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High Expression of Pregnancy Specific Beta-1-glycoprotein 1 Is Associated With Poor Gastric Cancer Prognosis

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Abstract. *Background/Aim: Pregnancy specific beta-1-glycoprotein 1 (PSG1) is a member of the immunoglobulin superfamily and associated with carcinoembryonic antigens. It has been reported to be highly expressed in variety of cancers. However, the role of PSG1 in gastric cancer remains unclear. The aim of our study was to examine the clinical significance and functional role of PSG1 in gastric cancer. Materials and Methods: We analyzed the association between PSG1 expression levels and clinicopathological features using Kaplan-Meier survival curves and publicly available microarray data. In gastric cancer cell lines, PSG1 expression levels were detected by polymerase chain reaction and western blot analysis. The functional role of PSG1 on the proliferation, migration and invasive abilities were also investigated using PSG1 siRNA or an over-expression plasmid vector through WST, transwell migration and invasion assays. Results: PSG1 expression levels were higher in gastric cancer patient tissues than in normal gastric tissues. Increased expression of PSG1 was associated with poor patient prognosis. Knockdown of PSG1 inhibited cell proliferation, migration, and invasion in gastric cancer cells. In contrast, over-expression of PSG1 enhanced cell proliferation, migration, and invasion. Conclusion: PSG1 is up-regulated in gastric cancer and may serve as an oncogene that promotes cell proliferation, migration, and invasion. PSG1 is an independent prognostic factor for the progression of gastric cancer and may be a potential biomarker and therapeutic target for gastric cancer.*

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Key Words: Pregnancy specific beta-1-glycoprotein 1 (PSG1), gastric cancer, cell proliferation, migration, invasion.

Gastric cancer (GC) is the fourth most common cause of cancer deaths worldwide (1, 2). In particular, it shows a specifically high incidence in northeast Asia (3, 4). Although GC is a major cancer with high incidence and death rates, studies on GC are lacking. Despite various efforts (resection, radiation, anti-cancer drugs, *etc.*) to treat cancer, the prognosis of GC patients is still poor (5-9). Therefore, continuous research on GC is needed. Early diagnosis of GC greatly improves patient survival rate. However, most GC patients are discovered after aggressive tumor progression. In this case, patients have poor prognosis due to resistance to chemotherapy or the development of metastatic ability (10, 11). Therefore, additional studies on early diagnosis of gastric tumor, chemoresistance and metastasis are essential to improve the prognosis of GC patients. Understanding the underlying mechanisms for the occurrence and progression of GC is one of these efforts. Our study has focused on finding molecules involved in these mechanisms.

Pregnancy-specific glycoproteins (PSGs) belong to the immunoglobulin superfamily and contain four immunoglobulin (Ig) domains (12, 13). There are 10 genes (PSG1-9, PSG11) encoding human PSGs (14). PSG is a placental protein mainly expressed during pregnancy and is known to play a role in pregnancy, such as immune regulation, angiogenesis, and platelet function (15, 16). Among them, pregnancy specific beta-1-glycoprotein 1 (PSG1 as known as SP1) is a pregnancy-related glycoprotein primarily expressed in the placenta. It has been proposed to play a role in various processes including implantation, trophoblast differentiation, activation of angiogenesis, and resolution steps during pregnancy (17, 18). PSG1 is related to TGF-β and mainly regulates the vascular endothelial growth factor family (VEGF A, B, C and D) and placental growth factor (PGF) (18, 19). Abnormal expression of PSG1 prevented stable pregnancy (20, 21).

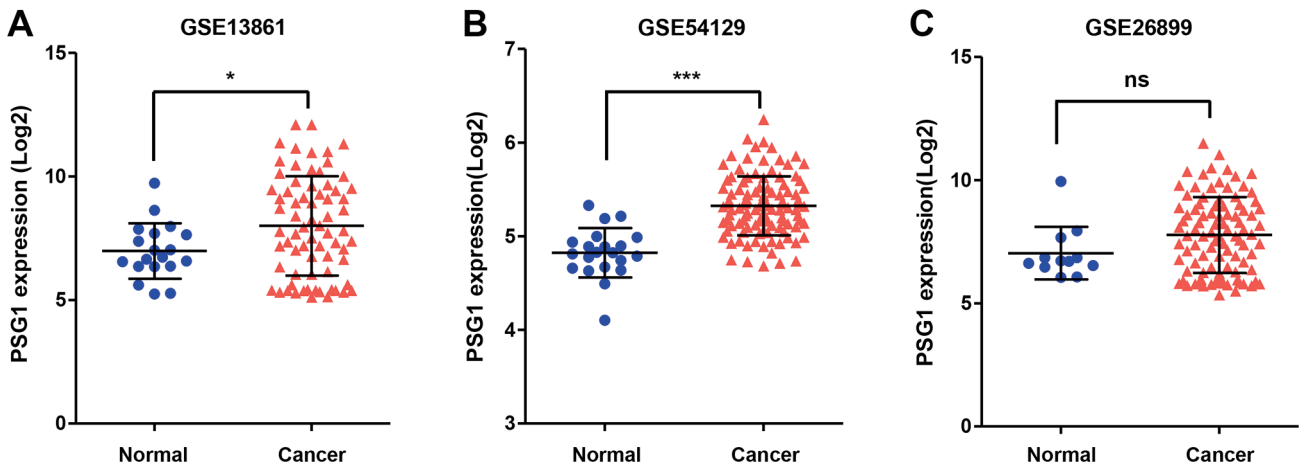

Figure 1. *Expression levels of PSG1 in GC patients. (A-C) mRNA expression levels of PSG1 in GC patient samples are presented as a scatter diagram using GEO datasets [GSE13861 (A), GSE54129 (B), GSE26899 (C)]. The significant differences are indicated by asterisks (***p<0.001, *p<0.05).*

PSG1 is associated with carcinoembryonic antigen (CEA) and has been reported to be expressed in various cancers (22, 23). In particular, PSG1 is up-regulated in GC and is used as a biomarker for diagnosis of GC. However, nothing is known about the role of PSG1 in GC. Therefore, we examined the role of PSG1 in the progression of GC.

Materials and Methods

Cell culture. Seven human GC cell lines (AGS, MKN-28, YCC-2, SNU-216, -601, -638 and SNU-668) and a human normal gastric epithelial cell (GES-1) were obtained from the Korean Cell Line Bank (Seoul, Korea) and cultured in RPMI-1640 media (Welgene, Gyeonsan, Republic of Korea) supplemented with 10% fetal bovine serum (FBS; Corning Inc., Corning, NY, USA) and 1% penicillin-streptomycin (antibiotics; Gibco, Waltham, NY, USA) in incubator with 5% CO_2 at 37˚.

Transfection and plasmid construction. Transfection of scrambled RNA (scRNA) or two human PSG1 siRNAs (PSG1 siRNA#1 and siRNA#2) was performed using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The sequences of PSG1 siRNAs were obtained from Genolution Inc. (Seoul, Republic of Korea) were as follows; #1: 5'-CGAAGUCUCUGGUAAGUGG -3' and #2 5'-CUCUUUAUCCGCCAUAUUA -3'. Human PSG1 cDNA was cloned into the pCMV–3Tag–1A plasmid between Hindlll and Xho1 restriction enzyme sites to obtain the pCMV–3Tag–1A_PSG1 construct. PSG1 coding sequence was amplified with PCR using the primers: 5'-AAGCTTATGGGAACCCTCTCAGCCCC -3' (Sense) and 5'- CTCGAGTCAGGGAACTGTCCAGTCAGAG -3' (Antisense).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from seven GC cell lines and normal gastric epithelial cells using RNA iso plus (Takara, Shiga, Japan) according to the manufacturer's protocol. Reverse transcription was carried out using a 5x RT Master Mix (Toyobo, Osaka, Japan), and PCR was performed using 2×TOP simple™ DyeMIX – nTaq polymerase (Enzynomics, Daejeon, Republic of Korea). The primer sequences were as follows; PSG1: 5'-GACTCCAGACGCAAGCTACC -3' (Sense) and 5'-GCACTCACTGGGTTCCGTAT -3' (Anti-sense); GAPDH: 5'-GGCTGCTTTTAACTCTGGTA -3' (Sense) and 5'-ACTTGATTTTGGAGGGATCT -3' (Anti-sense). GAPDH was used as an internal control.

Western blot analysis. Western blot analysis was performed using AGS and MKN-28 cell lysates. The detailed protocol has been described in a previous study (24). Anti-PSG1 and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Dallas, CA, USA). β-actin was used as a loading control. Antibodies were detected with ECL solution (Bio-Rad, Hercules, CA, USA) using Supernova-Q1800 (Centronics, Daejeon, Republic of Korea).

WST assay. GC cells were seeded in 96-well plates (each 5×10^3 cells). After incubation for 24 h, AGS cells were transfected with small interfering RNA (scRNA or two PSG1 siRNAs) and MKN-28 cells were transfected with an expression vector plasmid (pCMV–3Tag–1A_Empty or pCMV–3Tag–1A_PSG1). After 48 h of transfection, WST-8 solution (EZ-Cytox, DoGenBio, Seoul, Republic of Korea) was added to each well. The plates were re-incubated and absorbance was then measured every 30 min at 450 nm using a UV spectrophotometer (SPARK: Tecan, Menedorf, Switzerland).

Colony formation assay. Colony formation assay was performed to evaluate cell proliferation. GC cells were plated in 100-mm cell culture dishes. After incubation for 24 h, AGS cells were transfected with siRNA (scRNA or PSG1 siRNA) and MKN-28 cells were transfected with an expression vector plasmid (pCMV–3Tag–1A_Empty or pCMV–3Tag–1A_PSG1). After transfection for 24 h,

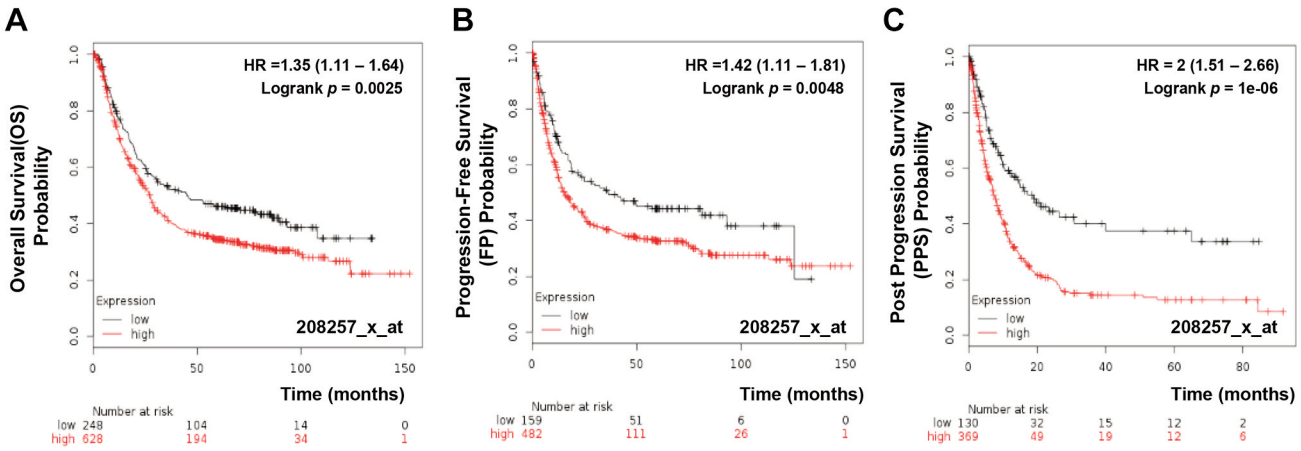

Figure 2. *Correlation between survival rate and PSG1 expression in GC patients. (A-C) Kaplan-Meier survival plots indicate the poor prognostic effect of PSG1 up-regulation, which correlated with a worse overall survival (OS, n=631), first progression (FP, n=499), post progression survival (PPS, n=876).*

cells were seeded in 60-mm cell culture dish (each 1×10^3 cells) and incubated in a 37˚C incubator with 5% CO_2. Media were changed to RPMI-1640 media supplemented with 10% FBS and 1% antibiotics every 3 days.

Migration and invasion assays. AGS cells were transfected with siRNAs and MKN-28 cells were transfected with an expression vector plasmid (pCMV–3Tag–1A_Empty or pCMV–3Tag–1A_PSG1). After 24 h of transfection, cell migration and invasion assays were performed using trans-well plates as described in the previous study (24). Plates were incubated for 16 h and H&E staining was carried out for quantification. After H&E staining, cells were counted in four randomly selected areas of each well using AXIO scope A1 microscope (Zeiss, Oberkochen, Germany). Data are expressed as mean±SD from four independent experiments.

Gene expression profiles using gene expression omnibus (GEO). GSE13861, GSE26899, and GSE54129 datasets were downloaded from the GEO database. GSE13861 includes 90 samples of primary gastric adenocarcinomas, gastrointestinal stromal tumors, and surrounding normal tissues. GSE54129 includes 132 samples of tumor and normal surrounding tissues in GC patients. GSE26899 includes 108 samples of GC and normal surrounding tissues.

Kaplan-Meier analysis. Kaplan-Meier analysis was performed using Kaplan-Meier Plotter. Survival rates were determined according to progression-free and post-progression variables as well as overall survival. To investigate the prognostic value of PSG1, the GC patient cohorts were divided into two groups (higher or lower) according to their median expression of PSG1. The survival plots of two patient groups were compared and hazard ratios with 95% confidence intervals and log rank *p*-values were calculated.

Statistical analysis. The expression of PSG1 in publicly available patient data was analyzed by Student's *t*-test using GraphPad Prism5. Results are presented as a mean±SD. Values of $p<0.05$ were considered statistically significant.

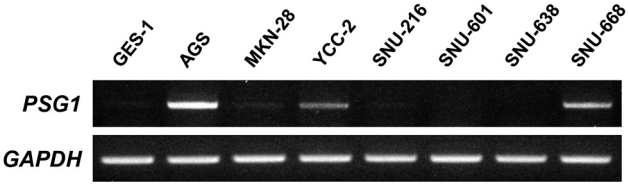

Figure 3. *mRNA expression basal levels of PSG1 in GC cell lines. PSG1 expression levels were evaluated in seven GC cell lines (AGS, MKN-28, YCC-2, SNU-216, -601, -638, and SNU-668). The gastric normal epithelial cell GES-1 is a negative control. mRNA expression levels were detected using RT-PCR.*

Results

Analysis of PSG1 expression and survival rate using public data of gastric cancer patients. We aimed to identify relationships between high expression of PSG1 and poor survival of GC patients with GC using GEO databases (Figure 1) and Kaplan-Meier plotter (Figure 2). We obtained publicly available datasets (GSE13861, GSE54129, and GSE26899) of GC patients and analyzed the expression levels of PSG1 in tumor and normal gastric tissues (Figure 1). Databases showed higher expression of PSG1 in cancer tissues than in normal tissues (Figure 1A and B). In addition, although not statistically significant, the expression of PSG1 tended to be higher in cancer tissues than in normal tissues (Figure 1C, $p=0.111$). Statistical analysis of survival rates using Kaplan-Meier plot for GC patients showed a correlation between high expression of PSG1 and a significantly lower probability of survival (Figure 2). Therefore, PSG1 up-regulated in cancer tissues indicates a poor prognosis for GC patients.

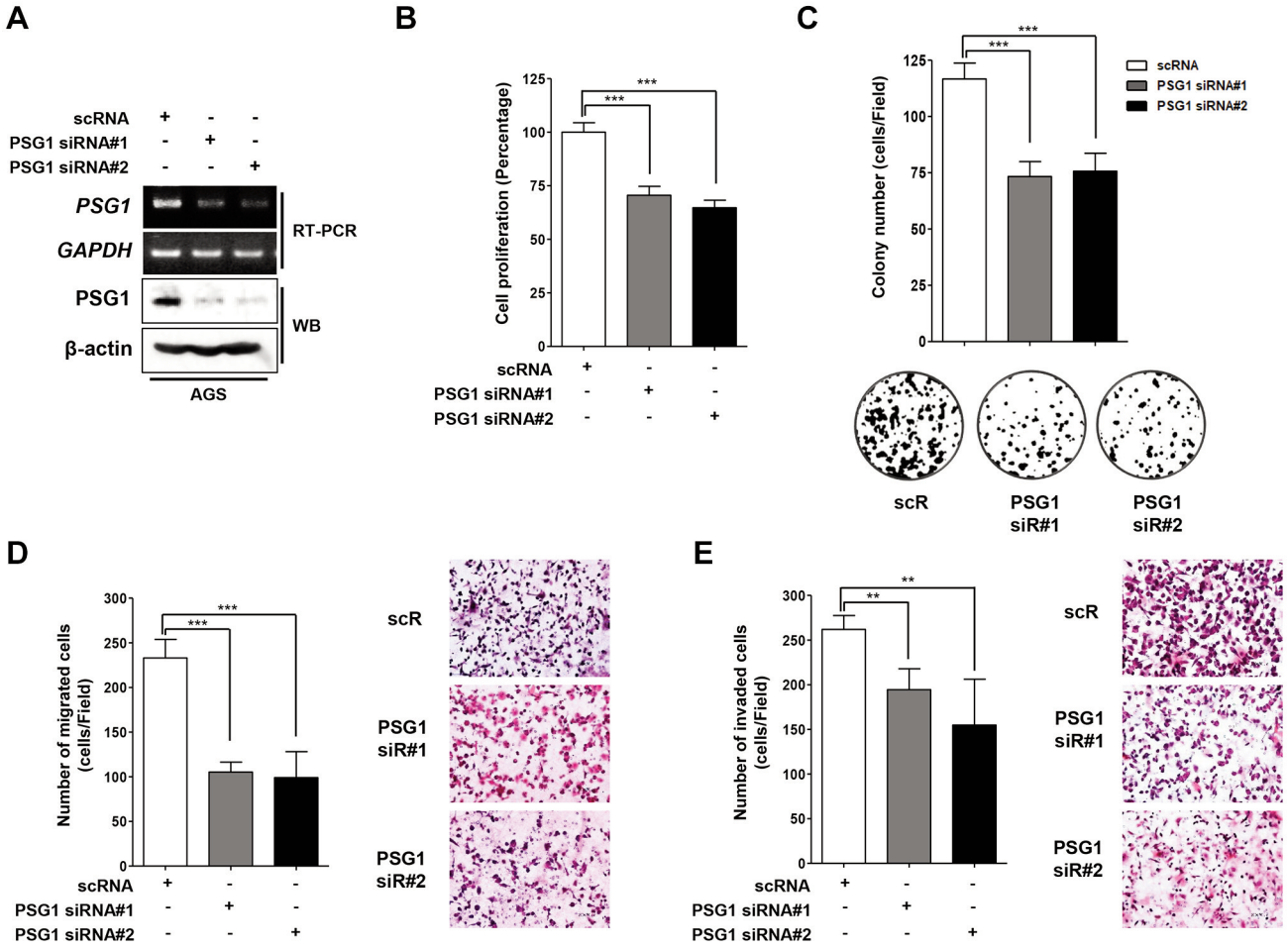

Figure 4. *Down-regulation of PSG1 reduces proliferation, invasion and migration in AGS cells. AGS cells were transfected with scRNA or two siRNAs specific for PSG1 (PSG1 siRNA#1 and siRNA#2). (A) Knock-down of PSG1 expression levels was shown using RT-PCR and western blot analysis. (B and C) Cell proliferation was examined using (B) WST assay and (C) Colony formation assay. (D and E) Transwell assay was performed to evaluate (D) migration and (E) invasion of cells (×200). The histograms represent the mean±SD (n=4). The significant differences are indicated by asterisks (***p<0.001, **p<0.01).*

Knockdown of PSG1 reduces the proliferation, migration, and invasion of gastric cancer cells. GC cell lines were used to confirm the role of PSG1 in GC. First, mRNA levels were measured using RT-PCR in seven GC cell lines (Figure 3). PSG1 was also expressed at high levels in GC cells. To investigate the effect of loss of function, two specific siRNAs were used to suppress the PSG1 expression in AGS cells. We confirmed that PSG1 expression was knocked down using RT-PCR and western blot (Figure 4A). After the knockdown of PSG1, we performed analysis for cell proliferation, migration and invasion. First, cell proliferation was measured using the WST assay (Figure 4B). Cell proliferation decreased in PSG1 knock-down AGS cells. In addition, knockdown of PSG1 reduced colony formation of AGS cells (Figure 4C). Next, transwell migration and invasion assays showed that migration and invasion was reduced when PSG1 expression was inhibited (Figure 4D and E).

Over-expression of PSG1 promotes proliferation, migration, and invasion of gastric cancer cells. Next, we examined the effects of gain of function of PSG1 in MKN-28 cells by over-expressing PSG1. MKN-28 cells were transfected with an empty vector (pCMV–3Tag–1A_Empty) or a PSG1 over-expression vector (pCMV–3Tag–1A_PSG1) for 48 h. Over-expression of PSG1 was confirmed by RT-PCR and western blot (Figure 5A). First, cell proliferation was measured using the WST assay (Figure 5B). PSG1 over-expressing cells showed more cell proliferation than the negative control (pCMV–3Tag–1A_Empty). Also, colony formation was increased when PSG1 was over-expressed (Figure 5C). Transwell migration

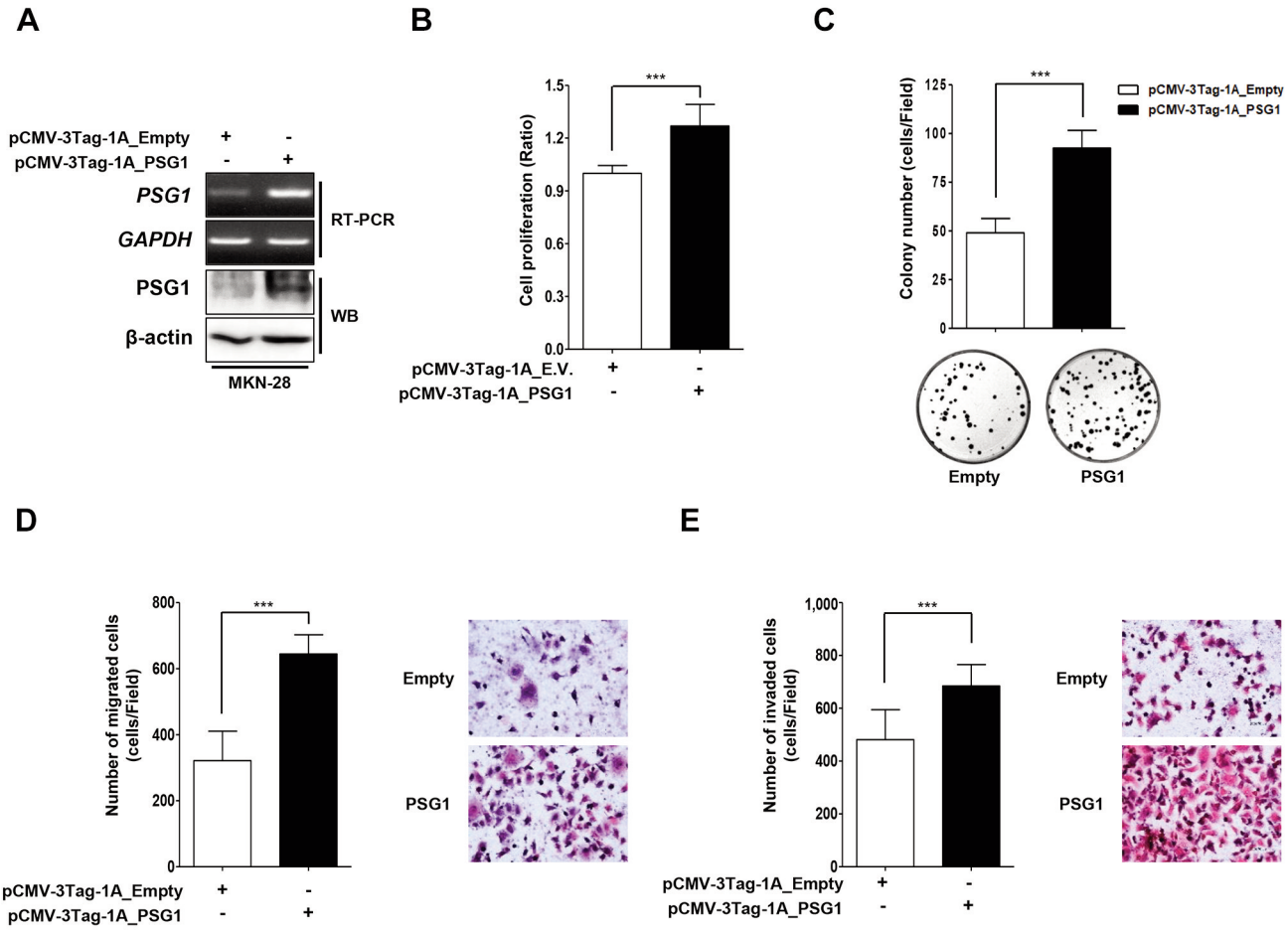

Figure 5. *Up-regulation of PSG1 promotes proliferation, invasion, and migration in MKN-28 cells. MKN-28 cells were transfected with an empty vector (pCMV–3Tag–1A_Empty) or PSG1 over-expression vector (pCMV–3Tag–1A_PSG1). (A) PSG1 expression levels were examined using RT-PCR and western blot analysis. (B and C) Cell proliferation is examined using (B) WST assay and (C) Colony formation assay. (D and E) Transwell assay was performed to evaluate (D) migration and (E) invasion of cells (×200). The histograms represented the mean±SD (n=4). The significant differences are indicated by asterisks (***p<0.001).*

and invasion assays showed that over-expression of PSG1 increased cell migration and invasion in MKN-28 cells (Figure 5D and E). These results suggest that PSG1 is involved in the progression of GC by regulating cell proliferation, migration and invasive abilities.

Discussion

PSGs have been detected not only in the placenta, but also in various types of tissue. There are recent reports of carcinogenesis and cancer progression of various tissues as well as functions associated with pregnancy. In particular, PSG1, a member of the Ig superfamily, exhibits structural similarity to CEA, and its high expression is functionally closely associated with a cancer-associated phenotype (25, 26). Several CEA-related proteins, such as PSGs, are secreted from various tumor tissues and used as serum biomarkers and therapeutic target molecules. PSGs have also been proposed as therapeutic targets and biomarkers for various cancers (27-29).

PSG1 has been reported to play a role in pregnancy (17, 18). It regulates TGF-β-mediated vascular endothelial growth factor (VEGF) and placental growth factor (PGF) (18, 19). It induces secretion of anti-inflammatory cytokines from monocytes and regulates T-cell activation and proliferation (30, 31). Also, PSG1 also contributes to lectin stabilization through binding to glycoprotein galectin-1 and regulates the interaction of various cell surface receptors (32). Among them, expression of various genes such as VEGF and galectin-1 play important roles in cancer progression (33, 34). Therefore, we hypothesize that PSG1 expression may be involved in cancer progression.

In addition, PSG1 was detected in the serum and various organs. Several studies have been conducted on the clinical correlation between PSG1 expression and cancer pathology in various types of cancer such as pancreatic, breast, and lung cancer (22, 25, 35-39). However, there has been no report on the role of PSG1 in GC. Therefore, this study investigated the association between PSG1 expression and GC.

We used publicly available databases to examine differentially expressed genes in normal and cancer tissues of GC patients. We confirmed that PSG1 is highly expressed in cancer tissues. In addition, the correlation between PSG1 and the survival rate of GC patients was analyzed using the Kaplan-Meier plotter. High expression levels of PSG1 were associated with short survival. That is, PSG1 is highly expressed in GC tissues and indicates a poor prognosis for patients.

Next, the role of PSG1 was investigated in GC cell lines. In AGS cells, downregulation of PSG1 inhibited cell proliferation, migration and invasive ability. In contrast, up-regulation of PSG1 enhanced cell proliferation, migration and invasion in MKN-28 cells. Therefore, PSG1 was highly expressed in GC patients, suggesting that it is related to GC progression.

PSG1 is a type of protein that is secreted unlike the common membrane attached protein CEA. This characteristic allows its detection in the patient's blood, which is one of the essential requirements for a diagnostic marker. However, further studies are needed to determine the potential of PSG1 as a diagnostic biomarker. Public databases have shown that PSG1 is highly expressed in GC, but the role of PSG1 in GC has not been well known. Our results demonstrated the role of PSG1 gene in the prognosis of GC. Therefore, PSG1 as a potential prognostic marker and target regulating GC progression.

Conflicts of Interest

All the Authors declare no conflicts of interest regarding this study.

Authors' Contributions

Conception, design, and funding: SJK; isolation, identification, and experiments: HGK; writing the manuscript: HGK and SJK; reviewing the manuscript: all Authors.

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