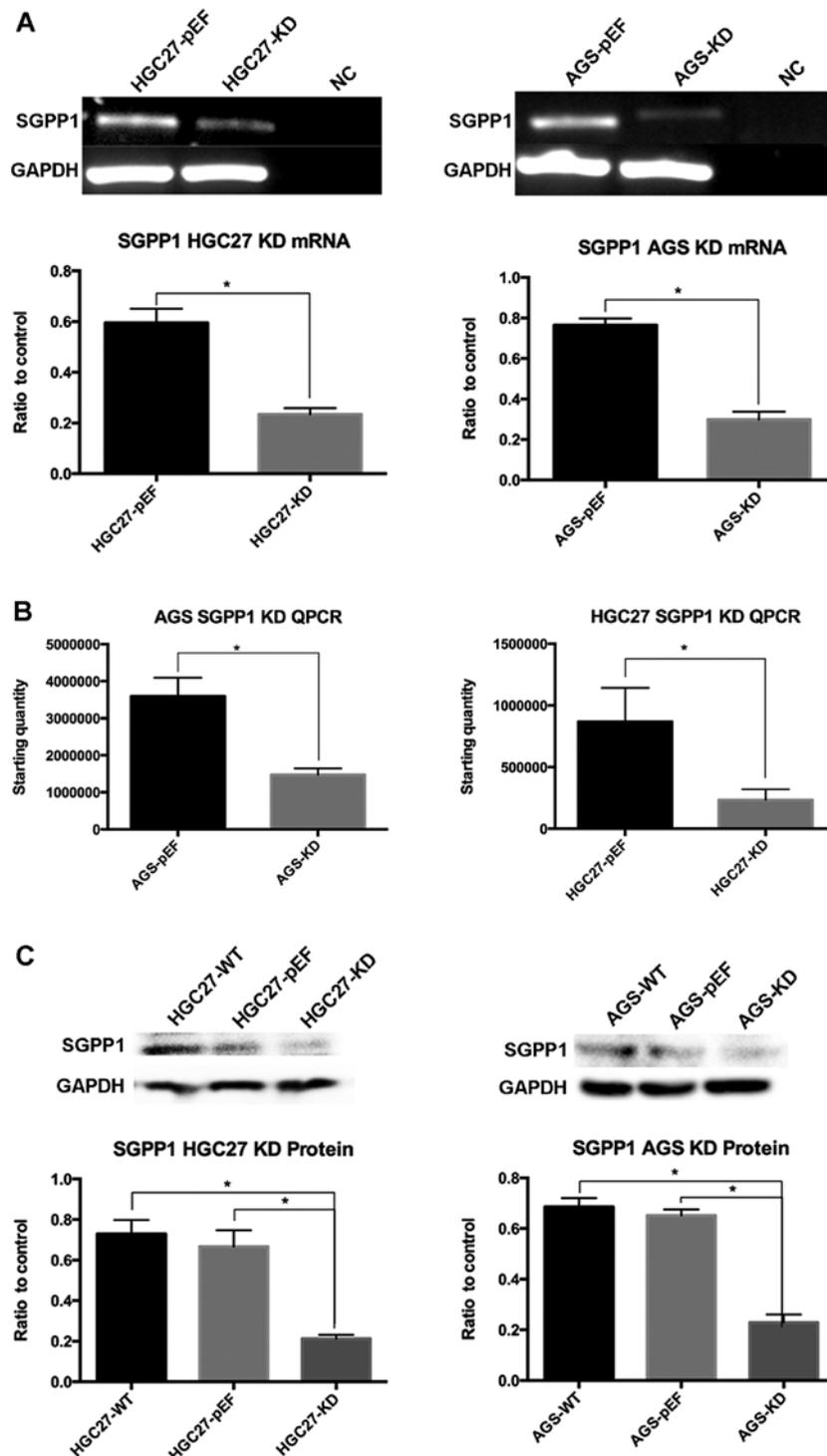


Figure 2. (A) Representative image of RT-PCR analysis of efficacy of SGPP1 knockdown in HGC27 and AGS cells, respectively, with correspondent densitometric analysis [presented as the relative ratio of *SGPP1* to GAPDH, (n=3)]. (B) RT-qPCR analysis confirmed *SGPP1* knockdown in HGC27 and AGS cells, respectively. (C) Western blot analysis of SGPP1 protein levels in HGC27 and AGS cells used in response to *SGPP1* knockdown. P<0.05, statistically significant. SGPP1, sphingosine-1-phosphate phosphatase 1.

such as, prohibitin 2 (27), TNF receptor-associated factor 2 (TRAF2) (28), and histone deacetylase (HDAC) (29). Through interaction of these intracellular targets, SIP regulates a wide variety of biological effects (30), such as cell movement, differentiation, survival, inflammation, immunity, calcium homeostasis, and angiogenesis.

SIP may be dephosphorylated by SGPPs which convert SIP to sphingosine. Previous studies have shown that SIP level

has a 2-fold increase in the SGPPs knockdown cell lines (8). There are two isoforms of SGPPs, *SGPP1* (31) and *SGPP2* (32). SGPPs can dephosphorylate SIP to regenerate sphingosine, leading to SIP inactivation. This, in turn, would lead cells to apoptosis as well as degeneration (9). SGPPs were highly selective for sphingolipid substrates (33). SGPPs have also been reported to be regulated by other factors. For example, laminar shear stress also downregulates the expression of *SGPP1* while

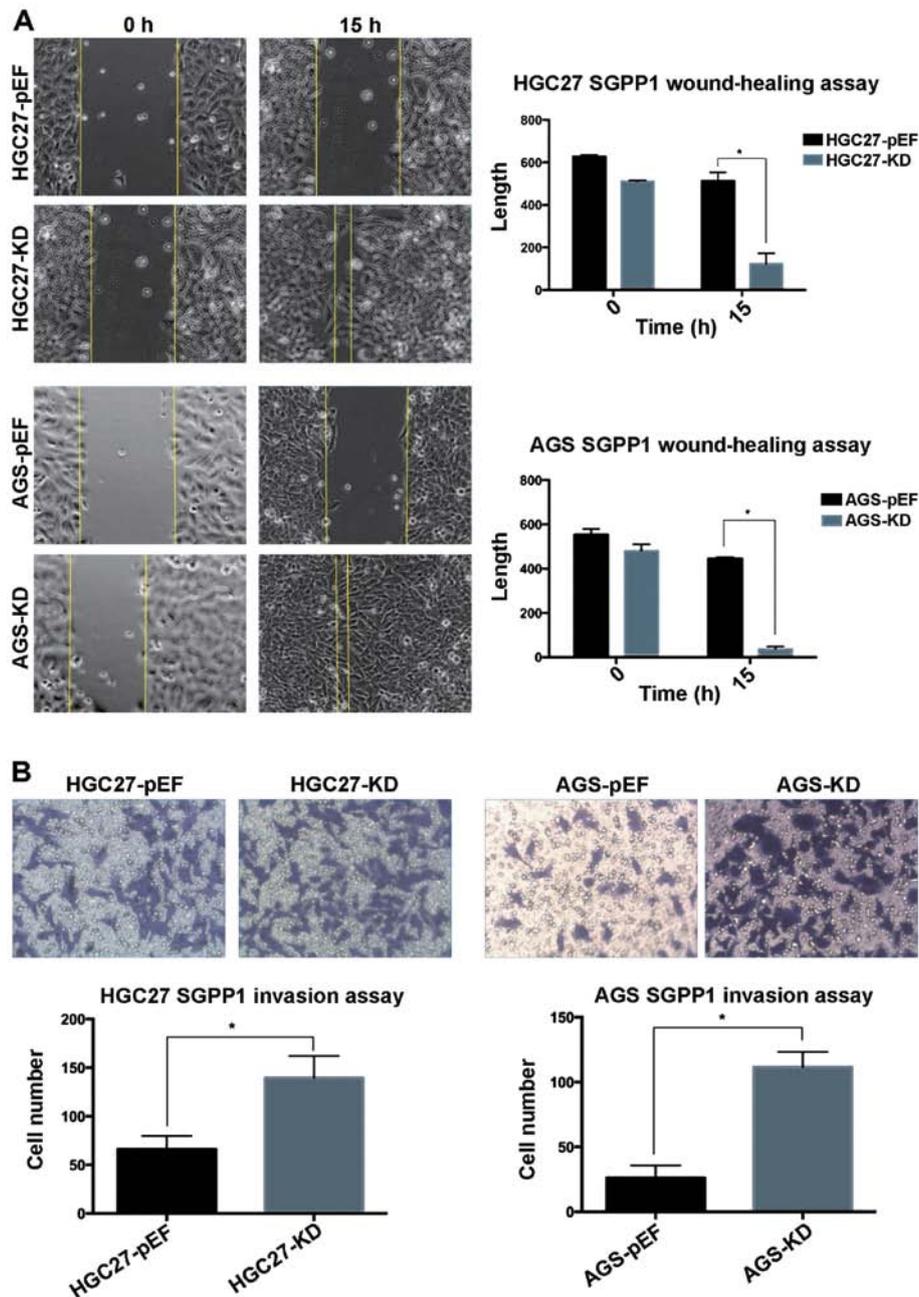


Figure 3. (A) Wound-healing assay was used to assess the effect of *SGPP1* knockdown on the migration ability of the gastric cancer cells. HGC27 cells (top panel) and AGS cells (bottom panel). (B) The influence of *SGPP1* knockdown on the invasive ability of HGC27/AGS cells. HGC27 cells (left panel) and AGS cells (right panel). Data shown are the mean value of three experiments in triplicate. Experiments were repeated at least three times. Data are presented as mean  $\pm$  SEM; \* $p < 0.05$ . *SGPP1*, sphingosine-1-phosphate phosphatase 1.

concomitantly stimulating *SIP* released from endothelial cells *in vitro* (34). *SGPP1* may also control the unfolded protein response and ER stress-induced autophagy (35), as well as vascular tone (36). Overexpression of *SGPPs* may elevate ceramide levels and provoke apoptosis, whereas knockdown of *SGPP1* enhanced resistance to  $TNF-\alpha$  and the chemotherapeutic agent daunorubicin (8) and radiotherapy (10) (Fig. 5).

In this study, we examined the function of *SGPP1* on gastric cancer cell lines as well as its clinical significance in gastric cancer progression. *SGPP1* is a potential biomarker candidate for early diagnosis and/or prognosis for patients with advanced gastric cancer. By accessing a relative large cohort of gastric

cancer, we retrospectively evaluated the relationship between the level of *SGPP1* expression and clinical characteristics. Our results showed that the patients who had a higher level of *SGPP1* expression had a longer OS and PFS compared with a lower level of *SGPP1* expression. Our study has demonstrated that a positive expression of *SGPP1* was an early diagnosis of improved clinical outcome in gastric cancer patients. In addition, the study showed that *SGPP1* expression was statistically associated with the location of tumor and lymph node metastasis in gastric cancer patients. Moreover, multivariate survival analysis showed that *SGPP1* was an independent prognostic factor. Based on these results, we suggest that *SGPP1* was a

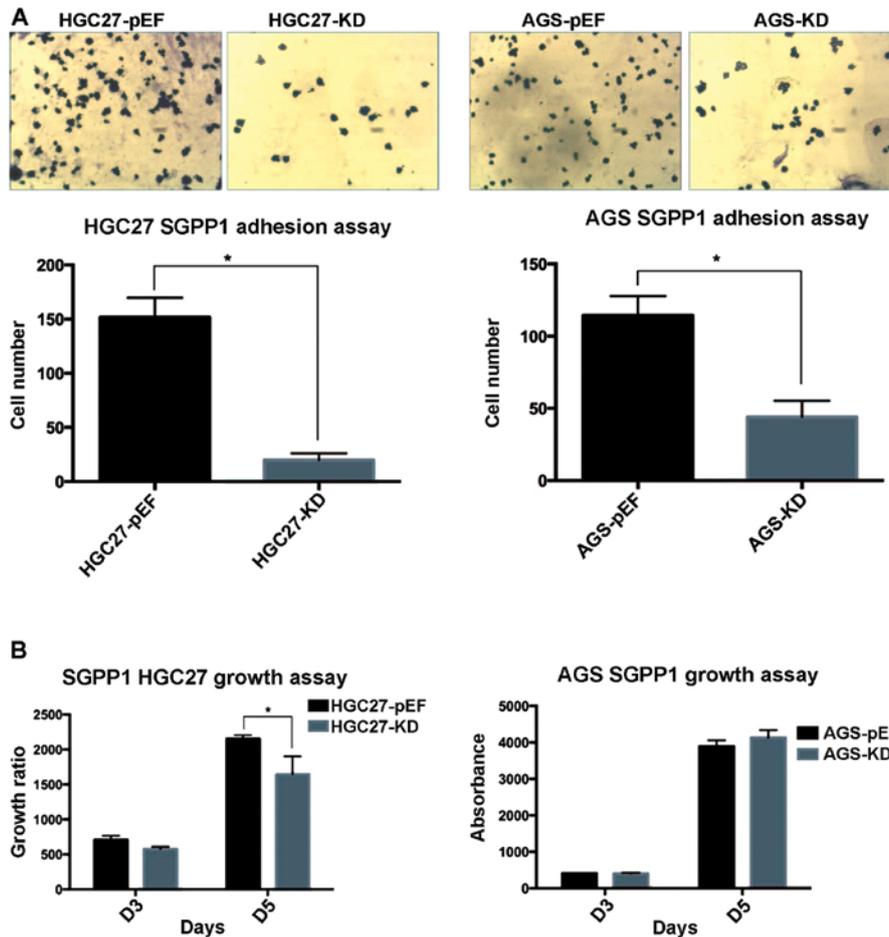


Figure 4. (A) *In vitro* adhesion test in AGS and HGC27 cell lines. Both HGC27 *SGPP1* KD cell (left panel) and AGS KD cell (right panel) showed less adhesion ability compared with the control cell lines. (B) *In vitro* growth test in AGS and HGC27 cell lines; HGC27 *SGPP1* KD cell grew slower than the corresponding control cell lines on day 5 (left panel), although no significant difference in AGS knockdown cell was observed (left panel). Experiments were repeated at least three times. Data are represented as mean  $\pm$  SEM; \* $p$ <0.05. *SGPP1*, sphingosine-1-phosphate phosphatase 1; KD, knockdown.

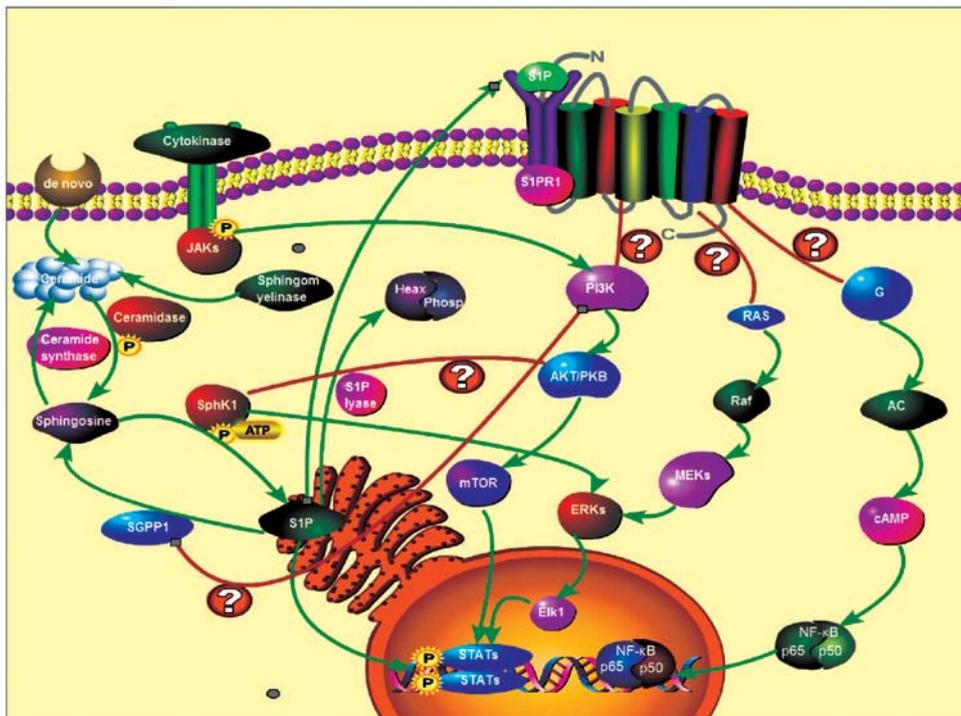


Figure 5. Diagram depicting S1P signaling pathway. S1P, sphingosine-1-phosphate.

novel molecular prognosticator in the evaluation of gastric cancer patient survival.

Invasion and metastasis are major obstacles in the effort to improve survival of gastric cancer patients (37). Our *in vitro* study showed that the cell ability of invasion and migration was markedly increased in the gastric cell lines which the expression of *SGPP1* is downregulated compared with the negative control. This phenomenon is similar to the observation in the data, showing a significant association with lymph node metastasis. Collectively, we suggest that *SGPP1* serves as a novel prognostic marker of cancer cell invasion and metastasis. In addition, we found that downregulating the expression of *SGPP1* affected the adhesion and growth ability in HGC27 cells but no significant change occurred in AGS cells. The reason for this phenomenon may be that the cancer cell decreases the ability of adhesion from the primary location and at the same time increases the ability of invasion and metastasis and epithelial-mesenchymal transition

In conclusion, the result show that *SGPP1* expression is significantly lower in tumor tissue than that in the normal paired tissue, while downregulation of *SGPP1* leads to an increase in cell migration and invasion in gastric cancer. A lower *SGPP1* expression is correlated with lymph node metastasis and *SGPP1* expression knockdown may lead to a more aggressive invasion and migration ability. Therefore, there is a significant correlation between *SGPP1* expression and OS, PFS in the gastric cancer cohort. Taken together, the results indicate *SGPP1* is a potential molecular marker that may be used to predict the effectiveness of prognosis in gastric cancer patients.

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